ADVANCES AND TRENDS IN POLYHYDROXYALKANOATE (PHA) BIOPOLYMER PRODUCTION

EDITED BY: Justyna Mozejko-Ciesielska, Prasun Kumar, You-Wei Cui and Paulo Costa Lemos

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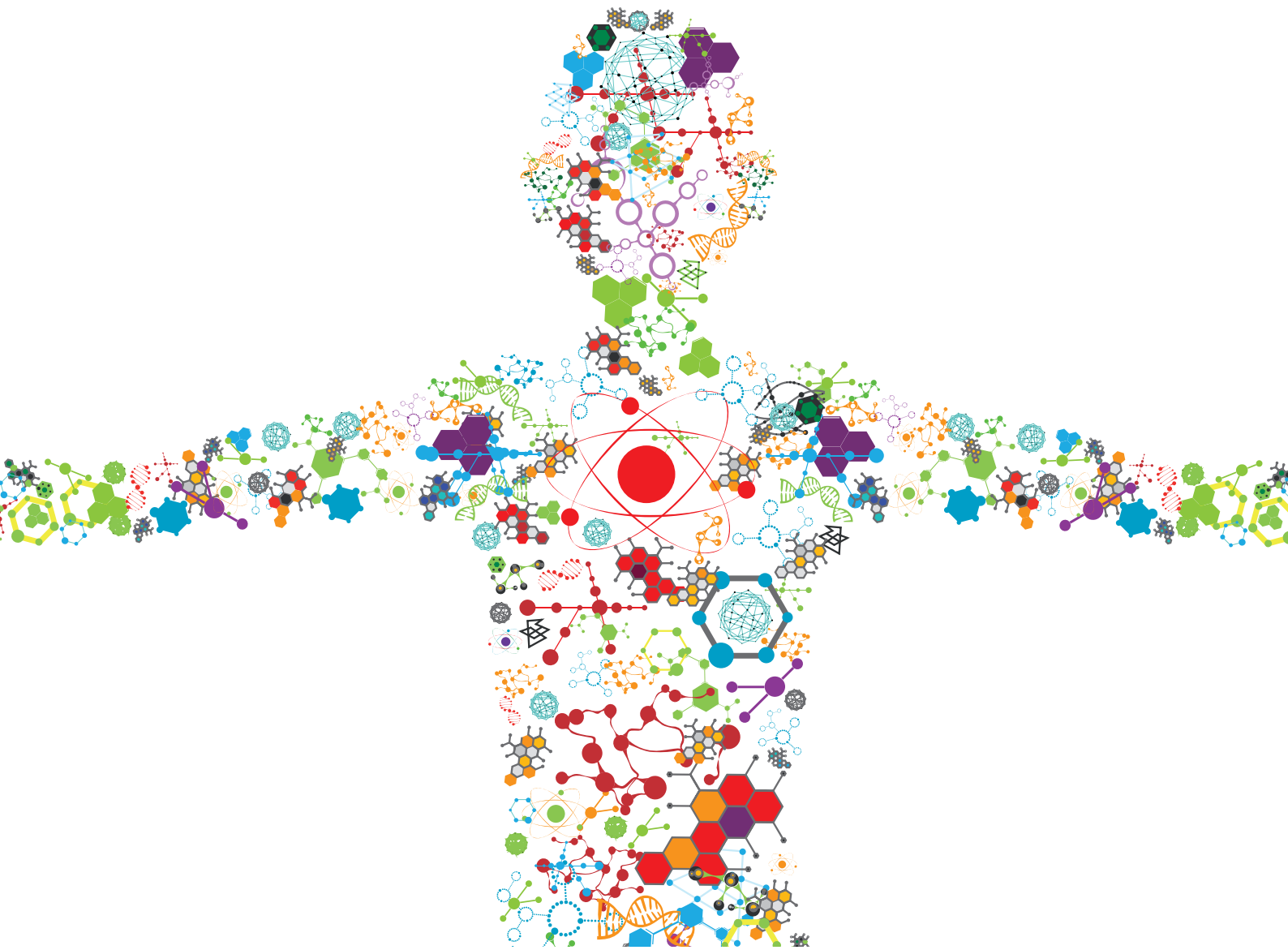

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ADVANCES AND TRENDS IN POLYHYDROXYALKANOATE (PHA) BIOPOLYMER PRODUCTION

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EDITORIAL
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Editorial: Advances and Trends in Polyhydroxyalkanoate (PHA) Biopolymer Production

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Keywords: high cell-density culture, polyhydroxyalkanoates, pure/mixed cultures, biodegradable polymer, co-polymer

Editorial on the Research Topic

Advances and Trends in Polyhydroxyalkanoate (PHA) Biopolymer Production

Plastics are key materials in delivering many benefits to society. Possessing a high level of strength, durability and lightness they have become versatile and innovative materials used in numerous products from nearly all industrial sectors. However, due to low degradability their production has led to serious environmental problems. Worldwide production of plastics is still increasing reaching 367 million tons in 2020, so 163 million tons more compared to 2002. It was estimated that in 2020 over 23% of plastic post-consumer wastes were discarded in the landfills (Plastics Europe, 2021). During their decomposition under the actions of biological, physical, and chemical processes, microplastics are formed which nowadays are a major global concern due to their threat to wildlife and humans. These small plastic particles have spread globally to even the most remote habitats becoming a new pollutant and one of the most important scientific issue in environmental science (Zhang et al., 2021). Furthermore, not only problems related to the utilization of plastic waste but also new regulations for its use and management, together with increasing concerns about global warming and climate change are reasons to search for biobased substitutes for petroleum-derived polymers. Moreover, the increased demand for sustainability on the utilization of plastics opens a new opportunity for a myriad of biopolymers that are generated in microbial processes. Over the past decades, great attention has been focused on polyhydroxyalkanoates (PHAs), environmentally friendly polymers with plastic-like properties.

The focus of this Special Issue is to highlight the latest advances in the microbial synthesis of PHAs. It addresses several distinguished aspects of the PHA production chain related to the development and design of novel bioprocesses towards the improvement of PHAs production, description of microbes as PHAs producers of industrial interest, molecular design of polymers with a variety of properties, solutions to decrease overall production costs of microbial PHAs.

This special issue consists of a total of six original articles, one brief research report, two specialized review papers, and one systematic review written by globally recognized experts who have had a significant impact on this research area already for a long time.

In the early 1990s, an exponential burst of scientific publications concerning research on PHAs started. Since then, different areas of exploration of these intriguing materials have been uncovered. However, to understand the field of these studies a systematic literature review is essential. Guzik et al. have performed an unbiased search of up-to-date literature to reveal trending topics in the research of PHAs over the past 3 decades by data mining of 2,227 publications. The conducted analysis allowed to identify eight main areas in PHA research that governed the 31 years of

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discoveries. The analysis of the articles published from 1988 to 2019 provides a comprehensive review of the trends and speculates where PHA research is heading.

In an attempt to increase PHA production, PHB biosynthesis operon of alkali tolerant *Cupriavidus necator* strain A-04 was expressed in *Escherichia coli* under the control of cold shock promoter (*cspA* promoter) by Boontip et al. The PHA synthase remained insoluble in the tested expression systems until it was co-expressed with TF chaperones. This study revealed that the *cspA* promoter in a cold-inducible vector can enhance PHA synthase expression and solubility without affecting polymer properties. The induction strategy was demonstrated on a 5-L fermenter showing a PHB production of 7.9 g/L with 89.8% PHB content in the cell dry mass. In another study, Napathorn et al. tested the expression system consisting of *araBAD* promoter and thioredoxin fusion proteins and compared it with the cold shock inducible expression system. This work demonstrated that both expression systems were able to yield high cell density and PHB production in fed-batch cultivation using a pH-stat feeding strategy. It was proposed that arabinose-based induction system would be a cheaper alternative to IPTG. Thus, *E. coli* XL1-blue expressing pBAD/Thio-TOPO-$phaCAB_{A-04}$ and pColdTF-$phaCAB_{A-04}$ possess a high potential for PHB production.

In the context of the potential to expand the molecular design of polymers with a variety of properties Arai et al. shows the capacity to incorporate glycolate (GL) units into the chains of PHAs since this monomer is not present in PHAs of natural occurrence. For that a new chimeric PHA synthase (PHACAR) containing the N-terminal region of PhaCAc derived from *Aeromonas caviae* and the C-terminal region of PhaCRe derived from *Cupriavidus necator* was constructed. This new class I PHA synthase was expressed together with propionyl-CoA transferase (PCT) in *Escherichia coli*. This microorganism was able to produce a random-homoblock PHA, P (GL-ran-3HB)-b-P (3HB) for the first time. The produced polymers were analyzed in terms of their thermal properties, solvent fractionation, and sequence heterogenicity. The simultaneous presence of random and block sequences in this biopolymer can be useful to control the physical properties of each segment and so to design new polymers with an array of different characteristics. The incorporation of the GL units can potentially manipulate the bioabsorption rate of PHAs, as hydrolytically degradable material, useful in biomedical fields.

Substrate cost contributes towards a major portion of incurred cost during PHA production. Several wastes have been explored for their suitability and potential for PHA production. Due to the flourishing biodiesel industry across the globe, the surplus amount of crude glycerol has emerged as a suitable feedstock for the bio-production of industrially important chemicals. In this context, algal biodiesel waste residue (source of crude glycerol) was tested as a substrate by halophilic PHA-producing bacteria by Dubey and Mishra. The authors proved that *Halomonas daqingensis* and *Halomonas ventosae* were capable of utilizing 3, 4% of algal waste residue and producing 68.96 and 72.41% in the cell dry mass, respectively. Similarly, Borrero-de Acuña et al. used biodiesel-derived crude glycerol in the cultivation of *Pseudomonas putida* KT2440 and its *ΔphaZ* mutant (PHA depolymerase deficient) for the production of mcl-PHAs under fed-batch conditions. Two different feeding approaches were compared to demonstrate that DO-stat fed-batch process is more suitable feeding strategy than the constant-feeding approach resulting in about 50% more PHA at the end of the fermentation (60 h) in the *ΔphaZ* mutant with a specific PHA volumetric productivity of $0.34\ g\ L^{-1}\ h^{-1}$. With a reduced fermentation period and unique feeding strategy, *P. putida* strains accumulated higher quantities of biopolymer with a monomeric composition largely consisting of 3-hydroxydecanoate. During the fermentation, citric acid was produced as a major co-product. This provides a scope of further improvement *via* metabolic engineering to decrease the carbon wastage and channelize the carbon flux for PHA biosynthesis.

In the past 2 decades, a lot of efforts have also been made to isolate and explore unique microbes that can produce large quantities of PHAs using cheap raw materials. However, very limited research has been tested on pilot-scale production and translated to produce PHAs on a large-scale. In this regard, photoautotrophic microalgae have recently emerged as one of the potent contenders for cost-effective and sustainable PHA production. They have minimal metabolic requirements, such as inorganic nutrients (CO_2, N, P, etc.) and light, and they can survive under adverse environmental conditions. Afreen et al. reviewed the potential of microalgae as a microbial factory for PHA production. In addition, PHA production by cyanobacteria (both wild-type and recombinants) such as *Nostoc*, *Arthrospira*, *Synechocystis*, and *Synechococcus* were presented in detail comparing the PHA yield and cultivation conditions. However, there are certain limitations–stringent management of closed photobioreactors, and optimization of monoculture in open pond culture. To overcome these limitations and reduce the feed cost incurred during PHA production, a "Hybrid Biological System" was suggested. It involves the participation of microalgae/cyanobacteria and bacteria to utilize the opportunities present in both photoautotrophic and heterotrophic cultivation with the following cultivation regimes: (A) Mixotrophy; (B) Photoautotrophic-heterotrophic consortium; and (C) Two module system. For the optimization of the process parameters, several relevant metabolic network modeling approaches were also discussed. This review emphasizes the intellectual but efficient use of biological systems and explores various attributes associated with photoautotrophic and heterotrophic organisms.

Similar to the hybrid biological system, open mixed microbial cultures had been favored for PHA production under non-axenic conditions. Such an approach involves enrichment of the PHA producers under specific conditions utilizing organic wastes as substrate. Thus, co-metabolic activities of microbes not only favour microbial growth but also alleviate the substrate cost. Lorini et al. conducted a unique study concerning PHA production at pilot scale exploiting different process configurations and organic waste streams such as the organic fraction of municipal solid waste and sewage sludge, cellulosic primary sludge, and fruit waste. Here, thermal drying and wet

acidification of the biomass at the end of the PHA accumulation process were used to preserve the intracellular PHA. The polymer was extracted using aqueous-phase inorganic reagents under optimized conditions. PHA extracted from wet-acidified biomass had higher viscosity average molecular weights compared to thermally stabilized dried biomass. This investigation shows the possibility to exploit organic biowastes as raw materials for PHA production. The stabilization method before extraction helped to preserve the polymer properties. On the other hand, the monomeric composition of the polymer was greatly influenced by the type of feedstock used. This study reveals that waste-derived biopolymers can achieve good characteristics and workability. Such information may play a pivotal role in future development and sustainable PHA production.

Furthermore, Santolin et al. proposed a novel strategy for reducing the dependency of a single feedstock during PHA production process. The authors described effective substrate-flexible two-stage fed-batch cultivations for the production of P(HB-co-HHx) copolymer using fructose during the biomass accumulation stage and rapeseed oil for polymer production. In order to achieve high space time yields a "drain and fill" modus was proposed for semi-continuous biomass production during the initial stage. Taking advantage of the high-cell-density achieved during the first stage, the second stage was run without previous sterilization of the in-series bioreactors. The obtained results suggested that applying the new method presented by the authors could contribute to reduce production costs and, in this way, accelerate the commercialization of a sustainable PHA-bioplastic.

In the field of PHA production, the recovery of the polymer from its intracellular environment is of major importance. This step impact is one of the major contributors to the high selling price of the polymers but also to its sustainability, due to the non-environmentally friendly nature of most of the processes used for its recovery. Delving into this topic, Pagliano and co-workers considered the different natural production of PHA either by pure or mixed cultures and some of the polymer characteristics as well as the utilization of either solvent-based or cellular-lysis-based methods for its recuperation. Parameters as the intracellular PHA content, the PHA recovery and its yield, the PHA purity, molecular weight (M_W), and polydispersity index (PDI) were used to compare the different methods. Considering the nature of the cultures used there is no rule of thumb to be applied. PHAs produced by MMC are the ones presenting a major variability in the considered parameters mostly due to the different PHA content observed as well as the chemical characteristics of the polymers produced by those cultures. Solvent extraction methods perform better in terms of higher purity and molecular weight of the recovered polymers, mostly for halogenated and carbonate solvents and to a lower extent to ketones and alcohols. Cellular lysis although provides a higher recovery and yield, can cause polymer degradation that will impact both M_W and PDI (polymer quality). The purity of the polymer will be affected (70–80%) which would have a major impact on its final utilization. Some techno-economics aspects of the extraction processes were provided that differentiate both methods. In terms of costs, cellular lysis would present lower costs at the expense of a low-quality polymer while solvent extraction, in particular halogenated ones, at a larger scale and considering quality aspects, would become more competitive.

The Guest Editors are thankful to all the submitting authors for considering this Special Issue as a forum for publishing their research work. The assistance of the reviewers, through their high-quality reports, was very helpful for choosing the papers which are suitable for this Special Issue. We hope that this Special Issue will attract the interest of the researchers which are involved in PHAs study and will be an inspiration for them to improve PHA-related processes.

AUTHOR CONTRIBUTIONS

JM-C conceived the idea of the Research Topic and served as editor. PK, PL, and YC served as editors. All authors contributed to the editorial and approved the submitted version.

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SYSTEMATIC REVIEW
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What Has Been Trending in the Research of Polyhydroxyalkanoates? A Systematic Review

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Over the past decades, enormous progress has been achieved with regard to research on environmentally friendly polymers. One of the most prominent families of such biopolymers are bacterially synthesized polyhydroxyalkanoates (PHAs) that have been known since the 1920s. However, only as recent as the 1990s have extensive studies sprung out exponentially in this matter. Since then, different areas of exploration of these intriguing materials have been uncovered. However, no systematic review of undertaken efforts has been conducted so far. Therefore, we have performed an unbiased search of up-to-date literature to reveal trending topics in the research of PHAs over the past three decades by data mining of 2,227 publications. This allowed us to identify eight past and current trends in this area. Our study provides a comprehensive review of these trends and speculates where PHA research is heading.

Keywords: polyhydroxyalkanoate, data mining, trends, composites, medicine, renewable production, polyhydroxyalkanoate (PHA) synthesis, biotechnology

INTRODUCTION

Polyhydroxyalkanoates (PHAs) have been researched since their discovery in 1920. An exponential burst of scientific publications started in the early 1990s. Since then, yearly, more and more scientific documents are appearing, increasing our knowledge in this realm of biopolymers. A systematic literature review is an important method of understanding the field of study. However, due to high labor intensity, it is usually limited to narrow subjects. They are, in most cases, narrative and qualitative (Tranfield et al., 2003). To study the whole field, it is necessary to adopt text-mining toolset and quantitative methods. It is relatively easy to perform analysis based on metadata related to publications, mostly titles, keywords, and abstracts. The required software is readily available. Unfortunately, research by Blake (2010) revealed that authors report <8% of scientific claims in abstracts. Moreover, keywords used in publications are in many cases limited or modified by publishers. Therefore, studies based on metadata cannot be treated as fully reliable. The following limitation of most text-mining studies is predefinition of themes, clusters, or categories. This can prevent the discovery of new topics that were not predefined by researchers. Additionally, in the case of qualitative methods, the risk of researcher bias grows significantly.

The approach offered in this study was designed to overcome such limitations. The analysis was performed using full texts of publications, and categories were discovered in data, not predefined. The well-known quantitative approach to text-mining was supplemented with new and original tools (for a full description, please see the Materials and Methods section). This allowed authors to identify not only categories but also trends. A diagram of the proposed approach compared with systematic literature review steps is presented in **Figure 1**. The procedure is consistent with general rules for systematic literature reviews (Ananiadou et al., 2009). In this article, we have sampled scientific publications from Web of Science Core Collection that were firmly related to PHA research and performed an *in silico* analysis of the most frequently appearing words within the main text. We have omitted on purpose reviews that are not original *per se*, thus focusing only on scientific publications concerning the generation of new data in areas related to PHAs at the time of their publications. This allowed us to identify scientifically relevant keywords that enabled us to group the publications into clusters, groups, and then trends that have been emerging since the late 1980s.

FINDING TRENDS

Collection of Publications

In order to collect publications, it was necessary to define search criteria, which included:

- the main subject of a publication related to PHA polymers (search topic term "polyhydroxyalkanoate"),
- availability of the full text of the publication,
- published between 1988 and 2018,
- only scientific publications were included.

The search was performed in the scientific database Web of Science Core Collection using the command "all fields." In the result, 2,432 publications were found. Publications from

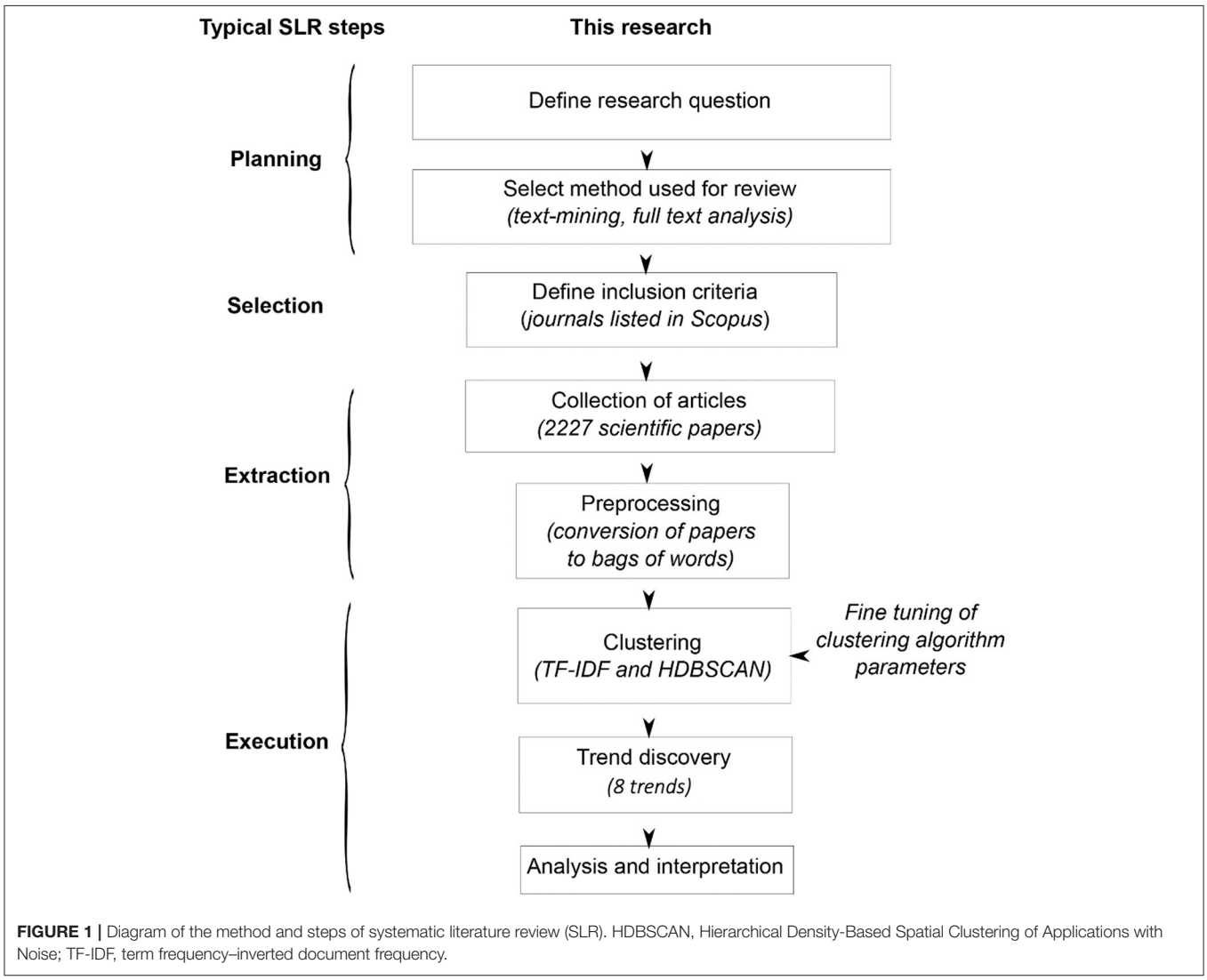

FIGURE 1 | Diagram of the method and steps of systematic literature review (SLR). HDBSCAN, Hierarchical Density-Based Spatial Clustering of Applications with Noise; TF-IDF, term frequency–inverted document frequency.

TABLE 1 | Number of publications in polyhydroxyalkanoate (PHA) research by journal.

Applied Microbiology and Biotechnology	139
Applied and Environmental Microbiology	108
Bioresource Technology	86
International Journal of Biological Macromolecules	69
Fems Microbiology Letters	68
Biomacromolecules	60
Journal of Biotechnology	57
Journal of Bioscience and Bioengineering	54
Polymer Degradation and Stability	46
Biotechnology and Bioengineering	36
Biotechnology Letters	35
New Biotechnology	34
Journal of Bacteriology	28
Journal of Polymers and the Environment	28
Water Research	28
Applied Biochemistry and Biotechnology	25
Journal of Applied Microbiology	25
Journal of Chemical Technology and Biotechnology	25
Water Science and Technology	25
Microbial Cell Factories	23
Journal of Industrial Microbiology Biotechnology	21
Macromolecular Bioscience	21
Canadian Journal of Microbiology	20
Polymers	19
Process Biochemistry	19
World Journal of Microbiology Biotechnology	19
Environmental Microbiology	18
Microbial Biotechnology	18
Plos One	18
Biomaterials	17
Journal of Applied Polymer Science	17
Biochemical Engineering Journal	16
Metabolic Engineering	16
Abstracts of Papers of the American Chemical Society	15
Frontiers in Microbiology	15
AMB Express	14
Bioscience Biotechnology and Biochemistry	14
European polymer Journal	14
Journal of Microbiology and Biotechnology	14
Microbiology Resource Announcements	14
Microbiology Sgm	14
Biotechnology Journal	13
Applied Food Biotechnology	12
Indian Journal of Microbiology	12
International Journal of Systematic and Evolutionary Microbiology	12
Biotechnology and Bioprocess Engineering	11
ACS Symposium Series	10
Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology	10
Bioprocess and Biosystems Engineering	10
Current Microbiology	10
Enzyme and Microbial Technology	10
Other Journals	765

1988 to 1994 were excluded from the analysis due to an overall small number over these years (34 publications). After removing duplicated, editorials, reviews, and other non-scientific publications, 2,227 studies were left. The number of publications ordered by journal title was presented in **Table 1**. Only publications from journals, which published more than 10 studies in total with relation to the keyword "polyhydroxyalkanoate" were presented in this table.

Each publication was converted into a text file and prepared for automatic analysis using computer algorithms. In this research, algorithms have been created using Python libraries, including grobid, nltk, scikit-learn, hdbscan, and scipy (Jones et al., 2001; Lopez, 2009; Pedregosa et al., 2011; McInnes et al., 2017).

The corpus of the research can be presented as a network of authors of publications and authors indicated by them in the references. This means that not only the most frequently quoted authors but also the relations between them can be discovered. The source of the relations, for example, includes similarity of covered topics, joint research, and long-term cooperation. The network of relations, therefore, helps to understand trends in the literature better. The network was prepared using Gephi (**Figure 2**). For the sake of clarity, only links with a number of citations >10 were presented. An analysis of all relations would contain over 60,000 authors and over 530,000 relationships. However, it would not significantly impact the list of the most often quoted authors and the most important relations.

Search for the Most Important Terms

The most important terms are not always those which are the most frequent. In English, the most frequent words are "the" and "of." In the case of this study, the word "polymer" can be found in every publication. Thus, it does not impact the results. The terms that exist in one publication only are important only for that publication. The most important terms are those which occur in a group of publications. They allow researchers to identify clusters and then trends (Salton and Yang, 1973; Cong et al., 2016). The term frequency–inverted document frequency (TF-IDF) method was designed to solve that problem. It takes into account the frequency of the term but at the same time includes several documents in which that term occurs. The following formula is used:

$$w_{i,j} = tf_{i,j} \cdot \frac{N}{df_i}$$

where:

$w_{i,j}$—result for term i in document j,
$tf_{i,j}$—number of occurrences of i in j,
df_i—number of documents containing i,
N—number of documents in corpus (set of documents).

In the result, each publication can be presented as a vector that consists of multiple dimensions. The number of dimensions is equal to the number of terms used in the analysis, usually several thousands. The similarity of the publications as vectors can be assessed using mathematical methods, e.g., cosine similarity

FIGURE 2 | The network of authors of the most frequently quoted in analyzed journals. From each publication, analyzed pairs were identified consisting of: (1) author of the paper and (2) author of referenced paper, represented by the lines in the graphic. All identical pairs in whole corpus were summed up. The greater the sum, the stronger the relation is, which is visualized by the increased font size in the iconographic. The network of relations allows to discover groups of cooperating scientists. These relations are probably based on common university, field of study, etc.

(Mihalcea et al., 2006). Some other methods can be used instead of TF-IDF, e.g., Latent Semantic Indexing (LSI) or Latent Dirichlet Allocation (LDA). They can explain the meaning of a text, but the format of results makes their use in further steps of our cluster analysis difficult.

Discovery of Thematic Groups (Clusters)

The similarity of the vectors can help discover thematic groups of publications. It can be done using partitioning or hierarchical clustering methods. As a result of partitioning, all elements of the corpus has to be assigned to one of the predefined partitions, even if it is not similar to the other elements. The hierarchical clustering includes in clusters only those elements that are similar. Therefore, hierarchical clustering is better when searching for trends. The examples of partitioning methods include k-means, affinity propagation, spectral clustering, and agglomerative clustering. In contrast, the examples of clustering are mean shift (based on k-means), DBSCAN, Optics, and HDBSCAN (McInnes et al., 2017).

We decided to use HDBSCAN (Hierarchical Density-Based Spatial Clustering of Applications with Noise), which is a relatively new method (McInnes et al., 2017). The algorithm takes each publication (vector) and checks at what distance it can find similar ones. Then it compares the results, and the densest areas

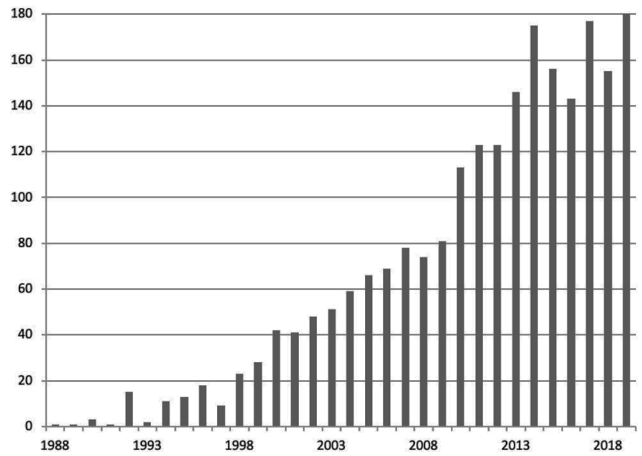

FIGURE 3 | Number of publications in polyhydroxyalkanoate research identified in Web of Science Core Collection for years 1988–2019.

are detected as clusters. The density and number of elements in the cluster can differ. The researcher has to define the minimum cluster size, which should be identified experimentally. In the case of this study, the minimum cluster size was set to 5.

The sample was divided into groups that contained publications published in 5-years overlapping periods starting with 1995–2000 and ending with 2014–2019. Each publication was assigned to all the groups into which it fitted. Cluster analysis was performed in every group separately. **Figure 3** presents the number of articles in each year.

Due to the limited length of the publication, it is not possible to describe HDBSCAN algorithm in detail. Full documentation with examples and comparison to other methods can be found on the dedicated website (hdbscan.readthedocs.io).

Discovery of Trends

Clusters require further analysis to discover trends. Definition of overlapping periods allows automatic detection of trends based on tracking of publications in subsequent corpora. A trend occurs when the average number of articles exceeds a certain expert-determined level per given time. Each step of the analysis leads to the discovery of slightly different clusters in which publication tracking is possible. Thanks to tracking, an evolution of clusters can be observed, and trends can be identified. As a result of the analysis, several types of trends can be discovered:

- long-lasting trends that exist and evolve during the studied period,
- declining trends which end during the studied period,
- emerging trends which begin during the studied period,
- ephemeris trends that begin and end during the studied period.

The final step is trends verification, description, and interpretation, which has to be performed by researchers without the help of algorithms. The researcher has to decide whether trends have been identified correctly. The algorithm usually identifies more trends, as it is very sensitive. They have to be merged by researchers. High sensitivity is intentional, as a lower one could lead to an unjustified merger. Trends can be described based on the most important terms found during the TF-IDF analysis of publications that constitute the trend. The interpretation phase should help to highlight changes within the trends and try to predict their future evolution.

TRENDS UNVEILED

The unbiased data mining allowed us to identify eight trends that have been spanning since 1988 (**Figure 4**). Below, the reader will find their brief description with links to the more specialist reviews and original publications. Some of them are continuing and will last into the future; the others have peaked for several years and are not anymore persistent in the published literature. Nevertheless, it does not mean that research in these areas is not conducted and probably more detailed and narrower studies, which polish the findings over the trend life span, are conducted.

Understanding and Manipulation of Genes Responsible for Polyhydroxyalkanoate Synthesis

Years 1988–2019

This trend is the longest appearing in the research of PHAs over the years. The first works relate to understanding the biosynthesis of PHAs in microorganisms, of which more than 300 have been investigated. Primary findings were described in 1988. Slater et al. (1988) succeeded in the expression of poly-3-hydroxybutyrate (PHB) synthesis genes in *Escherichia coli* by introducing plasmids; at the same time, Schubert et al. (1988) also synthesized PHB in *E. coli* using cosmids. Since then, several biosynthetic pathways have been discovered to date. One distinct was described for short chain length PHAs, the biosynthesis enzymes encoded in the *phaABC* operon, whereas for medium chain length PHAs, the most abundant are: pathways of metabolite shunts through β-oxidation, *de novo* fatty acid synthesis, acyl chain elongation, and others that provide precursors of (*R*)-3-hydroxyacyls from numerous vital biochemical pathways within a given microorganism (see review, Suriyamongkol et al., 2007; Lu et al., 2009). In parallel to the discovery of microbial enzymatic apparatus leading to the synthesis of these polyesters, three key enzymatic groups, directly responsible for PHA synthesis, are also in the spotlight: PHA synthases (see in Stubbe and Tian, 2003; Nomura and Taguchi, 2007), PHA depolymerases (see in Jendrossek, 1998; Ong et al., 2017), and granule associated proteins (see in Dinjaski and Prieto, 2015; Maestro and Sanz, 2017; Gonzalez-Miro et al., 2019). With the development of molecular genetic tools, the constantly discovered genes were being transferred to other model prokaryotes (e.g., *E. coli*, see trend *PHB production by genetically modified Escherichia coli*) and eukaryotes (yeasts, see Breuer et al., 2002, or plants, see Suriyamongkol et al., 2007) in order to increase production yields and thus lower the costs of PHAs. Moreover, directed evolution of enzymes, hybrids generation, and genome manipulations [i.e., through

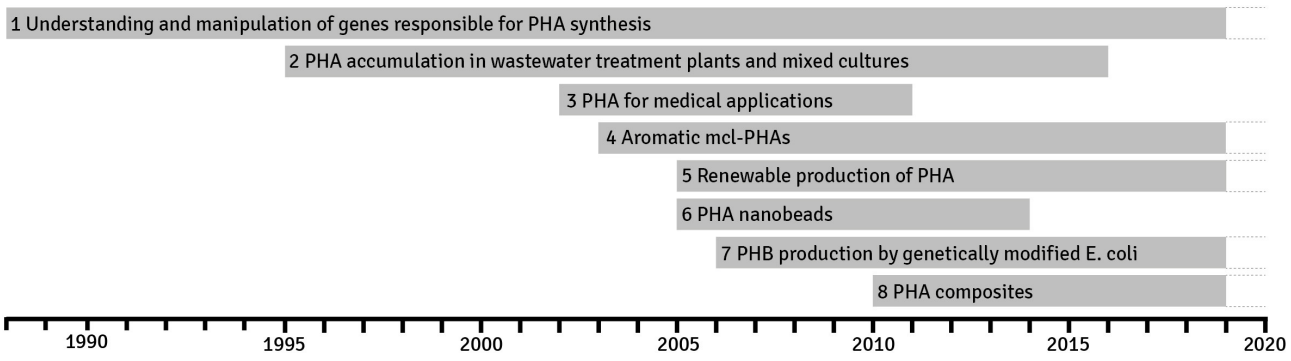

FIGURE 4 | Trends discovered in research on polyhydroxyalkanoates in years 1988–2019 based on the most important terms found during the term frequency–inverted document frequency (TF-IDF) analysis of publications. A trend occurs when the average number of articles exceeds a certain expert-determined level per given time.

CRISPR/Cas9-based systems (Qin et al., 2018)] not only led to the creation of new robust production strains characterized by good productivities but also allowed for the development of sophisticated polymer bead/enzyme-based platforms (see review Gonzalez-Miro et al., 2019, and also trend *PHA nanobeads*).

Polyhydroxyalkanoate Accumulation in Wastewater Treatment Plants and Mixed Cultures

Years 1995–2016

The increasing world population causes the production of large quantities of urban and industrial waste. Recycling waste and wastewater opens up an opportunity for reducing their quantity and the treatment costs (Cavaillé et al., 2013). Activated sludge can be an alternative to pure cultures for PHA production due to many PHA-producing bacteria present in activated sludge (Yang et al., 2013). In the wastewater treatment process, the microorganisms from activated sludge display the ability to transform a biodegradable carbon source into PHA before using them for growth (Qu and Liu, 2009). Industrial-scale PHA production is based mainly on pure-culture systems with refined feedstock and sterile cultivation conditions. Both features result in high-energy consumption, thereby strongly increasing production costs (Valentino et al., 2019).

Consequently, PHA cannot be cost-competitive to conventional petroleum-based plastics. An innovative approach has been proposed to combine PHA production with sludge minimization in municipal wastewater treatment. In the publications that make up this trend, both the impact of changing the approach to wastewater on the natural environment (Valentino et al., 2015) and the life cycle assessment (LCA) analysis of the production process (Morgan-Sagastume et al., 2016a), as well as specific examples of biochemical (Yang et al., 2013; Inoue et al., 2016) and industrial processes (Pittmann and Steinmetz, 2014; Valentino et al., 2019) resulting in biopolymers, are presented. In the publications, an economic analysis of the process on the example of selected European countries is also carried out (Pittmann and Steinmetz, 2017). The first reports of a significant relationship between PHA production and activated sludge were published in 1998. The issue of biodegradable polymer production with the use of sludge was raised in this publication, as well as the laboratory scale process was described along with performance analysis depending on the reaction conditions (Satoh et al., 1998). Later studies (after 2000) focus on parameters such as carbon and nitrogen sources, their mutual quantitative relations in the fermentation reaction, and the impact of these parameters on process efficiency (Khardenavis et al., 2005). Further analysis on the example of PHB production describes the impact of critical factors such as dissolved oxygen, pH, and food to microorganism (F/M) ratio in the batch reactor on the reaction efficiency (Qu and Liu, 2009). Researchers also carried out physiochemical properties analysis of the polymers PHB and P(HB-co-HV) to identify production conditions that directly affect final product mechanical properties (Wallen and Rohwedder, 1974; Patel et al., 2009). In works mentioned in this trend, the potential of mixed colony nitrogen-fixing bacteria cultures for producing biodegradable polymers with mechanical and chemical properties similar to those originating from pure culture counterparts is presented. In the latest reports (2019), a description of operating pilot plant designed for the production of biopolymer from wastewater can be found (Valentino et al., 2019).

As a summary, one can indicate that a more economical and much cheaper way of producing biopolymers does not necessarily mean a deterioration of their properties, which further indicates the high desirability of projects involving the study and optimization of the production process of bioplastics from sludge (Patel et al., 2009).

Polyhydroxyalkanoate for Medical Applications

Years 2002–2011

PHAs attract the attention of researchers and medical doctors mainly due to their biocompatibility. This feature allows for the creation of medical devices and implants that are completely safe for use in mammals, including humans. PHAs such as P(3HB) and its breakdown products, 3-hydroxy acids, have been

found in many organisms—from bacteria to higher mammals. Furthermore, (*R*)-3-hydroxybutyric acid is a natural blood component at concentrations between 0.3 and 1.3 mM. This means that PHAs are excellent biocompatible materials and can, therefore, be successfully used for the construction of cell scaffolds, biodegradable sutures, wound dressings, and drug delivery systems (Zinn et al., 2001; Chen and Wu, 2005). *In vitro* and *in vivo* studies have shown that among the different PHAs, P(3HB-co-3HHx) retains the chondrocyte phenotype, due to the support of chondrocyte-specific extracellular matrix (ECM), type II collagen, and the promotion of sulfated glycosaminoglycan (sGAG) production (Deng et al., 2003). The unquestionable advantage of a polymer such as P(3HB) is its piezoelectric properties, which are similar to those of bones (van der Walle et al., 2001). Therefore, PHAs have been tested for the use of bone loss engineering, and the results showed no chronic inflammation even after 1 year of use (Porter et al., 2013). It was also noted that PHA polymers are widely used in the construction of nerve tissue regeneration devices, dressing materials, cardiovascular patches, venous–arterial valves, orthopedic pins, adhesion barriers, tendon repair materials, bone marrow scaffolds, cardiovascular stents, and tissue engineering (Chen and Wu, 2005; Valappil et al., 2006; Hazer, 2010; Shrivastav et al., 2013). Due to the wide interest of the biomedical industry in PHA polymers, the chemocompatibility of these polymers was also evaluated by incubating P(3HB) and P(3HB-co-3HV) films with mammalian blood during which it was shown that P(3HB) and P(3HB-co-3HV) in contact with blood did not cause negative reactions (Shrivastav et al., 2013).

The first material made of PHA to receive a positive opinion from the US Food and Drug Administration (FDA) was TephaFLEX®, which is an absorbable surgical floss made of P(4HB). There are other commercially available products made of PHA, i.e., GalaFLEX®, which is made of P(4HB) and is designed for reconstructive surgery, plastic surgery, and soft tissue reinforcement, as well as MonoMax Suture for soft tissue regeneration, BioFiber for tendon repair, and Phasix Mesh for hernia regeneration (Williams et al., 2016). Due to their biocompatibility and the hydrophobicity of PHA, they can be used as drug delivery systems in the form of microcapsules, microspheres, and nanoparticles (Shrivastav et al., 2013). In the course of studies on the release of antibiotics such as gentamicin and sulperazone from sticks constructed with P(3HB-co-3HV), continuous release of these drugs over 2 weeks was observed, and it was proved that the content of longer PHA monomers is conducive to prolonging drug release time (Gursel et al., 2002). It has also been shown that lower crystallinity of PHA results in more controlled release of drugs to surrounding tissues. An example was a mixture of P(3HHx-co-3HO) with tamulosin, which was characterized by better penetration of the active substance into the skin, compared to a mixture of the drug with a short-chain, highly crystalline P(3HB) (Wang et al., 2003b). For further details, the reader is encouraged to see the following reviews: Chen et al. (2017), Lizarraga et al. (2018), Elmowafy et al. (2019), and Grigore et al. (2019).

Processing and Modifications of Polyhydroxyalkanoate Polymers

Years 2003–2019

A natural way to add desirable functionalities to PHA polymers is through monomer alterations. More than 150 monomers have been identified (Steinbüchel et al., 1995) that offer a broad range of physicochemical properties (Dinjaski and Prieto, 2015). Among these, aliphatic monomers are in the spotlight. However, these monomers offer only certain properties to scl-PHAs and mcl-PHAs. The other most researched group of PHA monomers is that of aromatic chemistry. We have identified this subtrend in our *in silico* analysis (2003–2009), where most of the publications regarding aromatic monomers were produced. However, the first report that disclosed a biosynthesized PHA bearing an aromatic group as a side chain was presented by Fritzsche et al. (1990) in 1990. In later reports, one can find various aromatic constituents being introduced into PHA chains through biosynthesis achieved mainly with use of *Pseudomonas* strains (Ishii-Hyakutake et al., 2018), recombinant *E. coli* and *Ralstonia eutropha* (Mizuno et al., 2018; Yang et al., 2018). Aromatic PHAs show different mechanical characteristics depending on the type of aromatic monomers incorporated in their structure (Ishii-Hyakutake et al., 2018). In numerous literature reports, the aromatic PHAs have been studied widely. Properties such as degradability (Olivera et al., 2001), surface structure (Takagi et al., 2004), solubility (Mizuno et al., 2017), and thermal behavior (Antoun et al., 1991) have been assessed, and their dependency on the polymer's structure was identified (Ishii-Hyakutake et al., 2018).

Another way to improve the properties of PHAs is to create composites with other materials by physical blending with nanoparticles and nanofillers, produce PHA-based multiphase materials, and perform chemical modifications (**Table 2**). Modifying PHA is justified with concern regarding features such as their durability, shelf lifetime, replacement, and maintenance costs (Visakh and Roy, 2015). Majority of works on improving and modifying PHAs aims to increase their competitiveness concerning traditional plastics both in terms of physicochemical properties and production costs (Pandey et al., 2005). Incorporating nanoparticles into PHA polymer expands the potential application area thanks to the significant enhancement of materials properties. The most commonly used nanofillers are silylated kaolinite (Zhang et al., 2009), carbon nanotubes (Misra et al., 2010), bioactive glass (Misra et al., 2007), nanoclay (Bordes et al., 2009), cellulose nanocrystals (Yu et al., 2011), calcium phosphates (Cichoń et al., 2019), and modified hydroxides (Dagnon et al., 2009).

To give PHAs new and unique properties, several multiphase materials have been developed, mainly by mixing PHB or P(HB-co-HV) with other products such as plasticizers, fillers, or other polymers (Visakh and Roy, 2015). The structure of a PHA can be modified to obtain a polymer with desired properties for niche applications by chemical processes such as carboxylation, hydroxylation, epoxidation, chlorination, and grafting reaction (**Figure 5**) (Hazer and Steinbüchel, 2007; Bassas-Galià et al., 2015; Antwi Peprah et al., 2018).

TABLE 2 | Most common methods used for mechanical modification of polyhydroxyalkanoate (PHA) polymers.

Modification method	PHA polymer used	Expected result	Potential application	References
Hyaluronan coating	P(HB-co-HHx)	Hydrophilicity ↑	Biomaterial design	Wang et al., 2003a
Blending	P(HB-co-HHx)	Biocompatibility ↑	Biomaterial design	Zhao et al., 2002
Crosslinking (γ-irradiation)	mcl-PHA	Biodegradability ↓ Young Modulus ↑	Biomedical devices design	Ashby et al., 1998
PEGMA grafting (UV irradiation)	mcl-PHA (PHO)	Blood protein adsorption ↓ Biocompatibility ↑	Blood contacting devices	Kim et al., 2005
OH- ion implantation	PHB	Bioactivity ↑ Wettability ↑	Cell culture scaffolds	Hou et al., 2006
O_2 plasma treatment	P(HB-co-HV)	Hydrophilicity ↑ Surface roughness ↓ Cell proliferation ↑	Retinal pigment epithelium cell culture scaffolds	Tezcaner et al., 2003
Electrospinning	lcl-PHA, mcl-PHA	Elasticity ↑ Wettability ↑	Tissue engineering	Kwon et al., 2007

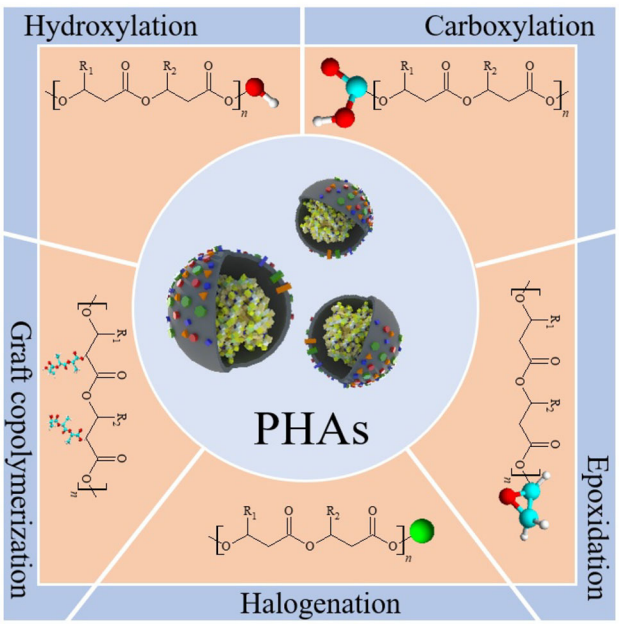

FIGURE 5 | Modification of a polyhydroxyalkanoate (PHA) structure through selected chemical reactions. Carboxylation by addition of carboxylic functional group to the polymeric macromer which can serve as functional binding sites for bioactive moieties. Hydroxylation resulting in shortened hydroxy-terminated PHA chains important in block copolymerization reactions. Epoxidation is useful in diverse reactions such as cross-linking, copolymerization, and bioactive moieties attachment thanks to the high reactivity of the epoxide group. Halogenation which relies on the addition of halogen atoms such as chlorine, bromine, or fluorine to the olefinic bonds of unsaturated PHA, being an excellent method in diversifying functions and applications of the polymer. Graft copolymerization results in the formation of a modified segmented copolymer. Chemical-, radiation-, or plasma discharge-induced reaction evokes improvement of selected polymer properties, i.e., increased wettability or mechanical strength.

A large group of PHA-based materials was obtained from hydroxylated PHAs and have been reviewed in numerous scientific reports (Cheng et al., 2013). An important factor applicable in PHA modification is high reactivity of epoxide group which can participate in numerous reactions like cross-linking (Visakh and Roy, 2015). PHA modification through epoxidation was reported in several cases, i.e., by Bear et al. (1997) and Park et al. (1999). In a different study, Lee et al. (1999) presented a cross-linking reaction of epoxidized PHA using hexamethylene diamine (HMDA). Another excellent method in diversifying the polymer functions and applications is its modification through halogenation (Arkin et al., 2000; Arkin and Hazer, 2002). In their study, Arkin and Hazer modified the PHA-Cl into ammonium salts, thiosulfate moieties, and phenyl derivatives (Arkin et al., 2000). A process of P(HB-co-HHx) direct fluorination was described by Samsuddin et al. (2013) PHA chemical modification by sulfanyl halogenation and its potential application in electrophotographic imaging were also reported and have been patented in 2008 (Mihara et al., 2006).

Enzymatic PHA modification is a mild, specific, and environment-friendly method widely discussed in the literature. The biopolymer can be modified on an enzymatic route by enzymatic degradation synthesis or by using the degradation products itself (Sato et al., 2008; Gumel et al., 2013; Kwiecień et al., 2015). Enzymatic catalyzed surface erosion of PHA can significantly enhance the surface roughness, thereby cell adhesion and proliferation. The raw PHA surface lacks a bioactive ligand to couple with molecules in targeting devices or biosensors (Visakh and Roy, 2015). Therefore, surface erosion and roughening are essential to immobilize bioactive molecules such as insulin, collagen, or fibronectin to expand the polymer's biomedical applications. The demand for customized biodegradable PHAs with unique features and the difficulty in their conventional production by biosynthesis have shifted the current interest

in polymer modification and functionalization *via* chemical, physical, and enzymatic processes (Visakh and Roy, 2015). By precise control over properties such as wettability, elasticity, biocompatibility, biodegradability, or Young's modulus value, one can get a set of smart materials with properties so diverse that they will not only be able to replace traditional polymers but can also contribute to developing new technologies and therapeutic techniques whose use was limited by the lack of material with desired functionality.

Renewable Production of Polyhydroxyalkanoate

Years 2005–2019

PHAs are a promising alternative to petroleum-based polymers; however, their production is still expensive and at industrial scale often competes with the food chain supply. Therefore, a trend emerged in the research of PHAs in 2005 that lasts up to date where research is focused on the search for sustainable production of PHAs from renewable resources. Two parallel platforms are constantly bettered, one that searches for natural wild-type strains capable of efficient conversion of substrates to PHAs and the other that seeks to modify microorganisms genetically. The process optimizations are achieved through not only genetic manipulations but also *via* different fermentation process developments. These include novel strategies for batch, fed-batch, and continuous fermentation optimization with the aid of mathematical modeling of single or mixed microbial cultures. Different strategies for limiting inorganic nutrients are also applied. However, the most crucial issue addressed by research is the application of sustainable carbon sources for the production of renewable PHAs. Therefore, many processes are developed to be integrated into biorefineries as they envisage the use of primary or secondary substrates of existing biotechnologies.

The primary carbon sources for the production of PHA can be various types of vegetable oils such as soybean, coconut, palm, rapeseed, or rubber, which are characterized by low production costs (Kahar et al., 2004; Lee et al., 2008; Kynadi and Suchithra, 2017). The fatty acids also are seen as primary resources used for PHA production—they are obtained from vegetable oils. They include, among others, palmitic, stearic, oleic, linoleic, as well as α-linolenic, caproic, caprylic, and myristic acids (Kynadi and Suchithra, 2017). Employing these substrates for PHA production, one can obtain polymers such as P(3HB-co-3HV), P(3HB-co-3HHx), P(3HB), or mcl-PHAs (Kahar et al., 2004; Ng et al., 2011).

The substrates of the second generation include food, industrial, forestry, and timber waste as well as municipal sewage. Various food waste and food by-products can be used as raw materials for the biotechnological production of PHAs. Mixed food waste is characterized by high complexity and variety of physical properties, particle size, and composition, so pretreatment of such raw material is a necessary step (Nikodinovic-Runic et al., 2013). The second stage of the process of PHA production from food bio-wastes is to obtain a sufficient amount of bacterial biomass capable of production and maximum accumulation of PHA inside the cells. One of the approaches is the use of pure bacterial cultures, their multiplication to an appropriate amount of active biomass on media with easily assimilated carbon source, and their acclimatization before the third stage of the process (PHA synthesis) (Cardozo et al., 2016; Schmidt et al., 2016). The last step usually takes place in a different reactor than the second stage. Still, with a strategy using pure bacterial culture, the approach of PHA synthesis in the same reactor as biomass multiplication is also used, where the polymer synthesis is driven by inorganic nutrient limitation (Rodriguez-Perez et al., 2018).

Wastes with more unified characteristics are also used in the production of PHA. These are, for example, industrial wastes mainly from food production and processing plants (sugar factories, distilleries, slaughterhouses, dairies and cold stores, and oil mills). Others are plant waste from crops, which are generated by agriculture and represent a considerable biomass resource. Agricultural residues include stems, leaves, and seed pods, while process waste also includes peelings, husks, seeds, marc, roots, bark, and sawdust. All these wastes are rich in cellulose, starch, and other carbohydrates. Examples of processes for PHA production based on bacterial monocultures from these substrates are collected in **Table 3**. The substrates either are fermented directly (glycerol, fatty acids) or require appropriate preprocessing (hydrolysate production). For details, please see the following reviews: Nikodinovic-Runic et al. (2013), Koller (2017), Koller et al. (2017), Kourmentza et al. (2017), Blunt et al. (2018), and Favaro et al. (2019).

In recent years, there was an increasing number of reports published that provide LCA for PHA-based products and process (Pietrini et al., 2007; Cristóbal et al., 2015; Morgan-Sagastume et al., 2016b; Nitkiewicz et al., 2020). Mainly these studies point toward the implementation of waste streams (resources) for the production of biopolymers. Further, they highlight also that the selection of materials and, eventually, the final product also plays a crucial role in both environmental and economic outcomes of the whole process. Furthermore, the LCA analyses conclude that PHA polymers can find their right place in a biorefinery context, where their production can be carried out in parallel to other biotechnologies, while sourcing substrates from coexisting blocks' waste streams. In this way, there would be not only complemented with zero-waste policies but also upcycling of the generated wastes within biorefineries to high-value products.

Polyhydroxyalkanoate Nanobeads

Years 2005–2014

Inspired by the creation of PHA granules *in vivo* by microorganisms, a trend emerged that encompassed technologies based on spherical nanobeads. First, reports reached for the science behind simple nano/microbeads creation where these vehicles were prepared by emulsification. Thanks to PHA being hydrophobic in nature, it was proposed to mix by physical blending bioactive compounds to produce nano/micro-preparations for antimicrobial and anticancer treatments. Moreover, technologies have been adapted from polymer chemistry in order to functionalize PHA polymers by graft and/or block polymerization with other polymers [e.g., with polyethylene glycol (PEG), poly(ethylene imine) (PEI), or Jeffamine] or covalently bind drugs to the polymeric chains

TABLE 3 | Examples of secondary raw materials and processes for polyhydroxyalkanoate (PHA) production.

PHA production strain	Resource	Resources pretreatment	References
Haloferax mediterranei	Whey	Acidic hydrolysis	Pais et al., 2015
Recombinant *E. coli* and *R. eutropha*	Rice bran	Acidic hydrolysis	Oh et al., 2015
Bacillus megaterium	Molasses	Enrichment of molasses with mono and disaccharides	Gouda et al., 2001
Burkholderia sacchari IPT 101	Bagasse	Filtration through activated carbon, acidic hydrolysis	Silva et al., 2004
Pseudomonas aeruginosa 42A2 NCIB40045	Used cooking oil	–	Fernandez et al., 2005
Cupriavidus necator IPT 029 or *Bacillus megaterium* IPT 429	Crude glycerol	–	Ribeiroa et al., 2016
Haloferax mediterranei	Starch	Hydrolysis	Chen et al., 2006
Burkholderia cepacia ATCC 17759	Wood hydrolysate	Hydrolysis	Pan et al., 2012
Haloferax mediterranei	Rice bran and starch hydrolysate	Enzymatic hydrolysis	Huang et al., 2006

and further create nano/micro-carriers. Recently, hollow or highly open porous microspheres based on PHA biocompatible polymers were constructed as cell carriers for targeted therapies. Tapping into molecular biology and material design, a platform was created that enabled the creation of functionalized nano/microbeads *in vitro* and *in vivo* by enzymatic apparatus of microorganisms. Several works were performed where a genetically modified PHA synthase was used to prepare functionalized PHA beads in a test tube from PHA monomers that either carried a bioactive drug inside a granule or the granule itself was decorated with engineered protein. Finally, a very sophisticated approach was proposed, where microorganism itself through genetic manipulations became able to synthetize a native PHA granule decorated with proteins of interest through their conjugation to granule-associated proteins (phasins or PHA synthetases), enabling the use of such PHA beads in the creation of vaccines, new methods in imaging, bioseparations, or protein purification. More details on the above technologies can be found in the following reviews: Li and Loh (2017), Michalak et al. (2017), Wei et al. (2018), and Gonzalez-Miro et al. (2019).

Poly-(*R*)-3-Hydroxybutyrate Production by Genetically Modified *Escherichia coli*

Years 2006–2019

PHAs are polymerized by synthases, which use various hydroxyacyl-CoAs as substrates. The PHB production from *E. coli* has been attracting attention, even though *E. coli* does not produce PHB naturally. However, its recombinant engineering allowed for the synthesis of this biopolymer. In publications making up this trend, various *E. coli* strain modifications are examined to improve and optimize both the fermentation process and the final product itself. In the majority of the studies, stress is put on the modification of bacteria for processing cheap and readily available carbon sources such as substrates deriving from biomass (Nikel et al., 2006; Park et al., 2012; Yang et al., 2014; Favaro et al., 2019).

The first reports of *E. coli* targeted recombination date back to 1999 when the first attempt of modification of metabolic network on PHB biosynthesis in recombinant *E. coli* was performed. The genes responsible for PHB biosynthesis in *R. eutropha* were cloned in *E. coli* and subsequently sequenced and characterized (Shi et al., 1999). An important source of carbon and nitrogen for bioplastic producing bacteria can be waste and agricultural by-products such as whey and steep corn liquor. It has been presented that PHB can be efficiently produced by the recombinant strain grown aerobically on a laboratory scale bioreactor on a medium supplemented with the agroindustrial by-products (Nikel et al., 2006). A recombinant *E. coli* strain containing the PHA biosynthetic genes from an *Azotobacter* species was specially prepared for producing PHB from milk whey (Nikel et al., 2006). Genetic engineering of *E. coli* can be used for PHA accumulation at low cost and high productivity. The biosynthesis of PHAs containing 2HB monomer from glucose by metabolically engineered *E. coli* strains has been reported (Park et al., 2012). In 2013, the whole process of PHB production from bacterial strain was modeled and simulated. A detailed analysis of each nutrient was performed using response surface methodology (Heshiki, 2013). Modification of metabolic pathways can be used not only to improve the production of known biopolymers but also to program microorganisms to perform a variety of functions such as producing new materials with unique properties (Rahman, 2014). *E. coli* was also metabolically engineered to synthesize copolymers such as [P(3HB-co-3HV)] from glucose (Yang et al., 2014). Most of the studies in this trend focus on the applications of modified *E. coli* bacteria to reduce the cost of PHA production and the possibility of using cheap carbon and nitrogen sources in the fermentation process (Mezzolla et al., 2017; Favaro et al., 2019).

Polyhydroxyalkanoate Composites—Organic and Nonorganic Blends

Years 2010–2019

High price, limited processing capabilities, and poor mechanical properties of PHAs restrain their use for everyday products such as food packaging, disposable cutlery, or device enclosures (Cunha et al., 2016). Additionally, tailored biopolymer

composites can play an important role in medical applications such as drug delivery systems, wound healing products, or surgical implant devices (Hufenus et al., 2012). Blending is a simple and effective approach for obtaining new polymeric materials with improved properties, and the drawbacks of the primary components can be eliminated. Typically, the mechanical properties of polymer blends can be easily tuned by varying the compositions of the blend and preparation conditions. Additionally, blends with biodegradable additives can enhance some of the PHA features, such as biodegradability and biocompatibility (Li et al., 2016). In studies included in this trend, several PHA blends were described.

PHA–PLA blends are the most common and the cheapest products for application replacing traditional, petroleum-based plastics (Long, 2009). Predominantly, reports described physical blending of scl-PHAs with PLA polymers which to some extent enhanced the single polymer properties. However, drawbacks such as high melting temperature and brittleness of these blends could be overcome by supplementing them with either mcl-PHAs or other bio-additives (Abdelwahab et al., 2012). This effect can also be achieved by blending Polycaprolactone (PCL) with scl-PHAs, which is an excellent option to improve the mechanical performance of both homopolymers (Visakh and Roy, 2015). Other biopolymers used to create blends with PHAs were cellulose (Zhang et al., 1997), starch (Visakh and Roy, 2015), and chitosan (Ikejima et al., 1999). These polymers have been shown to enhance the PHA properties by lowering the crystallinity of scl-PHAs and enhancing their biodegradability.

Creating blends with PHAs has gained popularity in current world trends. They serve both to reduce the cost of PHA production so that they can compete with petroleum-derived plastics and to improve their physical, chemical, or biological properties to expand the area of potential applications. Blending of PHA with other biodegradable polymers is particularly prevalent when it comes to the creation of novel materials suitable for specific applications.

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

The approach proposed in the study, which was based on the analysis of the full texts of articles, allowed to overcome the limitations of the traditional method based only on a systematic review of literature. The sample selected for analysis was divided into groups containing articles published in 5-years overlapping periods beginning in 1988 and ending in 2019. The analysis allowed to identify eight main areas in PHA research that governed the 31 years of discoveries. It should be noted that the indicated research groups do not exhaust all the conducted studies in the analyzed period. However, they present the research with the highest empowerment in identifiable scientific articles.

The most important outcome of the data-mining process was the identification of these research areas that are still trending in the scientific community and are of high probability to continue. Firstly, scientists try to understand genetics and biochemistry behind PHA synthesis. This leads to the identification of key enzymes responsible for PHA synthesis, thus to the creation of better production strains. Moreover, insight into PHA accumulation from a genetic perspective opens new routes for obtaining multipurpose bioactive granules. Secondly, it is visible from identified trends that a lot of work is being conducted on processing and modifications of PHA, leading to smart composites. These processes and products will lead directly to providing solutions to substitute petrochemical polymers and provide markets with a range of smart materials. Such an approach is also backed up by studies leading to the sustainable production of these biopolymers from renewable and cheap substrates, which directly can reduce their so far high production costs.

It needs to be emphasized that the indicated trends are to serve as a supportive tool for seeking further research directions, which should allow scientists to revise their research areas, improve the research process, and avoid duplication in studies, thereby increasing the efficiency of scientific work. Furthermore, it is vital to remember the given definition of a "trend." Trends were discovered with restricting criterion of at least 10 papers published in each 5-years period. The criterion was adopted to allow the presentation of the most popular trends. That does not mean that other trends are less important. Moreover, those less popular trends may become leading ones in the future. As cited here, some works, both original or reviews, were published post the trend apogee. They presented either a summary of a specific research area or further narrowed and specialized research continuing from a particular trend. It is also not a foregone conclusion that some of the presented trends will reemerge or new ones will manifest from this broad research on microbial polyesters.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SW designed and performed the *in silico* study, grouped literature, arranged, and technically analyzed it. MG and TW searched and provided raw data. SW, MW, and MS wrote methodology and part of introduction and edited the manuscript. MG, TW, and AS performed thematic description of trends, wrote part of introduction, the result, and discussion sections. MW provided funding for *in silico* analysis part. MG gave idea for the study and provided funding.

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Biosynthesis of Random-Homo Block Copolymer Poly[Glycolate-*ran*-3-Hydroxybutyrate (3HB)]-*b*-Poly(3HB) Using Sequence-Regulating Chimeric Polyhydroxyalkanoate Synthase in *Escherichia coli*

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Glycolate (GL)-containing polyhydroxyalkanoate (PHA) was synthesized in *Escherichia coli* expressing the engineered chimeric PHA synthase $PhaC_{AR}$ and coenzyme A transferase. The cells produced poly[GL-*co*-3-hydroxybutyrate (3HB)] with the supplementation of GL and 3HB, thus demonstrating that $PhaC_{AR}$ is the first known class I PHA synthase that is capable of incorporating GL units. The triad sequence analysis using ^1H nuclear magnetic resonance indicated that the obtained polymer was composed of two distinct regions, a P(GL-*ran*-3HB) random segment and P(3HB) homopolymer segment. The random segment was estimated to contain a 71 mol% GL molar ratio, which was much greater than the value (15 mol%) previously achieved by using $PhaC1_{Ps}$STQK. Differential scanning calorimetry analysis of the polymer films supported the presence of random copolymer and homopolymer phases. The solvent fractionation of the polymer indicated the presence of a covalent linkage between these segments. Therefore, it was concluded that $PhaC_{AR}$ synthesized a novel random-homo block copolymer, P(GL-*ran*-3HB)-*b*-P(3HB).

Keywords: sequence regulation, block copolymer, engineered PHA synthase, polyglycolic acid, chimeric enzyme

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are bacterial storage polyesters, which are currently used as a biobased replacement for some petroleum-derived plastics (Surendran et al., 2020; Zheng et al., 2020). PHAs have attracted considerable interest in recent years for their potential as a biodegradable material (Morohoshi et al., 2018, 2020), since the polymers degrade well in the

Abbreviations: GL, glycolate; 3HB, 3-hydroxybutyrate; LA, lactate; 2HB, 2-hydroxybutyrate; PCT, propionyl-CoA transferase.

environment by the action of PHA depolymerases (Juengert et al., 2018). PHAs possess diverse structures due to their variety in monomer constituents and their copolymerization. The primary structure of PHAs critically influences the physical properties of the material. For example, a random copolymerization of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx) units with controlled composition is effective for regulating the crystallinity of the polymer so that the P(3HB-*co*-3HHx) copolymer possesses a flexible mechanical property that can be adjusted for various uses, such as a mono-material (Wong et al., 2012), composite (Rebia et al., 2019), or blend (Ivorra-Martinez et al., 2020). Therefore, engineering the PHA structure is important for expanding its range of applications.

The glycolate (GL) unit is an unusual component of PHA, because it does not exist in naturally occurring PHAs (Matsumoto et al., 2011). As a typical example, P(GL-*co*-3HB) is a semitransparent material with pliable property (Matsumoto et al., 2017). In addition, non-enzymatic hydrolytic degradability is an important feature of P(GL-*co*-3HB). Chemically synthesized polyglycolide acid and poly(lactide-*co*-glycolide) are known as highly hydrolytic degradable materials, and they are used in biomedical fields as bioabsorbable materials (Shawe et al., 2006; Pervaiz et al., 2019). Although the bioabsorption of natural PHAs is very slow, natural PHAs have also been studied extensively for their applications in tissue engineering because of their biocompatibility (Basnett et al., 2018; Chen and Zhang, 2018). Artificial PHA P(GL-*co*-3HB) exhibits an intermediate hydrolytic degradability between P(3HB) and poly(lactide-*co*-glycolide), which potentially manipulates the bioabsorption rate of PHAs (Matsumoto et al., 2017).

PHA synthases are the key enzymes that determine the monomeric unit composition of a polymer (Tan et al., 2020). The engineered PHA synthase PhaC1$_{Ps}$STQK, which belongs to class II PHA synthases and has two point-mutations, possesses extremely broad substrate specificity and plays a central role in the biosynthesis of lactate-based and other 2-hydroxyalkanoate-based PHAs (Taguchi et al., 2008; Taguchi and Matsumoto, 2020). The finding of the glycolyl (GL)-CoA-polymerizing activity of PhaC1$_{Ps}$STQK was the first discovery of the biosynthesis of a GL-based PHA (Matsumoto et al., 2011). Nevertheless, only PhaC1$_{Ps}$STQK and a homologous enzyme with the same point mutations are known to produce GL-based PHAs to date. Therefore, new GL-CoA-polymerizing PHA synthase(s) are desired to expand the variety of GL-based PHAs.

The present study aims to examine the GL-incorporating capacity of an engineered PHA synthase, PhaC$_{AR}$. PhaC$_{AR}$ is a chimeric PHA synthase composed of the N-terminal region of PhaC$_{Ac}$ derived from *Aeromonas caviae* and the C-terminal region of PhaC$_{Re}$ derived from *Ralstonia eutropha* (*Cupriavidus necator*) (Matsumoto et al., 2009). The junction site of the chimeric enzymes is chosen in their highly conserved and putative random coil regions of each protein and they are fused without inserting a linker region. PhaC$_{AR}$ is the first known class I PHA synthase that can efficiently incorporate 2-hydroxybutyrate (2HB) units (Matsumoto et al., 2018a), whereas PhaC$_{Re}$ exhibits very little activity toward 2HB-CoA (Han et al., 2011). Moreover, PhaC$_{AR}$ possesses a unique function for synthesizing block copolymers (Matsumoto et al., 2018a). *Escherichia coli* expressing PhaC$_{AR}$ and propionyl-CoA transferase (PCT) spontaneously produced P(2HB-*b*-3HB) from the mixture of 2HB and 3HB precursors supplemented in the medium. P(2HB-*b*-3HB) is the first structure-proven block PHA. Therefore, the monomer sequence of the obtained polymer is of interest in the attempt to incorporate GL units using PhaC$_{AR}$. Indeed, we found PhaC$_{AR}$ is the first known class I GL-CoA-polymerizing PHA synthase and that the obtained polymer possesses a unique block sequence consisting of a random segment and a homopolymer segment.

MATERIALS AND METHODS

Plasmids and Culture Conditions

pBSP$_{Re}$phaC$_{AR}$pct harboring the *phaC*$_{AR}$ and PCT genes from *Megasphaera elsdenii* under the control of the *R. eutropha phb* operon promoter and pBSP$_{Re}$phaC1$_{Ps}$STQKpct harboring the *phaC1*$_{Ps}$*STQK* instead of the *phaC*$_{AR}$ were constructed in a previous study (Matsumoto et al., 2018a). pBSP$_{Re}$phaC$_{AR}$pctAB, which is a pBSP$_{Re}$phaC$_{AR}$pct derivative harboring the *phaA* and *phaB* genes from *R. eutropha*, was constructed by inserting the *Sac*I fragment of pGEM'-phbCAB (Matsusaki et al., 2000), which contains the *phaAB* genes, into the *Sac*I site of pBSP$_{Re}$phaC$_{AR}$pct (**Supplementary Scheme S1**). *E. coli* JM109 strains harboring these plasmids were cultivated on a 100 mL Luria–Bertani (LB) medium containing 2 wt% glucose, 100 mg/L ampicillin, 5 g/L sodium (*R,S*)-3HB and varied concentrations of sodium GL in a 500 mL shake flask at 30°C for 24 h with reciprocal shaking at 120 rpm.

Capillary Electrophoresis

The concentration of monomer precursors in the medium was measured using Agilent 7100 CE, a capillary electrophoresis (CE) system, equipped with a capillary tube (HPCE standard cap 50 μm id, 72 cm). The sample was injected for 4 s under a pressure of 50 mbar. The electrophoresis was performed at 25°C and -15 kV, with α-AFQ108 used as the running buffer (Otsuka Electronics Co., Ltd., Osaka, Japan). The sample was detected by a diode array at 400 nm.

Gas Chromatography

The lyophilized cells (approximately 10 mg) were treated in a solution of 0.5 mL chloroform and 0.5 mL 15% sulfuric acid in ethanol at 100°C for 2 h. The obtained ethyl esters were analyzed by gas chromatography (GC) as described previously (Taguchi et al., 2008).

Analytical Methods of Polymers

The polymer was extracted from lyophilized cells with chloroform at 60°C for 48 h in a test tube with a screw cap. The cell debris was removed through a 0.2-μm-pore-size polytetrafluoroethylene (PTFE) membrane filter. The chloroform extract was concentrated under air flow in a fume hood. The excess amount (approximately 10 times) of methanol was added to the extract at room temperature to precipitate the polymer. This purified polymer was applied in further analyses.

A differential scanning calorimetry (DSC) sample was prepared on an aluminum pan. A polymer chloroform solution containing approximately 3 mg polymer was applied to the pan and air dried for 2 h, then subsequently *in vacuo* for 24 h at room temperature. The DSC data were recorded on a DSC3+ differential scanning calorimeter (Mettler Toledo). The samples were heated from -50 to 210°C at 20°C/min (the first heating scan). After rapid quenching at -50°C and isothermal incubation for 5 min, the samples were heated to 210°C at 20°C/min for a second heating scan.

Nuclear magnetic resonance (NMR) analysis of the polymers was performed using approximately 5 mg/mL of a polymer solution in $CDCl_3$ containing tetramethylsilane, which was dissolved at 60°C and passed through a 0.2-μm-pore-size PTFE membrane filter. The NMR data were recorded on JEOL ECX-400 and ECS-400 spectrometers (JEOL, Japan). The same solution was analyzed using size-exclusion chromatography equipped with two tandem high-performance liquid chromatography columns of Shodex GPC K-806L (Shodex, Japan). The flow rate was 0.8 mL/min; the column oven was kept at 40°C; and the sample volume was 100 μL.

Solvent Fractionation

The purified polymer samples were fractionated into two fractions via solubility to distinguish a polymer blend and block copolymer (Matsumoto et al., 2018a). The 10 mg polymer samples were dissolved in 4 mL chloroform at 60°C for 15 min. After the solution was cooled to room temperature, 0.5 mL methanol was added to the solution and incubated at room temperature for 1 h. This step was repeated until precipitation was visually observed. As the results, total 5.0 and 4.5 mL methanol was added to the P(GL-*co*-3HB) and P(3HB) solutions, respectively. Then, the suspension was filtrated through a 0.1-μm-pore-size PTFE membrane filter. The precipitated fraction refers to the polymer on the filter that was recovered in chloroform, whereas the soluble fraction was the polymer solution that dried up after passing through the filter.

RESULTS

Production of the GL-Containing Polymer in *E. coli* Harboring the *phaC$_{AR}$* Gene

The attempts to synthesize P(GL-*co*-3HB) in *E. coli* JM109 tested the GL-incorporating capacity of PhaC$_{AR}$. Two 3HB-CoA supplying pathways were used: the CoA transferring pathway catalyzed by PCT from the 3HB supplemented in the medium (pBSP$_{Re}$phaC$_{AR}$pct) and the well-known dimerization pathway of acetyl-CoA catalyzed by β-ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaB) (pBSP$_{Re}$phaC$_{AR}$pctAB). First, the cells harboring the pBSP$_{Re}$phaC$_{AR}$pct were grown on the GL and 3HB. The *phaC1$_{Ps}$STQK* gene served as a control. As a result, a polymer containing GL units was obtained in both constructs (**Table 1**), which indicates that PhaC$_{AR}$ possesses GL-CoA-polymerizing activity. In addition, the GL molar ratio in the polymer synthesized by PhaC$_{AR}$ was higher than that of PhaC1$_{Ps}$STQK. Therefore, PhaC$_{AR}$ has greater GL-incorporating capacity than that of PhaC1$_{Ps}$STQK. The slightly decreasing trend of the cell dry weight (CDW) with an increase in the GL concentration was due to the toxicity of the GL in the medium and a decrease in polymer production. A GL concentration of 4 g/L was used in further study, because a greater increase in the GL concentration (12 g/L) exhibited no significant effect on the GL molar ratio. *E. coli* did not grow at 20 g/L GL concentration (data not shown).

The cells harboring pBSP$_{Re}$phaC$_{AR}$pctAB produced P(8 mol% GL-*co*-3HB) from the glucose and GL (**Supplementary Table S1**), which indicates that the supplementation of 3HB in the medium can be replaced by PhaAB. In further investigations of the present study, however, 3HB supplementation was used because of its higher GL molar ratio in the polymer, which facilitated the structural analysis of the polymers.

The Time-Course of GL-Based Polymer Production Using PhaC$_{AR}$

The time-course of GL-based polymer synthesis using PhaC$_{AR}$ was monitored. The GL molar ratio was relatively low at 12 h and exhibited an increasing trend corresponding with the cultivation time (**Figure 1A**). This indicates that a P(3HB)-like polymer was synthesized during the first 12 h. The concentrations of the precursors in the medium did not significantly decrease during cultivation (**Figure 1B**). Thus, the change in the monomer composition was not due to the precursor concentrations in the medium. PhaC1$_{Ps}$STQK exhibited a similar trend during the time-course of polymer production (**Supplementary Figure S1**).

To eliminate the time-dependent change of the monomer composition, 3HB was added at 12 h (**Figure 1C**). Under these conditions, no polymer was produced during the initial 12 h. Consequently, the monomer composition stayed nearly constant throughout the cultivation process (**Figure 1C**), 41 mol% GL at 18–24 h). The GL molar ratio in the polymer considerably increased compared to that under the conditions shown in **Table 1**. The precursor consumptions in the medium were almost negligible (**Figure 1D**). Thus, the precursor concentrations did not influence the monomer composition. The obtained polymer was used in further studies.

Under the condition of **Figure 1C**, no polymer was detected at 12 h in the chloroform extract, which could be due to the insolubility of the polyglycolate homopolymer in chloroform. Therefore, the whole cell at 12 h was subjected to GC analysis. As a result, 0.05 ± 0.01 g/L GL was detected in the 2.5 ± 0.0 g/L CDW, which suggests that a small amount of polyglycolate was synthesized, although the product was not contained in the extracted polymer samples. Further analysis is needed to characterize the product.

Differences in the ^{1}H NMR of the Copolymer Synthesized by PhaC$_{AR}$ and PhaC1$_{Ps}$STQK

GL-based polymers synthesized by PhaC$_{AR}$ and PhaC1$_{Ps}$STQK were analyzed by ^{1}H NMR. The resonance at 5.2–5.4 ppm

TABLE 1 | Glycolate-based polymer synthesis in *E. coli* JM109 expressing different PHA synthases, PhaC1$_{Ps}$STQK and PhaC$_{AR}$[a].

PHA synthase	GL-Na (g/L)	CDW (g/L)	Polymer production (g/L)	Monomer composition (mol%)	
				GL	3HB
PhaC1$_{Ps}$STQK	0	3.7 ± 0.14	0.41 ± 0.01	0 ± 0	100 ± 0
	4	3.3 ± 0.04	0.31 ± 0.07	17 ± 1.4	83 ± 1.4
	12	2.8 ± 0.06	0.36 ± 0.01	16 ± 0.7	84 ± 0.7
PhaC$_{AR}$	0	3.8 ± 0.07	0.56 ± 0.08	0 ± 0	100 ± 0
	4	3.6 ± 0.08	0.42 ± 0.12	20 ± 3.0	80 ± 3.0
	12	3.4 ± 0.12	0.45 ± 0.01	23 ± 0.5	77 ± 0.5

[a] *The data are the average ± standard deviations of three independent trials.*

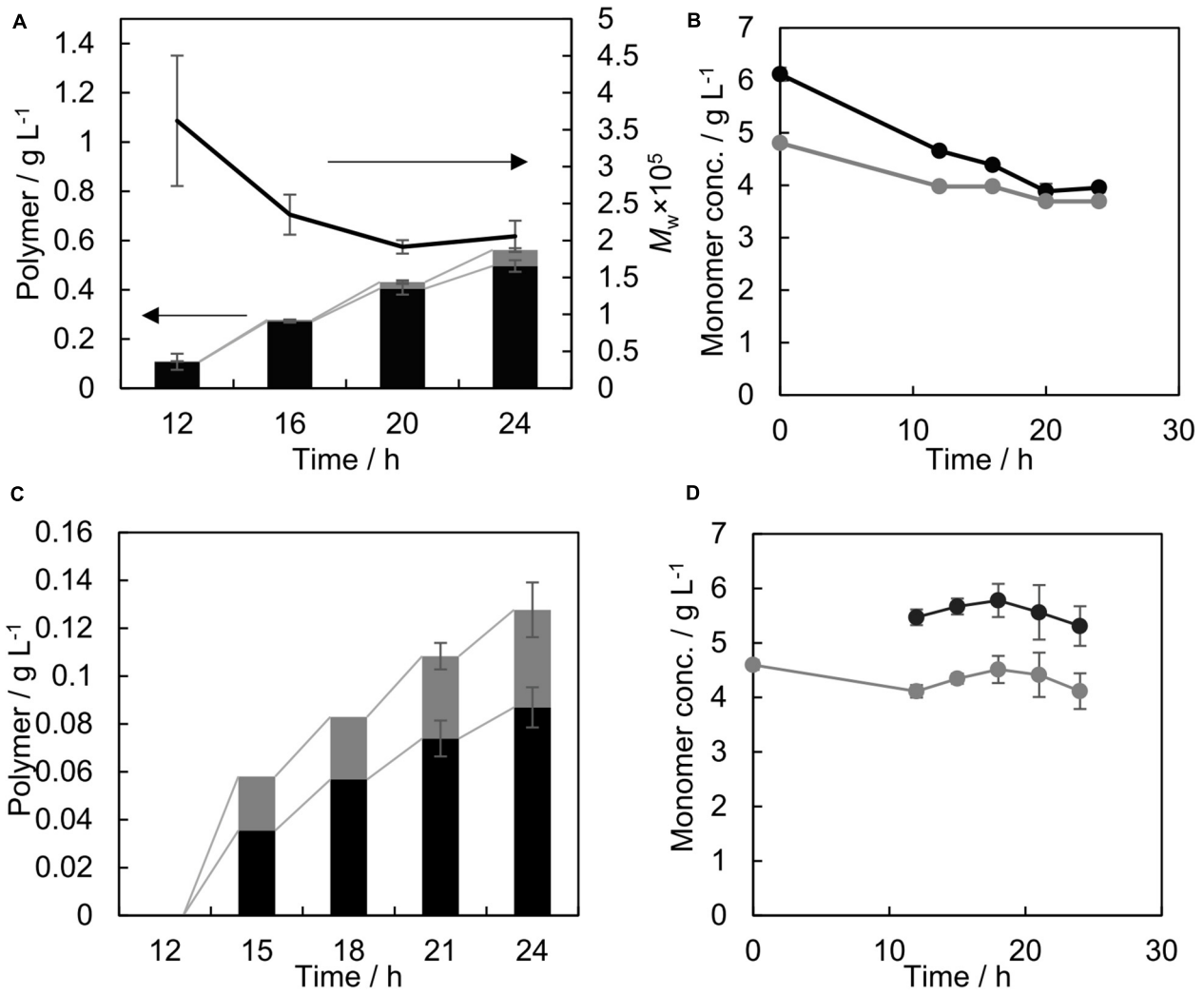

FIGURE 1 | Time course of GL-based polymer production by PhaC$_{AR}$ **(A,C)** and the concentration of monomer precursors in the medium **(B,D)**, with the precursor added at different times. Black and gray bars indicate 3HB and GL units in the polymer, respectively, and the line graph indicates the weight-average molecular weight of the polymer **(A)**. Black and gray circles indicate the concentrations of 3HB and GL in the medium, respectively **(B,D)**. Both precursors 3HB and GL were added at 0 h **(A,B)**; GL was added at 0 h and 3HB was added at 12 h **(C,D)**. A 12 h sample was taken immediately after the 3HB addition.

was ascribed to the methine proton of 3HB units. The low-field shift of the resonance was due to the presence of a GL-3HB* dyad in the polymer. The methylene proton of the GL units exhibited four characteristic resonances at 4.5–4.9 ppm. Based on the analogy to poly(lacatate-*co*-3HB) (Yamada et al., 2009), these four resonances were ascribed to GL-GL*-GL (a), GL-GL*-3HB or 3HB-GL*-GL [(b) or (c)], and 3HB-GL*-3HB (d), respectively (Matsumoto et al.,

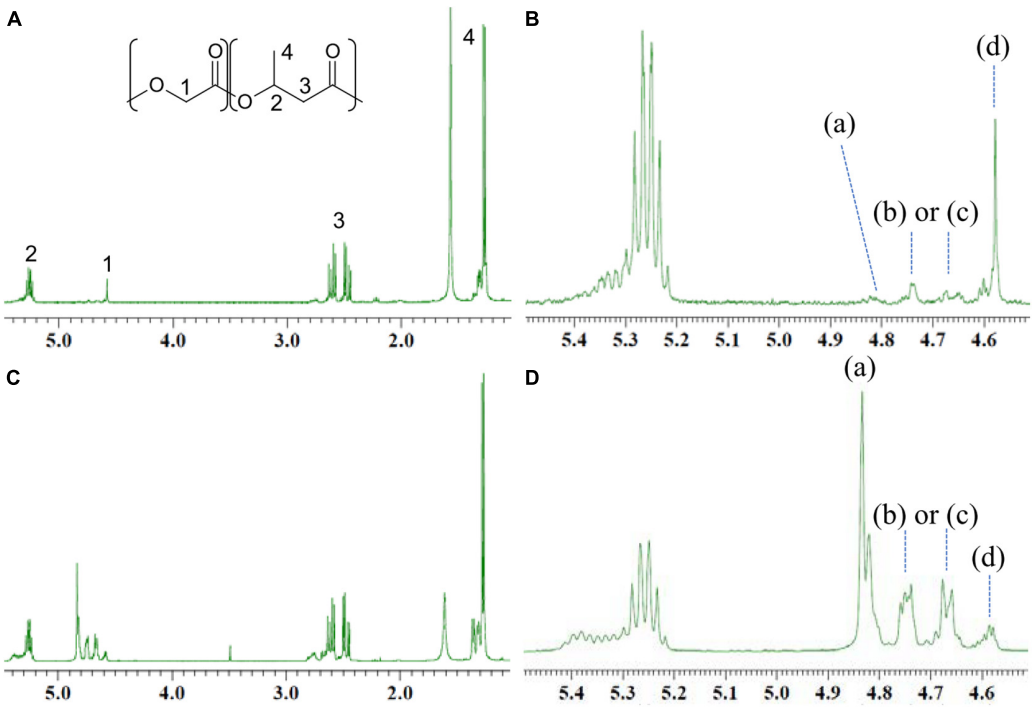

FIGURE 2 | ^{1}H NMR analysis of the GL-based copolymers containing 11 mol% GL synthesized by PhaC1$_{Ps}$STQK **(A,B)** and 40 mol% GL synthesized by PhaC$_{AR}$ **(C,D)**.

2017). Notably, the intensity of resonance (d) was highest among the signals in the polymer synthesized by PhaC1$_{Ps}$STQK (**Figure 2B**), which indicates that the 3HB-GL-3HB triad is abundant in the copolymer. In contrast, resonance (a) was strongest in the polymer synthesized by PhaC$_{AR}$ (**Figure 2D**), which indicates the abundance of the GL-GL-GL triad in the copolymer. A similar resonance pattern was observed when 3HB was supplied at 0 h (**Supplementary Figure S2**B under the conditions shown in **Figure 1A**). These results indicate that the copolymers synthesized by PhaC$_{AR}$ and PhaC1$_{Ps}$STQK differed in terms of the monomer sequence in the polymer chain. Furthermore, the copolymer synthesized by PhaC$_{AR}$ contains an irregularly heterogenous structure, which was revealed by the detailed analysis of the results as discussed below.

Sequence Heterogeneity Analysis of the Copolymers

The ^{1}H NMR pattern of the GL units can be used to determine the heterogeneity of the monomer sequence in a polymer chain. For example, the strong intensity of the GL-GL-GL triad indicates the presence of a GL-rich region in the polymer chain. The local GL molar ratio in the GL-rich region can be quantitatively estimated based on the relative intensity of the four triad signals (**Table 2**).

The value ***a*** is defined as the relative intensity of the GL-GL-GL triad over the total peak intensity ascribed to the GL units (**Figure 2**). The function *Area* is defined as the ^{1}H NMR peak area of the molecular species. The values ***b***, ***c***, and ***d*** are similarly defined based on the peak intensities of (b), (c), and (d) (**Table 2**).

$$\boldsymbol{a} = \frac{Area[(a)]}{Area[(a)+(b)+(c)+(d)]} (0 \le a \le 1)$$

Given an ideal random copolymer with a GL rate of ***x*** (mol/mol) over the total polymer, the abundance of the four triad sequences ***a***–***d*** is calculated by the following equations. Here, 1-***x*** indicates the 3HB rate (mol/mol) over the total polymer.

$$\boldsymbol{a} = x^2 \quad (1)$$

$$\boldsymbol{b}\ and\ \boldsymbol{c} = x(1-x) \quad (2)$$

$$\boldsymbol{d} = (1-x)^2 \quad (3)$$

Based on the ^{1}H NMR of P(40 mol% GL-*co*-3HB) synthesized by PhaC$_{AR}$, the ***a***∼***d*** values were experimentally determined (**Table 2**, No. 1). Surprisingly, these values were nearly identical to those of an ideal random copolymer with 71 mol% GL (**Table 2**, No. 2, calculated using formulas (1)–(3) when ***x*** = 0.71). This means that the P(40 mol% GL-*co*-3HB) contains a considerably rich GL region, which is a nearly ideal random copolymer with a local GL molar ratio of 71 mol%. Thus, the GL-rich region is referred to as the P(GL-*ran*-3HB) segment. Consequently, it was rationally presumed that the copolymer contains a region(s) composed of mostly 3HB units, which is referred to as the P(3HB)

TABLE 2 | Monomer sequence analysis of P(GL-*co*-3HB)s synthesized by PhaC$_{AR}$ and PhaC1$_{Ps}$STQK based on the ^{1}H NMR intensity.

No.	Polymer	GL ratio (mol%)	3HB ratio (mol%)	Relative ^{1}H NMR intensity of GL units in the triad sequences			
				a	***b***	***c***	***d***
1	P(GL-*co*-3HB) synthesized by PhaC$_{AR}$ (determined)	40 ± 4	60 ± 4	0.51 ± 0.02	0.21 ± 0.01	0.21 ± 0.01	0.08 ± 0.009
2	Ideal random copolymer (calculated)	71	29	0.50	0.21	0.21	0.08
3	P(GL-*co*-3HB) synthesized by PhaC1$_{Ps}$STQK (determined)	11 ± 1	89 ± 1	0.07 ± 0.10	0.15 ± 0.01	0.12 ± 0.00	0.66 ± 0.014
4	Ideal random copolymer (calculated)	18	82	0.03	0.15	0.15	0.67

The determined data are the average ± standard deviations of three independent trials.

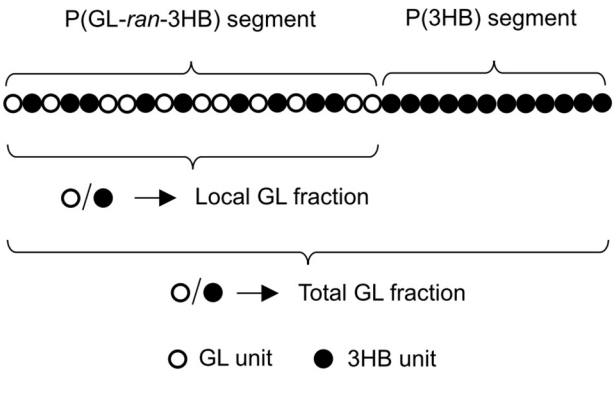

FIGURE 3 | The proposed random-homo block monomer sequence of P(GL-*co*-3HB) synthesized by PhaC$_{AR}$.

segment. The molar ratio of the P(3HB) segment over the total polymer was calculated by subtracting the amount of 3HB units in the P(GL-*ran*-3HB) segment from the total 3HB units as shown in the following equation. Here, function [*n*] indicates the molar ratio of *n*. $\boldsymbol{x}$ is the local GL rate (mol/mol) in the P(GL-*ran*-3HB) segment; and 1-$\boldsymbol{x}$ is the local 3HB rate (mol/mol) in the P(GL-*ran*-3HB) segment.

$$[total\ 3HB] - [total\ GL] \times \frac{1-x}{x} = [P(3HB)segment]$$

Using the values of 0.71 for $\boldsymbol{x}$, 0.6 for [total 3HB], and 0.4 for [total GL], the [P(3HB) segment] was estimated to be 0.44 (mol/mol). The high intensity of the resonance occurring at 5.2 ppm, which is ascribed to the methine proton of the 3HB units in the 3HB-3HB* dyad, is consistent with the interpretation. Overall, it was concluded that the copolymer synthesized by PhaC$_{AR}$ is composed of two segments, P(71 mol% GL-*ran*-3HB) and P(3HB), and the ratio of these segments is 56:44 (mol/mol) (**Figure 3**).

In contrast to PhaC$_{AR}$, PhaC1$_{Ps}$STQK synthesized the copolymer with a large ***d***, which indicates an abundant 3HB-GL-3HB triad in the polymer chain (**Table 2**, No. 3). The ***a–d*** values were close, but not equal, to the values that were calculated from an ideal random copolymer with 18 mol% GL ($\boldsymbol{x}$ = 0.18) (No. 4). The calculated value (18 mol%) was slightly greater than the actual GL molar ratio (11 mol%). These results indicate that the copolymer synthesized by PhaC1$_{Ps}$STQK is a nearly random copolymer, but the monomer sequence is not ideally random and contains a slightly GL-rich region.

Thermal Property Analysis of P(GL-*co*-3HB)s Synthesized by PhaC1$_{Ps}$STQK and PhaC$_{AR}$

The ^{1}H NMR analysis indicated that P(GL-*co*-3HB)s synthesized by PhaC1$_{Ps}$STQK and PhaC$_{AR}$ has distinct structures in terms of the monomer sequence. The interpretation was verified by the thermal properties of the polymers. The whole polymer without solvent fractionation was used for the analysis. P(11 mol% GL-*co*-3HB) synthesized by using PhaC1$_{Ps}$STQK exhibited a small melting peak at 126°C (**Figure 4A**), which was lower than that of P(3HB) (176°C) (Yamada et al., 2009), and no melting peak was detected in the second heating scan. The low and slow crystallization agreed with the randomly copolymerized structure indicated by the ^{1}H NMR analysis. The copolymer exhibited a glass transition temperature (T_g) at 7.6°C, which is consistent with the previous result (Matsumoto et al., 2017).

In contrast, the copolymer synthesized by using PhaC$_{AR}$ exhibited a larger melting peak at 151.4°C, and a melting peak was also detected in the 2nd heating scan (**Figure 4B**). The high and fast crystallization of the polymer should be due to the presence of a P(3HB) segment in the polymer. The degree of crystallinity of the polymer synthesized by PhaC$_{AR}$ (24%), which was estimated using the enthalpy of fusion of 100% P(3HB) crystal (146 J/g) (Barham et al., 1984), was 1.5-fold greater than the crystallinity of the polymer synthesized by PhaC1$_{Ps}$STQK (16%). The copolymer synthesized by PhaC$_{AR}$ exhibited a complicated T_g shift (**Figure 4B**), which indicates that the polymer possesses multiple T_gs. These results show a good agreement with the model in **Figure 3**. In fact, the crystalline property of the polymer synthesized by PhaC$_{AR}$ was observed as a film opacity, which was in contrast with the semitransparent P(GL-*co*-3HB) film synthesized by PhaC1$_{Ps}$STQK with similar GL molar ratio (**Figure 5**), and a low extensio to break (7%, **Supplementary Figure S3**).

Solvent Fractionation

The copolymer synthesized by using PhaC$_{AR}$ contained two distinguishable segments. To determine whether these segments

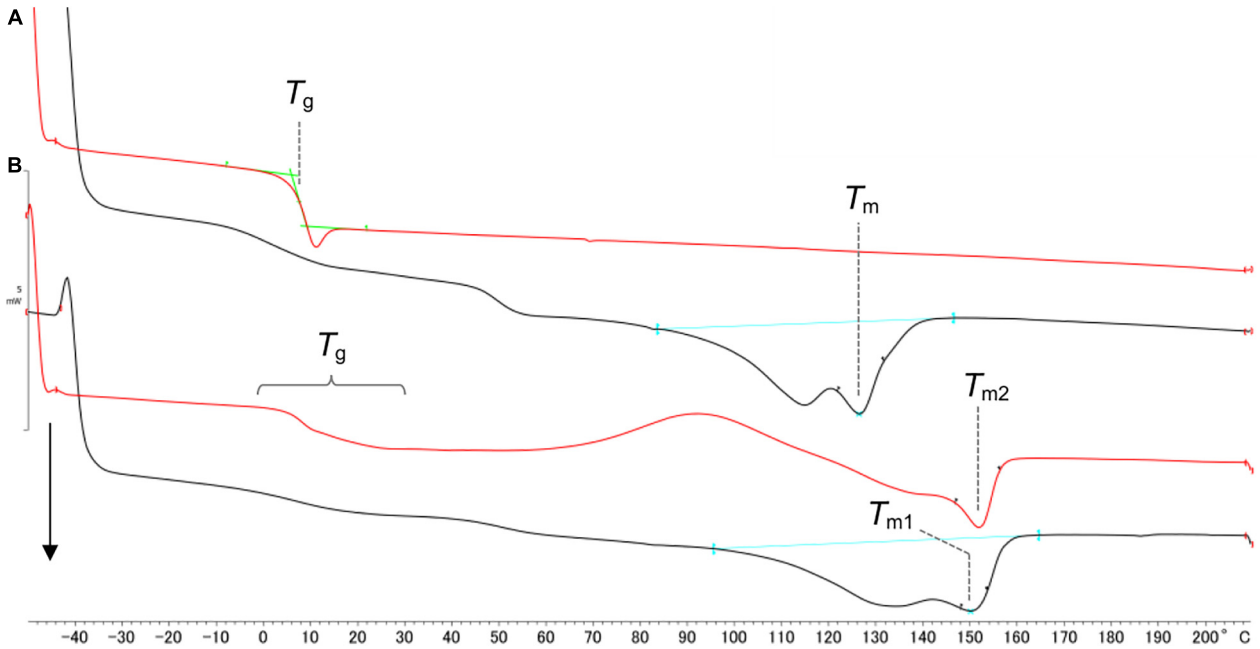

FIGURE 4 | DSC analysis of P(GL-*co*-3HB)s synthesized by PhaC1$_{PS}$STQK **(A)** and PhaC$_{AR}$ **(B)**. The black line indicates the 1st heating scan. The red line indicates the 2nd heating scan. The arrow indicates endothermic reactions. **(A)** T_m = 126.3°C, ΔH_m = 23.0 J/g and T_g = 7.6°C. **(B)** T_{m1} = 151.4°C, ΔH_m = 34.6 J/g (1st heating scan), and T_{m2} = 151.6°C (2nd heating scan). 3HB was supplemented at 12 h to produce a polymer by PhaC$_{AR}$.

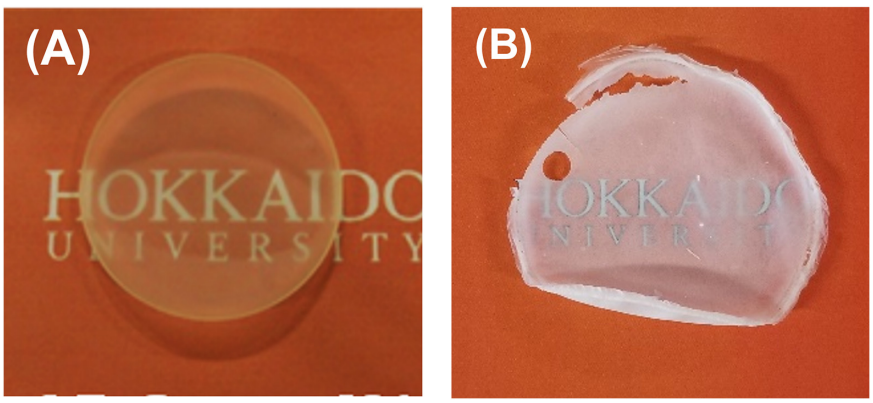

FIGURE 5 | Solvent-cast films of P(GL-*co*-3HB)s. P(16 mol% GL-*co*-3HB) synthesized by PhaC1$_{PS}$STQK **(A)**. P(14 mol% GL-*co*-3HB) synthesized by PhaC$_{AR}$ **(B)**.

could be a block copolymer or polymer blend, the copolymer was subjected to solvent fractionation. Because no sufficient difference in the solubility was found between P(GL-*ran*-3HB) and P(3HB), the polymer solution in chloroform was partially precipitated by adding methanol. This fractionation method is based on the principle that the solubility of polymers in organic solvents is dependent on their molecular weight. In fact, the insoluble fraction of the partially precipitated P(3HB) possesses a higher molecular weight than that of the soluble (non-precipitated) fraction (**Table 3**).

After the addition of methanol, P(GL-*co*-3HB) synthesized by PhaC$_{AR}$ was separated into two fractions by solvent fractionation: precipitated and soluble fractions. The precipitated fraction showed higher total and local GL molar ratios, which was determined based on the ^{1}H NMR (**Supplementary Figure S4**) using the same method as shown in **Table 2**, than those of the soluble fraction (**Table 3**), which indicates that GL units promoted the precipitation of the polymer. This result agrees with the fact that polyglycolic acid is not soluble in chloroform and in methanol. The precipitated and soluble fractions both contained the P(3HB) segment. Notably, the molecular weight of the precipitated fraction (M_w = 80,000) was lower than that of the soluble fraction (M_w = 210,000). The postulation that the sample is a blend of P(GL-*ran*-3HB) and P(3HB) leads to a contradicting interpretation that P(3HB) with relatively low M_w was readily precipitated. Therefore, it is likely that the P(3HB) segment in the

TABLE 3 | Solvent fractionation of P(GL-*co*-3HB) synthesized by PhaC$_{AR}$.

Sample		Recovered amount (mg)	M_w (×10^5)	M_w/M_n	Total GL ratio (mol%)	Local GL ratio (mol%)	P(3HB) segment (mol%)
P(3HB)	Before fractionation		1.8	2.3			
	Precipitated fraction	2	2.0	1.8			
	Soluble fraction	9	1.7	2.0			
P(GL-*co*-3HB) synthesized by PhaC$_{AR}$	Before fractionation		1.9	4.6	30	68	55
	Precipitated fraction	2	0.8	2.1	53	75	30
	Soluble fraction	8	2.1	5.0	24	64	63

*Ten milligrams of polymers were used for each condition. The GL molar ratio and P(3HB) segment were determined by ^{1}H NMR analysis (**Supplementary Figure S3**).*

precipitated fraction is covalently linked to the P(GL-*ran*-3HB) segment.

Considering the results of the solvent fractionation and thermal property analysis together, it was concluded that the copolymer synthesized by PhaC$_{AR}$ is a random-homo block copolymer, P(GL-*ran*-3HB)-*b*-P(3HB) (**Figure 3**). Currently, the number of segments per polymer chain, namely diblock, triblock, etc., is unknown.

DISCUSSION

The present study demonstrates that PhaC$_{AR}$ is the first known GL-incorporating class I PHA synthase. It was concluded that PhaC$_{AR}$ possesses a higher GL-incorporating capacity than that of PhaC1$_{Ps}$STQK, because PhaC$_{AR}$ synthesized the P(GL-*ran*-3HB) segment, which was estimated to contain 71 mol% GL, whereas PhaC1$_{Ps}$STQK can incorporate a much lower GL molar ratio (**Table 2**). The contrasting results of two PHA synthases suggest that the high local GL ratio is due to the enzymatic properties of PhaC$_{AR}$ because the promoter, monomer supplying enzyme and culture conditions are the same. The incorporation of GL units by PhaC$_{AR}$ had no considerable effect on the molecular weight of the polymer (**Table 3**) in contrast to the case of PhaC1$_{Ps}$STQK, in which the molecular weight decreases as GL ratio increases (Matsumoto et al., 2017).

Moreover, the obtained polymer was a unique block PHA, P(GL-*ran*-3HB)-*b*-P(3HB). PhaC$_{AR}$ was previously shown to synthesize P(2HB-*b*-3HB), which is composed of homopolymer segments. These results raise a question about why a random segment was generated by the same set of enzymes PhaC$_{AR}$ and PCT. One possible factor for the generation of a random segment is the copolymerization kinetics of PHA synthase. It was previously demonstrated that the *in vitro* activity of PhaC1$_{Ps}$STQK toward lactyl-CoA was lower than that toward 3HB-CoA under single substrate conditions. However, when two substrates (each 0.2 mM) were combined, lactyl-CoA was consumed faster than 3HB-CoA (Matsumoto et al., 2018b). A similar mechanism could take place in the GL-CoA and 3HB-CoA copolymerization by PhaC$_{AR}$. An *in vitro* analysis of PhaC$_{AR}$ will be needed in a future study. The T_g of the polymer product also influences polymer synthesis. Our recent study revealed that P(2HB) biosynthesis is efficient when the cultivation temperature is higher than the T_g of the polymer (Matsumoto and Kageyama, 2019). Given the fact that the random copolymer possesses a lower T_g than that of polyglycolate (35–40°C), P(GL-*ran*-3HB) chains are more readily synthesized than the homopolymer. In other words, cultivation temperature could be a parameter for regulating GL ratio in P(GL-*ran*-3HB) segment.

In general, characteristic and useful properties of block copolymers are attributable to the linkage between segments with contrasting properties, such as soft and hard segments (Lendlein et al., 1998). In fact, studies on chemically synthesized block copolymers typically select segments with distinct structures; for example, polyester and polysaccharide segments (Isono et al., 2018). PHAs were also chemically conjugated with PHAs (Dai et al., 2009) and/or other types of polymers, such as poly(ε-caprolactone), to improve the flexibility of the material (Saad et al., 1999). In contrast, wholly biosynthesized PHAs are all composed of hydroxyalkanoate units linked via ester bonds. Therefore, the selection of segments with distinct properties is a challenging requirement in the molecular design of block PHAs. In this light, the technique to synthesize a block PHA containing a random segment is useful to control the physical properties of each segment. However, P(GL-*ran*-3HB)-*b*-P(3HB) was processed into a stiff film as was P(3HB) (**Figure 5** and **Supplementary Figure S3**). The stiff property was presumably due to the high ratio of the P(3HB) segment. Therefore, the regulation of P(3HB) segment ratio is next target to improve the physical property of the material.

The promoter used in this study is derived from *phb* operon of *R. eutropha*, which expresses in *E. coli* without induction under the conditions used in this study. The use of inducible promoter might be useful for optimizing the polymer production. The expression levels of PHA biosynthetic genes reportedly influenced the production and molecular weight of P(3HB) (Hiroe et al., 2012). In our previous study, we utilized the *lac* promoter for production of P(lactate-*co*-3HB) in *E. coli*. Interestingly, the highest polymer production was achieved with relatively low expression conditions (Hori et al., 2020). Although the mechanism behind the phenomenon has not been elucidated, the results suggest that the expression level of PHA biosynthetic enzyme is not a rate-determining step of P(lactate-*co*-3HB) production. Further study will be needed for efficient production of the block PHAs.

CONCLUSION

PhaC$_{AR}$ was found to be the first known class I GL-CoA-polymerizing PHA synthase. PhaC$_{AR}$ synthesized the unique random-homo block PHA consisting of P(GL-*ran*-3HB)-*b*-P(3HB). The finding of the random segment-containing block PHA has the potential to expand the molecular design of polymers with a variety of properties. In addition, the GL-rich structure synthesized by PhaC$_{AR}$ is useful as hydrolytically degradable material.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SA performed the experiments and wrote the original draft. SS and RM performed the experiments. TO and MZ contributed to the scientific discussion and the manuscript writing. KM conceived and designed the study and wrote the manuscript. All authors revised the manuscript and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.612991/full#supplementary-material

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REVIEW
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Recovery of Polyhydroxyalkanoates From Single and Mixed Microbial Cultures: A Review

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An overview of the main polyhydroxyalkanoates (PHA) recovery methods is here reported, by considering the kind of PHA-producing bacteria (single bacterial strains or mixed microbial cultures) and the chemico-physical characteristics of the extracted polymer (molecular weight and polydispersity index). Several recovery approaches are presented and categorized in two main strategies: PHA recovery with solvents (halogenated solvents, alkanes, alcohols, esters, carbonates and ketones) and PHA recovery by cellular lysis (with oxidants, acid and alkaline compounds, surfactants and enzymes). Comparative evaluations based on the recovery, purity and molecular weight of the recovered polymers as well as on the potential sustainability of the different approaches are here presented.

Keywords: polyhydroxyalkanoates, extraction methods, green solvents, mixed microbial cultures, single microbial strains, surfactants, cell lysis

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INTRODUCTION

Polyhydroxyalkanoates are a family of bio-polyesters constituted by 3-hydroxy acid monomers (e.g., mainly 3-hydroxybutyric acid copolymerized with longer monomers as 3-hydroxyvaleric acid) and produced by bacterial fermentation as intracellular carbon and energy storage. Despite their potential in the scenario of fossil plastic replacement due to thermo-physical properties like petrochemically-derived plastics, PHAs are currently more costly than standard petrochemical plastics (1.18–6.12 €/kg *vs.* <1 €/kg, Saavedra del Oso et al., 2020) and cover niche-market high-value applications (Samorì et al., 2015b; Valentino et al., 2017). The combination of two factors determines the current PHA high production cost: upstream costs and downstream costs. A huge effort has been devoted at reducing the upstream costs by investigating both the suitability of cheap raw materials as feed for PHA-producing bacteria and the suitability of alternative microbial consortia for fermenting unusual feedstock; to this purpose different kinds of wastewater streams (food waste, sugar cane molasses, olive mill wastewater, waste activated sludge, paper mill wastewater, cheese whey, Mannina et al., 2019) have been used for feeding mixed microbial cultures (MMC) that proved to have wider metabolic potential than single strains and to be cheaper in terms of operative costs (Dias et al., 2006; Serafim et al., 2008a; Mannina et al., 2020).

On the other hand, downstream processes (PHA recovery and purification) are among the least investigated aspects of the whole PHA production chain but the most impacting ones in terms of economic weight (Jiang et al., 2015; Koller et al., 2017; Saavedra del Oso et al., 2020). High energy consumption is intrinsic in the overall PHA production life cycle, especially during the PHA downstream processing, and this aspect is

clearly highlighted by life cycle assessment (LCA) studies that compare PHA and fossil plastics, having the firsts a higher carbon footprint despite being biobased and biodegradable (Saavedra del Oso et al., 2020).

The use of (i) appropriate organic solvents to extract PHA granules from inside the bacterial cells, or (ii) additives/chemical agents to disrupt cellular matrix (dissolution of non-PHA cell mass, NPCM) and releasing intracellular PHA, are the two approaches of choice in the downstream phase, often coupled with pretreatments (e.g., with an oxidant, or thermally assisted) to enhance the permeability of cellular membranes. Both are characterized by peculiar advantages and undeniable drawbacks (Samorì et al., 2015b): PHA-friendly solvents, especially the chlorinated ones, provide high extraction yields and high polymer quality (high molecular weight and low impurities) but they are often hazardous for the environment and for the operators. Moreover, the solvent-based processes require relevant operational costs due to the high quantity of solvents that has to be used (up to 20-folds the PHA-rich biomass) and the high amount of energy employed for solvent evaporation and partial/total water removal from bacterial biomass to improve the contact between hydrophobic solvents and PHA granules stored inside the cells (Koller et al., 2013; Madkour et al., 2013). The additives for achieving cellular lysis (enzymes, surfactants, oxidants, alkali) can be directly applied to microbial cultures, by-passing the drying of the biomass, but they can affect the characteristics of the extracted polymer, leading to PHA degradation and reduction of molecular weight (Kosseva and Rusbandi, 2018; Mannina et al., 2020). Moreover, these agents are often non-recyclable and consequently, the remaining aqueous solutions are wasted and must be treated.

The search for more sustainable alternatives for PHA extraction that could decrease both the environmental and economic impact of the current approaches has been investigated in the last years: linear and cyclic carbonates (Fiorese et al., 2009; Samorì et al., 2015a,b), ethyl acetate (Riedel et al., 2013), methyl isobutyl ketone (Riedel et al., 2013), ionic liquids, and supercritical fluids (Hampson and Ashby, 1999) are some examples of solvents used in the first approach, whereas recyclable surfactants are among the alternatives developed within the second approach (Samorì et al., 2015b; Mannina et al., 2019). In this framework it has also emerged that PHA-accumulating bacteria do not behave in the same way toward organic solvents or chemical additives: MMCs, cheaper in terms of upstream than single strains, seem more resistant to cell hydrolysis probably because of strong and complex extracellular biomass matrix that contains the PHA accumulating cells (Patel et al., 2009; Samorì et al., 2015b). This aspect poses a further complication in making the PHA-production process economically competitive with respect to fossil-based plastics and other bioplastics already on the market.

In this review, an overview of the various downstream approaches applied to single strains and MMCs for recovering PHAs is presented. These methods have been divided into two main categories: recovery with solvents and recovery by cellular lysis. A comparative assessment among them was shown by considering the recovery, purity, molecular weight, and polydispersity index of the recovered polymer. The last three properties, in fact, are among the most important parameters for determining the applicability of each specific PHA in the various fields; if PHA purity is strictly correlated to human-related applications (e.g., biomedical ones), PHA molecular weight, and polydispersity index are detrimental for PHA processability. In fact, mechanical properties of polymers (e.g., the tensile strength) are affected by their molar mass; molecular weight values above 0.5 MDa and polydispersity index below 3 are usually considered acceptable thresholds for these thermoplastic polymers, being typical of quite homogeneous chain lengths that can be processed through injection molding techniques without any compromising reduction in the total polymer length (Fiorese et al., 2009). Some studies have reported for example that a molecular weight below 0.1 MDa causes severe deterioration of the mechanical properties for P(HB-HV) (Burniol-Figols et al., 2020).

It is worth mentioning that PHAs are currently used as a chemically-extracted bulk material. However, it has been demonstrated that the extraction process, independently by being a solvent-based or a cellular lysis approach, has a crucial role in determining the properties of the extracted PHA, both in terms of crystallinity and purity, altering the original morphology of PHA granules. In fact, storage PHB (high molecular weight polymer with $> 10^3$ 3-HB units) and related PHA are accumulated intracellularly in the form of granules whose surface is surrounded by a considerable number of proteins (about 1.9 wt%), much more than those essential for PHA synthesis (Jendrossek and Pfeiffer, 2014). These structural, biosynthetic, catabolic, and even regulatory proteins, embedded in lipid monolayer (Merrick and Doudoroff, 1964; Mayer and Hoppert, 1997), create a surface layer around the polymer core (Jendrossek and Pfeiffer, 2014), and this complex structure have suggested a wider function of PHB/PHA granules (thus named "carbonosomes," Jendrossek, 2009) a part being an energy and carbon storage. When PHA granules are exposed to (bio)chemical (e.g., extraction with alkaline compounds, solvents, enzymes) or physical processes (freezing, pelleting by centrifugation), they rapidly undergo denaturation processes (Merrick and Doudoroff, 1964) and become more crystalline (typical degree of crystallinity 50–60%, Jendrossek, 2007) than the native PHB granules (in which the polymer chains are in an amorphous state due to a certain amount of water that acts as a plasticizer and prevents crystallization, Grage et al., 2009); moreover, the extracted PHA granules seem to retain the proteinaceous surface layer typical of the native PHB granules (Kuchta et al., 2007), meaning that a 100% purity of the granules is hard to be achieved. To avoid denaturation during the isolation process, it has been claimed that native PHA granules must be recovered by using mechanic (e.g., French Press) or enzymatic cell lysis followed by density gradient centrifugation; in this way, the particular spherical structure of native PHA granules and their shell-core composition could be maintained and thus exploitable in a broad range of applications in biotechnology and medicine, from protein purification to drug delivery (Grage et al., 2009). Although this approach could drastically increase the applicability of PHA in unexplored fields due to the extraction of

such peculiar PHB-carbonosomes, this review will mainly focus on PHA as low-medium cost bulk material, potentially capable of playing a role in the future bioplastic scenario.

PHA EXTRACTION: PROCESS STEPS AND ISSUES

Methodologies to Determine the PHB/PHA Content Inside Microbial Cells, Recovery, Purity, Molecular Weight and Polydispersity Index

This review aims at comparing various extraction approaches by considering four different parameters: polymer recovery, purity, molecular weight, and polydispersity index of the recovered polymer. Therefore, their definitions and the most common methodologies to quantify all of them have been initially reported in this section.

PHA Recovery (%)

The recovery of PHA is directly correlated to PHA extraction yield, purity of the extracted PHA, and initial PHA content inside microbial cells as follows:

$$[\text{PHA recovery (\%)} = \frac{\text{PHA yield (wt\%)*PHA purity(\%)}}{\text{PHA amount in the microbial cells (wt\%)}}]$$

PHA Yield (wt%)

The yield of the extracted PHA is usually calculated gravimetrically on a microbial biomass weight basis (wt%). The weight of the recovered polymer can be achieved after: (i) evaporating the solvent used for the extraction until reaching a constant PHA weight, (ii) adding an anti-solvent to the solvent used for the extraction (e.g., EtOH added to chlorinated compounds), (iii) lysing the microbial cells. If an anti-solvent or a cell-lysis approach is used, the PHA granules are recovered by centrifugation, washed with the anti-solvent or H_2O, and then dried until reaching a constant weight.

PHA Purity (%)

The purity of the extracted PHA can be determined by various approaches that fall into two main categories:

(i) methods to quantify the impurities associated with the extracted polymer, typically oriented toward specific classes of bacterial contaminants like proteins, endotoxins, or lipids:

- Proteins (about 1.9 wt%) are strictly associated to PHA granules, forming the so-called "carbonosomes" structure (Jendrossek, 2009) that is recalcitrant independently by the recovery approach (solvents or lytic agents); this residual content is directly quantifiable through the Lowry method (Lowry et al., 1951) or indirectly estimable through elemental analysis from the N amount of the sample (assuming that proteins have a mean nitrogen content of about 6%, Sosulski and Imafidon, 1990). A possible strategy to reduce protein residual content is the application of proteases after the extraction process.
- Pyrogenic lipopolysaccharides (LPS) form part of the cell wall of Gram-negative bacteria and are released into the environment when the membrane of these bacteria is broken; LPS represent the so-called "endotoxins" and are associated to the extracted PHA independently by the recovery approach; however, it has been proved that solvents extraction (e.g., with chloroform) gives lower endotoxin level (three orders of magnitude) than cell-lysis (Lee et al., 1999). LPS residual content can be semi-quantitatively determined by *ad hoc* tests like the Limulus Amebocyte Lysate (LAL) test, whose principle is based on the gelation process that occurs from the coagulation of the proteins caused by the presence of endotoxins (Lee et al., 1999). Possible strategies to reduce LPS residual content are the application of alkaline or oxidizing post-treatments to the extracted PHA (however causing the hydrolysis of PHA itself), repeated polymer dissolution-precipitation cycles (however causing a massive solvents consumption), or filtration through charcoal (however causing a considerable loss of PHA, Wampfler et al., 2010a; Koller et al., 2013).
- Lipids are usually found associated with extracted PHA if solvent approaches are used; this occurs because of the "like dissolves like" rule of thumb, according to which non-polar solvents used to solubilize PHA are also capable of dissolve non-polar solutes like lipids. Lipidic residual content is directly quantifiable through various techniques like thin-layer chromatography (TLC), gas chromatography (GC), liquid chromatography (LC), enzyme-linked immunosorbent assays (ELISA), nuclear magnetic resonance (NMR), and mass spectrometry (MS) (Li et al., 2014). Due to the similar chemical nature of lipids and PHA, post-treatment purification is quite hard; however, it can be useful to pretreat (degrease) the microbial biomass with solvents like methanol, ethanol, acetone, or supercritical CO_2 ($scCO_2$) that are scarcely suitable for PHA but not for lipids, weakening at the same time the cell envelops (Koller et al., 2013).

(ii) methods to quantify the PHA material in the mass recovered through solvent extraction or cell-lysis like methanolysis coupled to chromatographic analysis or thermogravimetric (TGA) analysis (see below). The main issue of the methods that calculate the purity level from the PHA content in the recovered material is that, even with a minor relative standard deviation (RSD) of the replicas, the absolute error becomes intrinsically comparable to the distance between measured purity and 100%. Gas chromatographic analysis, for example, typically brings 1–2% RSD due to imprecisions in weighing or dilution, thus more than 4–6 replicas (of a perfectly homogenous material) are needed to reduce the 95% confidence range of the data to 1%. If the confidence range is 1%, 99% purity can be investigated.

Given that pretreatment or derivatization procedures usually add a significant variability to the methods (RSD can exceed 5%) the number of replicas required to prove higher purities (>99%) becomes unfeasible. TGA analysis is characterized by higher precision (RSD < 1%), therefore it can be easily used (with a few replicas) to assess purity levels until 99%. Nonetheless, given the limitations highlighted above, all known methods that quantify the PHA material in the mass recovered cannot be used for a reliable determination of purities higher than 99%. When the interest is in determining a minimal amount of impurities, which is the case of higher purity investigation, direct quantification of impurities is markedly more efficient.

PHA Amount in Microbial Cells (wt%)

Several methods have been suggested in the last 50 years to quantify the PHA amount inside microbial cells and their monomeric composition (e.g., chromatographic, turbidimetric, spectrophotometric approaches) (Williamson and Wilkinson, 1958; Ward and Dawes, 1973; Braunegg et al., 1978; Gorenflo et al., 1999). Among them, methanolysis coupled to chromatographic analysis is considered one of the most reliable and accurate methods (Braunegg et al., 1978). It basically consists of a transesterification reaction (depolymerization) in methanol (>3 h at 100°C) catalyzed by H_2SO_4 to give the methylesters of PHA monomers (e.g., methyl 3-hydroxybutyrate from PHB), that are extracted by chlorinated solvents (e.g., dichloromethane or chloroform) and then analyzed by gas chromatography. The quantification of the methylesters by using an internal standard or a calibration curve gives the PHA content on a bacterial biomass weight basis. Despite being the method of choice in the field of PHA quantification, this procedure is far from being considered "green" (according to the modern concept of Green Analytical Chemistry, Gałuszka et al., 2013) since it is based on a lengthy transesterification reaction that needs the use of a considerable amount of harsh and harmful reagents (e.g., H_2SO_4 and chloroform) for forming and isolating the analytes. The need for fast routine solvent-less methods to reduce sample pretreatment, speed up the analysis and decrease the overall costs has brought toward the birth of a new generation of quantitative analysis based on the exploitation of the thermal properties of PHA (Morikawa and Marchessault, 1981; Hahn and Chang, 1995; Aoyagia et al., 2002; Li et al., 2003). PHA is thermally unstable above 180°C and this behavior can be exploited to depolymerize PHA and give specific analytes (namely 2-alkenoic acids) that can be used as markers for quantifying intracellular PHA and their monomeric composition. Thermal treatments like thermogravimetric analysis (TGA, Hahn and Chang, 1995), pyrolysis (Morikawa and Marchessault, 1981; Torri et al., 2014), or low-temperature thermolysis (Abbondanzi et al., 2017) have been exploited to this purpose, representing a faster and equally reliable alternative to conventional methanolysis-chromatography approach.

Molecular Weight and Polydispersity Index

Number and average molecular weight, as well as polydispersity index (a proxy of the distribution of the molecular weight and, thus, the heterogeneity of the polymer), are usually determined in chloroform solution by a comparative technique like gel permeation chromatography (GPC) using a single concentration detector (typically refractive index, RI). Care should be taken since some PHA tend to form gels in chloroform (as in some other "super-solvents" like γ-valerolactone, Samorì et al., 2016), hence their concentration should be kept below the gel point.

General Process Steps

The main steps involved in the two strategies (recovery with solvents and recovery by cellular lysis), as well as the most relevant technical/environmental issues that should be particularly considered (in red) are highlighted in **Figures 1**, **2**.

Solvent extraction of PHA from dry or wet microbial biomass (e.g., microbial slurry in which the content of biomass could be around 10–20 wt%) typically includes the following steps:

(1) Putting in contact biomass and admixing it with solvent; this is a relatively low energy consumption operation.
(2) Heating up the mixture to the desired extraction temperature. Such a step involves sensible heat that can be reliably recovered (more than 80%) through counter-current heat exchangers. The order of magnitude of the energy consumption for reaching the extraction temperature could be calculated from the heat capacities of the solvent and microbial biomass. Since the extraction is typically performed at a temperature lower than the PHA decomposition temperature (<200°C), the heat capacity of solvents/biomass ranges between 1–2 kJ kg^{-1} K^{-1} and the heat capacity of water (eventually present if a microbial slurry with 10–20 wt% of biomass is subjected to the extraction) is equal to 4.2 kJ kg^{-1} K^{-1}. The required energy for this operation is usually low (0.5–2 MJ kg^{-1} PHA) and it is not considered a critical issue.
(3) Separating the extraction residues (non-PHA biomass and eventually water) from the PHA-enriched phase (usually the organic solvent). This step can be accomplished through filters or other solid/liquid separations (centrifuge, settling) which typically give a PHA-enriched liquid phase (PHA dissolved in the solvent) with a low amount of residual solvent (that can be recovered up to a certain level). This step is usually favored when water-insoluble organic solvents with low viscosity are used since they can be easily separated from the aqueous slurries.
(4) Separating PHA from the solvent by evaporation of a low boiling solvent or by PHA precipitation followed by solid/liquid separation. Evaporation of the solvent can be made by distillation (providing heat) or by low-pressure evaporation (providing electrical energy). PHA precipitation can be obtained by changing the physical properties of the solvent (e.g., temperature) or by admixing it with a counter-solvent that dissolves the solvent but not PHA. Solvent evaporation is the simplest and most widely applied solution, but it represents a costly step. Thermal energy consumption is directly proportional to the Latent Heat of Evaporation (LHE, ranging from 2.3 MJ kg^{-1} for water to 0.4–1 MJ kg^{-1} for most common organic

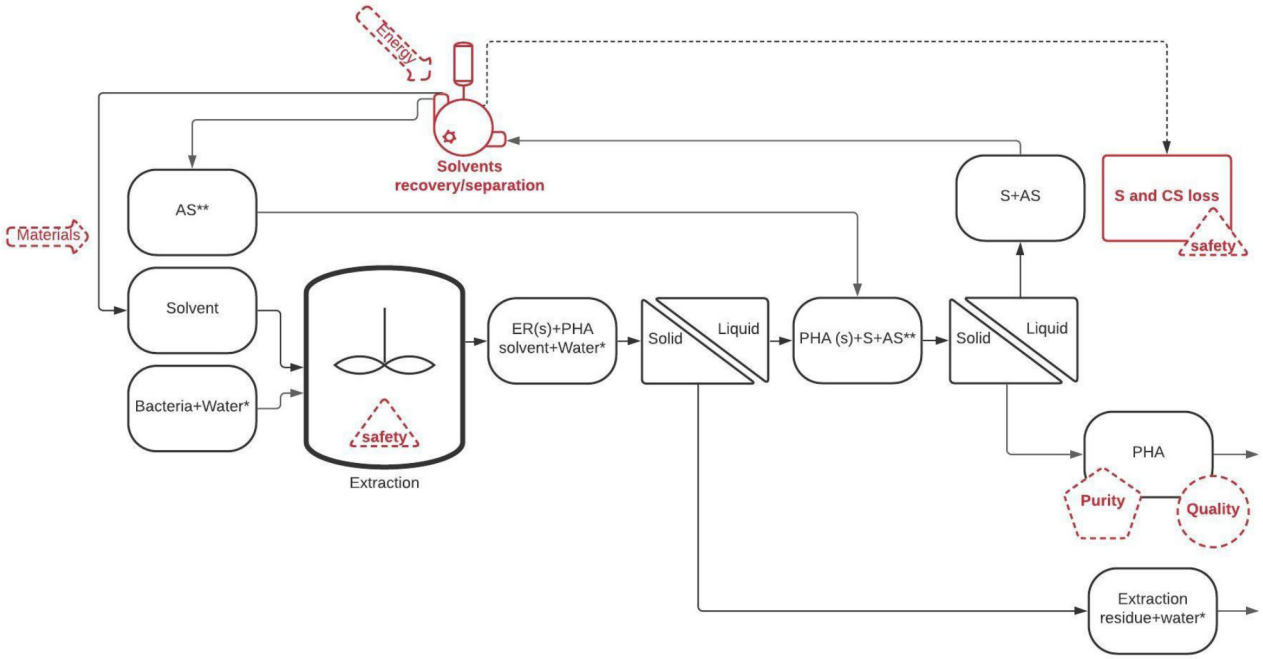

FIGURE 1 | General scheme representing the solvent-based approach for recovering PHA from PHA rich bacteria. AS, anti-solvent; S, solvent; PHA, polyhdroxyalkanoate; PHA (s), polyhydroxyalkanoate in suspended solid form; ER (s), extraction residue as suspended solids. *If the extraction is performed directly on wet microbial slurry. **If an anti-solvent is used.

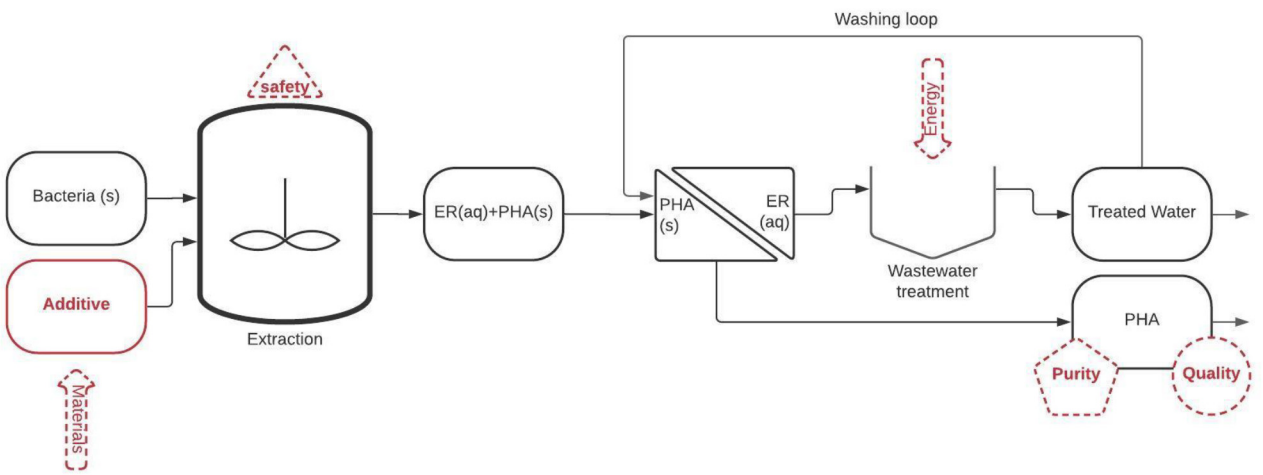

FIGURE 2 | General scheme representing the cellular lysis approach for recovering PHA form PHA rich bacteria. ER (aq), extraction residue dissolved in water; PHA, polyhydroxyalkanoate; PHA (s), polyhydroxyalkanoate in suspended solid form.

solvents and CO_2), but "quality" of such thermal energy and therefore costs are related to the boiling temperature. This means that the organic solvents with low boiling point and low LHE are the easiest solvents to be removed.

(5) Re-obtaining of the solvent with a quality (purity) comparable with that of fresh solvent used for the extraction. This means that the solvents (or mixtures of solvent and antisolvent) should be processed to recover the largest portion in relatively clean form. For that purpose, precipitation of PHA has the unique feature to provide a direct recovery of the solvent during the separation stage. Otherwise, in most approaches both solvent and additional antisolvent can be recovered by condensation of the solvent vapors eventually assisted by a membrane separation or pervaporation (e.g., for the separation of miscible solvents). As a general rule-of-thumb, recovery by simple condensation is technically easier for compounds with high boiling points. When more complex methods, as

membrane separation and pervaporation, are used to assist the solvent recovery, the efficiency is case-by-case related to the available membrane and affinity with solvents and potential interfering substances.

Due to the complexity of the process, four common/key critical issues can be identified in each solvent-based PHA extraction method:

(1) The efficiency related to solvent consumption: organic solvents cannot be 100% recovered because of leaks, losses (solvents remaining in PHA and extraction residues) and unavoidable safety needs. Even when a closed loop of solvent flow is used, a certain degree of system purging is required to avoid the formation of an explosive atmosphere.
(2) The efficiency related to the energy applied for solvent recovery: it is possible to significantly increase the solvent recovery by using higher energy and more complex equipment. The main drawback of this approach is the net energy consumption of the process as for the solvent evaporation under vacuum or the use of pervaporation through membranes.
(3) The solvent contaminations in the recovered PHA: whereas solvent extraction can be selective and could discriminate between PHA and other (polar) cellular constituents, the intrinsic affinity of the solvent for the polymer could hamper a complete solvent removal from the final product. Such aspect is quite relevant due to problems during the subsequent PHA processing (damage of injection molding equipment) and PHA utilization in the case of toxic solvents.
(4) Safety issues: all solvents but water is at least flammable and, in some cases, volatile. This means that they can accidentally form an explosive atmosphere, they can exert toxicity toward humans through respiratory systems or, being Volatile Organic Compounds (VOCs), they can cause air pollution.

The main steps involved in cellular lysis methods are presented in **Figure 2**.

(1) Mixing of PHA containing slurry and an additive (e.g., alkali, surfactants, or oxidants), eventually assisted by heating to improve the solubilization of non-PHA constituents. This operation produces slurry in which PHA becomes the main insoluble constituent.
(2) Separation of the slurry into a liquid solution and solid PHA usually recovered as a wet powder that must be dried and subsequently purified.
(3) Treating of the liquid solution, containing additives and solubilized bacterial biomass, as wastewater.

The main challenges of cellular lysis methods are:

(1) The soluble biomass constituents end up in the final PHA products, and, given that the dissolution process cannot be 100% efficient, the purity of the final PHA is typically the most relevant issue of such methods. This is especially important considering that impurities (e.g., endotoxins) could exert a negative effect on PHA applications (especially biomedical ones): pyrogenic endotoxin in fact causes fever if introduced into the bloodstream of humans or other animals and thus should be kept below a set limit. According to the U.S. Food and Drug Administration guideline, the upper pyrogen limit is 5.0 endotoxin units (EU)/kg (body weight) per injection.
(2) Whereas the energy input is typically lower than that necessary for solvent extractions, material consumption can be comparable or even larger than in solvent extraction methods. It follows that the recovery or saving of lytic agents through innovative approaches represents one of the challenges of the applied research concerning PHA extraction.

Techno-Economics of the Extraction Process

The PHA extraction process is strictly ruled by operational costs (OPEX) and fixed cost (CAPEX) of the extraction apparatus like all the other industrial processes. Beyond standard solid/fluid handling systems, which give a minor contribution to the system, the extraction apparatus is constituted by functional elements with a characteristic size (e.g., volume, power, or throughput capacity) that determine the CAPEX, and consumptions (power or material consumption) that determine the OPEX.

The OPEX (€/y) of a specific extraction system with a known capacity (kgPHA/y) are the actual yearly costs due to the equipment running, like electrical energy (purchased at 0.12€/kWh) or thermal energy (purchased at 0.06 €/kWh). Such values can be calculated from basic assumptions of plant economics in chemical engineering.

The capital expenditure (CAPEX) consists of the purchasing costs of the extraction equipment (€) and increases the "financial" yearly cost depending on the maintenance costs, equipment depreciation, amortization, and interest rate. Maintenance costs are a tangible value related to the fact that any equipment requires to be maintained, and the cost of maintenance is proportional to the CAPEX. Maintenance costs are estimated at 3–7% of the CAPEX, with lower maintenance costs for mature technologies and large-scale plants, and higher maintenance costs for new technologies and small-scale equipment. Such values should be integrated with the other financial aspects (e.g., amortization over 20 years of plant operation) which add a value of 5–8% depending on the interest rate. As general rules of thumb, 1 M€ of additional CAPEX implies a yearly cost equal to 70–150 k€/y. Therefore, estimating the order of magnitude of the CAPEX of a certain extraction technology has pivotal importance for the early development stage of the technology itself. Consistent delivery of this information is not trivial and requires crossing the information from general plant economics with additional information from gray literature, namely quotations and opinion of experts working in similar fields. The scope of this review is to provide a comparison of methods from several points of view, therefore, some general rules to estimate the costs of an extraction process were drawn. This task was performed by focusing on the major (from the point of view of cost) extraction

steps shown in **Figures 1**, **2**: (i) the material input needed for the extraction (e.g., solvents or additives), (ii) the extraction reactor, (iii) the solid/liquid separator, and (iv) the solvent/water recovery/removal unit.

Material Input

The first relevant cost of each extraction procedure is related to the material input (additive, solvent or anti-solvent), named $OPEX_M$ (€/kg_{PHA}):

$$[OPEX_M = M_{cost} \cdot \frac{Ext_{ratio} \cdot Ext_{loss}}{X_{PHA} \cdot R_{yield}}]$$

Where M_{cost} is the market price (€/kg) of solvents/additives used, Ext_{ratio} ($kg_M/kg_{feedstock}$) is the specific amount of material used per kg of feedstock (e.g., biomass slurry), X_{PHA} is the PHA content of the feedstock ($kg_{PHA}/kg_{feedstock}$), R_{yield} is the efficiency of extraction and Ext_{loss} is the amount of material that is lost at the end of the extraction process ($kg_{M,lost}/kg_{M,used}$). Ext_{loss} ranges between 1 (e.g., a single-use surfactant) and 0 (when the solvent is completely recovered without any loss). For solvents, Ext_{loss} depends on leaks (usually less than 1%, Kemper, 1997) and the amount of residual solvent in the extracted product (solvent in PHA) and byproducts (solvent in the microbial residue). Overall Ext_{loss}, which usually falls in the 0.005–0.05 range, but is deeply influenced by the chemistry of the system and process configuration.

Extraction Reactor

The extraction reactor consists of devices that pretreat/mix bacteria, solvents, and/or additives and manages the heating/cooling of the mixture, usually through electrical heating and a heat exchanger (since the extraction process is not significantly exo- or endothermic). The cost of the extraction reactors is mostly a function of the reactor volume (L) and relative operating pressure (bar). Being mixing almost negligible for the energy balance, the operating costs of the extraction reactor are mainly due to the heating of the mixture. Such aspects have been included in empirical relationships shown in the following equations, which can be used as a rule of thumb in the early design of new extraction processes. The CAPEX related cost (actualized considering depreciation and maintenance) of extraction vessel can be estimated as $CAPEX_{extr.vessel}$ (€/kg_{PHA}):

$$[CAPEX_{extr.vessel} = \frac{0.1}{8760} \cdot \frac{(1 + 0.0376P)}{\sqrt{PHA_{out}}} \cdot 528 \left(\frac{RT_{ext} \cdot (1 + Ext_{ratio})}{X_{PHA} \cdot R_{yield} \cdot \rho_{mix}}\right)^{0.5}]$$

Where P is the relative pressure of extraction process (bar), PHA_{out} is the absolute size of the system expressed as PHA output capacity (kg_{PHA}/h), RT_{ext} is the residence time in extraction vessel, Ext_{ratio} ($kg_M/kg_{feedstock}$) is the specific amount of material used per kg of feedstock (e.g., biomass slurry), X_{PHA} is the PHA content of the feedstock ($kg_{PHA}/kg_{feedstock}$), R_{yield} is the efficiency of extraction ($g_{PHA,extracted}/g_{PHA}$), and ρ_{mix} (kg/L)is the density of the mixture in the extraction vessel (e.g., 0.7–1.5 kg/L water and organic solvents).

The specific $OPEX_{extr.vessel}$ (€/kg_{PHA}) of the extraction reactor running is mainly related to the heating or pretreatment of the mixture as follows:

$$[OPEX_{extr.vessel} = \frac{ThE_{price} \cdot \Delta T \cdot C_p(1 + Ext_{ratio})}{3.6 \cdot 10^6 \cdot X_{PHA} \cdot R_{yield}} + \frac{ElE_{price} \cdot En_{req} \cdot RT_{ext}(1 + Ext_{ratio})}{3.6 \cdot 10^6 \cdot X_{PHA} \cdot R_{yield} \cdot \rho_{mix}}]$$

Where ThE_{price} and ElE_{price} are respectively the thermal and electrical energy costs (€/kWh), ΔT is the difference between the inlet and outlet temperatures (equal to the reaction temperature) and minus environmental temperature if heat recovery is not applied, C_p is the specific heat capacity of extraction mixture, Ext_{ratio} ($kg_M/kg_{feedstock}$) is the specific amount of material used per kg of feedstock (e.g., biomass slurry), X_{PHA} is the PHA content of the feedstock ($kg_{PHA}/kg_{feedstock}$), R_{yield} is the efficiency of extraction, En_{req} is the electrical energy required per unit of volume (W L^{-1}, e.g., heat dissipation, stirrers, mixers or ultrasound treatment), RT_{ext} is the residence time in extraction vessel, ρ_{mix} is the density of the mixture in the extraction vessel (e.g., 0.7–1.5 kg/L water and organic solvents). $OPEX_{extr.vessel}$ is not intrinsically related to the scale of the extraction, even if En_{req} can be affected by the scale, with a general lower volumetric energy requirement for larger reactors.

Solid/Liquid Separator

A solid/liquid separation can be performed using tangential filtration, dead-end filtration (e.g., industrial filter press), or centrifugation. Each separation technique is characterized by different applicability and economic features. Tangential filtration is usually characterized by high OPEX, mainly due to recirculation pumping required, and low fixed cost, consisting of filtration elements. As a comparison, filter presses and centrifuges are characterized by high CAPEX and lower OPEX, and for this reason are the best options at a larger scale, when scale factor decreases selectively the CAPEX related cost.

The tangential filtration unit is formed by filters and recirculation pump needed for tangential low through the filters and can be used as a general model for a preliminary cost evaluation. The cost of such systems is mainly related to the CAPEX and can be easily modeled as an example technology for S/L separation:

$$[CAPEX_{S/L} = \frac{0.1}{8760} \cdot \frac{3000}{PHA_{out}^{0.23}} \cdot \left(\frac{Filtrate_{ratio} \cdot (1 + Filtrate_{wash\ loop})}{Filter_{rate} \cdot X_{PHA} \cdot R_{yield} \cdot \rho_{filtrate}}\right)^{0.77}]$$

Where PHA_{out} is the absolute size of the system expressed as PHA output capacity (kg_{PHA}/h), $Filtrate_{ratio}$ is the specific amount of external input (e.g., solvent or aqueous additive) that has to be obtained as filtrate through S/L module, $Filtrate_{washloop}$ is the

times that the solid has to be washed to get an acceptable purity, $Filter_{rate}$ ($L/m^2/h$) is the specific filtration capacity of the filtration equipment, which depends on the filter (mainly the size of pores) and liquid viscosity, X_{PHA} is the PHA content of the feedstock ($kg_{PHA}/kg_{feedstock}$), R_{yield} is the efficiency of extraction, $\rho_{filtrate}$ ($kg\ L^{-1}$) is the density of filtrate. Typical values of $Filter_{rate}$ in microfiltration (the most used system for separation of cells or cellular debris in aqueous slurries) is around 35 $L/m^2/h$ and the updated 2020 cost for microfiltration elements is around 3000 $€/m^2_{filtration\ area}$.

$OPEX_{S/L}$ (€/kg_{PHA}) of tangential filtration are mainly related to the recirculation pump which is about 200-folds larger than feed pumps and determines most of the energy consumption of the system:

$$[OPEX_{\frac{S}{L}} = \frac{ElE_{price} \cdot PW_{E,reg} \cdot TFlow_{ratio} \cdot (1 + Filtrate_{wash\ loop})}{3.6 \cdot 10^6 \cdot X_{PHA} \cdot R_{yield} \cdot \rho_{filtrate}}]$$

Where ElE_{price} is respectively the electrical energy price (€/kWh), $PW_{E,req}$ is the work required for pumping 1 L of slurry (e.g., 360–600 J/L for large volumetric pumps working at differential pressure of 2–4 bar), $TFlow_{ratio}$ is the ratio between tangential flow and filtrate flow required to avoid plugging of filters (-e.g., typically in the 100–300 range), Ext_{ratio} ($kg_M/kg_{feedstock}$) is the specific amount of material used per kg of feedstock (e.g., biomass slurry), X_{PHA} is the PHA content of the feedstock ($kg_{PHA}/kg_{feedstock}$), R_{yield} is the efficiency of extraction, $\rho_{filtrate}$ ($kg\ L^{-1}$) is the density of filtrate.

Solvent Recovery or Drying Unit (SRU)

Solvent recovery (or water removal) can be accomplished through atmospheric distillation, vacuum distillation or pervaporation through a membrane. Atmospheric distillation is characterized by high OPEX and low CAPEX, whereas vacuum distillation or pervaporation save OPEX at the expense of higher CAPEX. Despite the large variability of technologies, a base-case in which the solvent is recovered by distillation at atmospheric pressure can be considered: such conventional evaporation of organic solvents can remove a solvent from a solid material (e.g., solvent + PHA) and requires an amount of energy slightly higher than the latent heat of evaporation due to solvent losses. An important aspect is that, when distillation is applied to a solid-free mixture (e.g., solvent + anti-solvent mixture) and especially with water, multiple-effect distillation can be used, decreasing the energy requirement down to 4% of the latent heat of evaporation.

For simpler evaporators that can be used for both water evaporation and solvent recovery the $CAPEX_{evaporator}$ (€/kg_{PHA}) is intrinsically related to the amount of heat exchange required, and ultimately on the surface area of heat exchangers. Such size is proportional to the amount of heat involved in the evaporation and the number of effects used in multiple effect evaporators. The 2020 cost of evaporators with a 0.5–0.6 MW latent heat capacity (0.7–0.8 ton_{H2O}/h) is in the 1.2–1.8 M€ range. Taking average values from quotation and, including some empirical assumptions, the CAPEX related costs associated with that step can be estimated as follows:

$$[CAPEX_{evaporator} = \frac{0.1}{8760} \cdot \frac{72}{PHA_{out}^{0.23}} \cdot \left(\frac{n_{eff} \cdot L_v \cdot Ext_{ratio}}{3600 \cdot X_{PHA} \cdot R_{yield}}\right)]$$

Where n_{eff} is the number of effects used if multiple-effect evaporation is used (n_{eff} equal to 1 for simple drying processes), L_v is the latent heat of evaporation (e.g., 2.2 10^6 J/kg for water), Ext_{ratio} ($kg_M/kg_{feedstock}$) is the specific amount of material used per kg of feedstock (e.g., biomass slurry), X_{PHA} is the PHA content of the feedstock ($kg_{PHA}/kg_{feedstock}$), R_{yield} is the efficiency of extraction. $OPEX_{evaporator}$ (€/kg_{PHA}) is described by the following equation:

$$[OPEX_{evaporator} = \frac{ThE_{price} \cdot L_v \cdot Ext_{ratio}}{3.6 \cdot 10^6 \cdot X_{PHA} \cdot R_{yield} \cdot n_{eff}}]$$

Where ThE_{price} is the thermal energy cost (€/kWh), L_v is the latent heat of evaporation (e.g., 2.2 10^6 J/kg for water), Ext_{ratio} ($kg_M/kg_{feedstock}$) is the specific amount of material used per kg of feedstock (e.g., biomass slurry), X_{PHA} is the PHA content of the feedstock ($kg_{PHA}/kg_{feedstock}$), R_{yield} is the efficiency of extraction, n_{eff} is the number of effects used if multiple-effect evaporation is used (n_{eff} equal to 1 for simple drying processes).

Both $CAPEX_{evaporator}$ and $OPEX_{evaporator}$ are proportional to L_v and Ext_{ratio}. For solvent extraction, this means that such type of costs is minimized with the use of a solvent with low L_v (e.g., weak intermolecular bonds) and high efficiency (which means lower Ext_{ratio}). Increasing n_{eff} decreases the $OPEX_{evaporator}$ but increases $CAPEX_{evaporator}$ and this means that this parameter should be optimized according to the size of the equipment.

Applying the abovementioned formulas to some PHA extraction examples gives a general idea of the main cost issue of different extraction procedures. Nonetheless, it should be pointed out that this is just an example of a possible application of an economic analysis that can be used to evaluate the order of magnitude of costs in the infancy of process development at low Technology Readiness Level (TRL), to drive the design toward more economically sustainable processes. At high TRLs, a more detailed analysis is mandatory to provide a go-not-go decision.

A comparison of the estimated costs for two hypothetical PHA extraction processes (extraction with chloroform at 60°C, with $CHCl_3$: biomass ratio of 20:1; cellular lysis with NaClO 1.9 M at 37°C for 1 h) is reported in **Figure 3**. *Ex ante* evaluation of the cost of the two extraction processes gives values of 0.7–1.3 €/kg, close to the values obtained through a more detailed economic analysis performed by Fernández-Dacosta et al., 2015. Summing up this value with 0.8–1 €/kg_{PHA} feedstock cost (e.g., PHA from 0.4 €/kg glucose with 30–40% yield) and cost for single strain cultivation (0.8–1.8 €/kg_{PHA}), we could calculate the PHA production cost within 2.3–4.3 €/kg_{PHA} range. Such data fit with a 1.4–4 €/kg_{PHA} realistic market price using current technologies.

This calculation highlights that cellular lysis can be considered as a low-cost extraction process that can be applied to produce low-cost and low-quality PHA even if the quite high cost of the material input. On the other hand, chlorinated solvents (characterized by the high cost of SRU) become competitive at a large scale and when PHA quality requirement prevents the

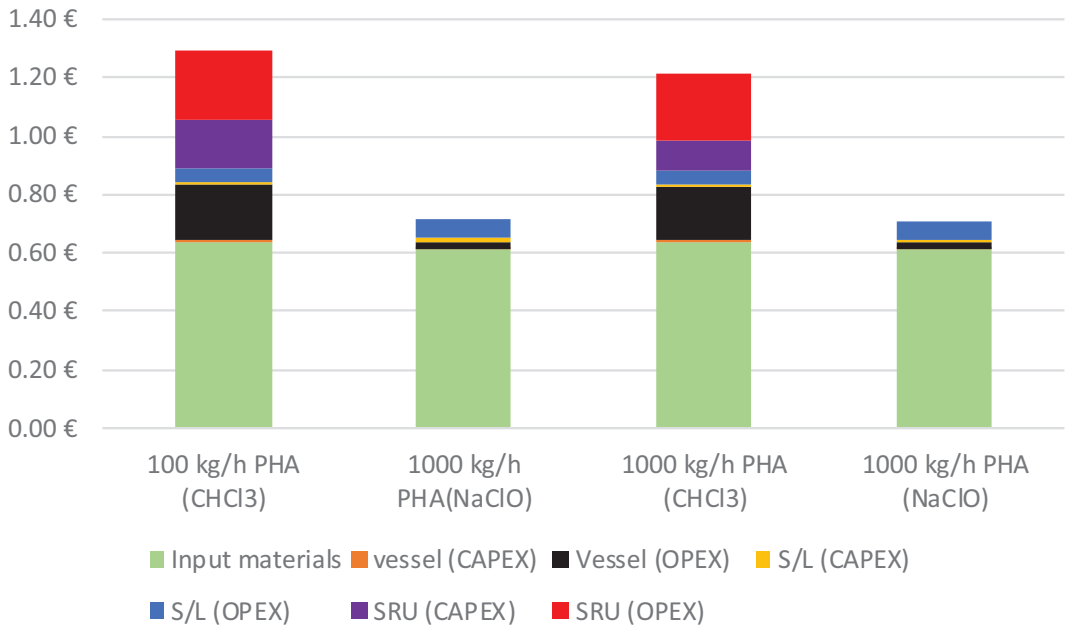

FIGURE 3 | Analysis of major sources of costs of two PHA extraction processes (extraction with $CHCl_3$ and cellular lysis with NaClO).

use of harsh cellular lysis methods. This is in line with life cycle costing (LCC) and LCA studies focused on PHA recovery from single microbial strains reported in the literature (Saavedra del Oso et al., 2020). They reveal that:

- methods based on cellular lysis with alkaline compounds or surfactants have lower costs (1.02–5.23 €/kg$_{PHA}$) than those based on solvent extraction (1.95–6.61 €/kg$_{PHA}$),
- methods based on cellular lysis have better environmental performance (0.81–4.16 kg CO_{2eq}) than those based on solvent extraction (3.93–12.96 kg CO_{2eq}),
- chemicals and heat production cover more than 50% and 20–30%, respectively, of the total operating costs and are the main contributors to environmental impacts of both processes, underlying the need of reducing energy use, employing greener energy sources, and introducing chemicals recovery units where it is possible.
- PHA extraction through solvents needs large amounts of energy, especially for solvent-recovery; thus, it seems a suitable approach just for these applications in which high-quality PHA is required. However, economic and environmental performance can be optimized by employing more easily recoverable solvents, utilizing the residual heat of the PHA production plant, or process byproducts exploitable as solvents.

EXTRACTION OF PHA FROM SINGLE MICROBIAL STRAINS

The research on PHA production has been focused from the beginning on the utilization of single strain cultures, and the few industrial realities that are currently producing PHA for the market are based on this approach (e.g., Biomer, Kaneka, Danimer Scientific). Different bacterial strains have been successfully used to synthesize biopolymers such as *Alcaligenes eutrophus*, *Alcaligenes latus*, *Azotobacter vinelandii*, *Azotobacter chroococcum*, *Azotobacter beijerincki*, methylotrophs, *Pseudomonas* spp., *Bacillus* spp., *Rhizobium* spp., *Nocardia* spp., and recombinant *Escherichia coli* (Pagliano et al., 2017). These bacteria can accumulate PHAs under extreme conditions such as excess carbon source and/or nutrient (nitrogen, phosphorus, sulfur, or oxygen) starvation (Reddy et al., 2003). Single strains have the double advantage to be able to accumulate a high PHA amount (above 80% of the cell dry weight) (Reis et al., 2003) and to reach high cell densities. On the other hand, such fermentations have high operating costs mainly associated with raw material and sterilization phase (Morgan-Sagastume et al., 2010).

Solvent Extraction Methods Applied to Single Microbial Strains

Halogenated Solvents

Chloroform, dichloromethane, 1,2-dichloroethane, 1,1,2-trichloroethane, and 1,1,2,2-tetrachloroethane are the solvents of choice for PHA extraction since very pure polymers (more than 90%) with high molecular weight (up to 1.2 MDa) can be easily obtained (**Table 1**); therefore halogenated compounds are often considered as the benchmark solvents for PHA recovery (Koller et al., 2013). On the other hand, a quite wide polydispersity index range is often observed (1.75–4.50). The extraction yields seem influenced by two factors, even if a clear independent effect of all of them is difficult to be highlighted:

TABLE 1 | PHAs extraction from single strains with solvents.

Solvent class	Solvent type and extraction conditions	PHA type and content (wt%)	Recovery (%)	Purity (%)	Molecular weight (MDa)	Polydispersity index (PDI)	References
Halogenated solvents	$CHCl_3$ (25°C)	PHB (50)	27	95	1.2	–	Ramsay et al., 1994
	$CHCl_3$ (61°C)	PHB (50)	55	92	0.9	–	Ramsay et al., 1994
	$CHCl_3$ (61°C, acetone pretreatment)	PHB (50)	68	96	0.9	–	Ramsay et al., 1994
	$CHCl_3$ (60°C)	PHB (50)	–	–	0.6–1.2	2.6–4.6	Mothes et al., 2007
	$CHCl_3$ (4°C)	PHB (62)	–	–	0.8–1.0	3.1–3.7	Cavalheiro et al., 2009
	$CHCl_3$ (37°C)	PHB (38)	31	92	0.8	2.6	Valappil et al., 2007
	$CHCl_3$ (37°C, NaClO pretreatment)	PHB (38)	27	99	1.1	1.7	Valappil et al., 2007
	$CHCl_3$ (38°C, NaClO pretreatment)	PHB (38)	30	95	0.9	3.1	Valappil et al., 2007
	$CHCl_3$ (25°C, NaClO pretreatment)	PHB (65)	90–95	95	0.6	4.5	Berger et al., 1989
	$CHCl_3$ and NaClO (30°C)	PHB (67)	–	97	1.0	2.0	Hahn et al., 1993
	$CHCl_3$ (NaClO and $NaHSO_3$ pretreatment)	PHB (55)	–	97	0.6	–	Suk Roh et al., 1995
	$CHCl_3$ and NaClO (Al–, Fe-coagulants pretreatment)	PHB	90–94	98–99	–	–	Ryu et al., 2000
	$C_2H_4Cl_2$ (83°C)	PHB (50)	54	92	0.8	–	Ramsay et al., 1994
	$C_2H_4Cl_2$ (83°C, acetone pretreatment)	PHB (50)	69	97	0.8	–	Ramsay et al., 1994
	CH_2Cl_2 (rt)	P(HH-HO) (30)	86	78	–	–	Furrer et al., 2007
	CH_2Cl_2 and NaClO (37°C)	PHB (65)	<90	99	0.4	2.6	López-Abelairas et al., 2015
	CH_2Cl_2 (25°C)	PHB (50)	26	96	1.2	–	Ramsay et al., 1994
	CH_2Cl_2 (40°C)	PHB (50)	15	89	1.05	–	Ramsay et al., 1994
	CH_2Cl_2 (40°C, acetone pretreatment)	PHB (50)	24	95	1.05	–	Ramsay et al., 1994
	CH_2Cl_2 (60°C)	P(HB-HV) (40)	–	98	0.9–1.2	2.7–3.3	Zinn et al., 2003
	CH_2Cl_2	PHB (80)	–	99	–	–	Hrabak, 1992
	CH_2Cl_2 (35°C)	PHO (15–17)	15–20	–	0.1	3.8	Wampfler et al., 2010b
	CH_2Cl_2 (37°C, HCl pretreatment)	PHA	–	97	0.2–0.5	–	Martínez et al., 2011
Alkanes	Hexane (rt)	P(HH-HO) (30)	53	93	0.2	1.3	Furrer et al., 2007
	Hexane (30°C)	PHB (60)	49	>99	0.1	–	Manangan and Shawaphun, 2010
	Hexane (50°C)	PHB (98)	3	89	–	–	Aramvash et al., 2018
Alcohols	Medium chain-length alcohols	PHB (65–70)	95	>98	0.2–0.4	–	Nonato et al., 2001
	EtOH/H_2O (30°C)	PHA (30)	96	81	0.3	2.6	Mohammadi et al., 2012a

(Continued)

TABLE 1 | Continued

Solvent class	Solvent type and extraction conditions	PHA type and content (wt%)	Recovery (%)	Purity (%)	Molecular weight (MDa)	Polydispersity index (PDI)	References
	MeOH (50°C, NaClO pretreatment)	PHB (98)	81	99	–	–	Aramvash et al., 2018
	PrOH (100°C, NaClO pretreatment)	PHB (98)	28	97	–	–	Aramvash et al., 2018
	PrOH (rt)	P(HH-HO) (30)	23	66	–	–	Furrer et al., 2007
Esters	Butyl acetate (100°C)	P(HB-HH) (76)	42	>99	–	–	Riedel et al., 2013
	Ethyl acetate (100°C)	P(HB-HH) (76)	99	>99	–	–	Riedel et al., 2013
	Ethyl acetate (60°C)	P(HB-HH) (50)	–	–	1.0	–	Chen et al., 2001
	Butyl acetate (103°C)	PHB (7)	96	98	1.4	–	Aramvash et al., 2015
	Ethyl acetate (25°C)	PHB (7)	87	86	–	–	Aramvash et al., 2015
	Ethyl acetate (49°C)	PHB (7)	33	99	–	–	Aramvash et al., 2015
	Ethyl acetate (35°C)	PHO (15–17)	10–15	–	0.1	3.7	Wampfler et al., 2010b
	Ethyl acetate (rt)	P(HH-HO) (30)	80	92	-	-	Furrer et al., 2007
	γ- butyrolactone (120°C)	PHB (82)	45	97	0.2	2.2	Jiang et al., 2018
Carbonates	Ethylene carbonate (100°C, NaClO pretreatment)	PHB (98)	90	99	1.3	–	Aramvash et al., 2018
	1,2-propylene carbonate (130°C)	PHB (71)	95	84	0.7	3.1	Fiorese et al., 2009
	1,2-propylene carbonate (130°C, heat and pH pretreatment)	PHB (71)	95	88	1.3	3.5	Fiorese et al., 2009
	1,2-propylene carbonate (130°C)	PHA (72)	75	–	0.2	2.9	McChalicher et al., 2010
	Dimethyl carbonate (90°C)	PHB (74)	94	93	1.1	2.7	Samorì et al., 2015b
Ethers	Anisole (125°C)	PHB (58)	97	98	0.7	2.3	Rosengart et al., 2015
	Phenetole (125°C)	PHB (58)	39	–	0.5	2.2	Rosengart et al., 2015
	MTBE (35°C)	PHO (15–17)	15–20	47	0.1	3.9	Wampfler et al., 2010b
	Tetrahydrofuran (rt)	P(HH-HO) (30)	80	84	–	–	Furrer et al., 2007
Ketones	Acetone (120°C)	P(4HB-HV) (88)	92	98	1.0	1.5	Koller et al., 2013
	Acetone (22°C, NaOH pretreatment)	PHA (66)	85	78	0.08	1.8	Jiang et al., 2006
	Acetone (25°C)	PHO (60)	94	98	–	–	Elbahloul and Steinbüchel, 2009
	Acetone (30°C, NaOH pretreatment)	P(4HB) (52)	95	–	0.5	3.1	Gorenflo et al., 2001
	Acetone (rt)	P(HH-HO) (30)	83	92	–	–	Furrer et al., 2007
	Acetone (35°C)	PHO (15–17)	10–15	–	0.1	3.9	Wampfler et al., 2010b
	MIBK (100°C)	P(HB-HH) (76)	55	99	–	–	Riedel et al., 2013
	MEK (100°C)	P(HB-HH) (76)	95	>99	–	–	Riedel et al., 2013
	MEK (60°C)	P(HB-HV) (65)	94	91	0.4	2.8	Yang et al., 2015
	Cyclohexanone (125°C)	PHB (58)	93	98	0.8	1.5	Rosengart et al., 2015
	Cyclohexanone (120°C)	PHB (82)	99	99	0.2	2.1	Jiang et al., 2018
Others	DMSO (150°C)	PHB (98)	61	95	–	–	Aramvash et al., 2018
	DMF (150°C)	PHB (98)	30	97	–	–	Aramvash et al., 2018
	Acetone, EtOH and propylene carbonate	PHB (62)	85	92	1.4	–	Fei et al., 2016

The investigated parameters are the solvents used for the extraction, the initial content of PHA in bacteria (wt% by dry weight) and the recovery (%), and the properties of the extracted PHA (purity%, molecular weight in MDa and polydispersity index).

1. the solvent type: the recovery of PHA from single strains with a polymer amount of 50% is similar if 1,2-dichloroethane or chloroform are used under their reflux temperature, whereas the recovery with dichloromethane under the same conditions is almost four-times lower (Ramsay et al., 1990).
2. the temperature of the extraction process: Ramsay et al. (1990) demonstrated that the extraction of single strains containing 50 wt% of PHA with chloroform doubles by increasing the temperature from rt to the reflux one, but the same is not true when dichloromethane is used (results achieved at rt or under reflux are around 20%).

Many attempts have been done to improve the extraction process mediated by halogenated solvents; the application of a pre-treatment by employing chemical (e.g., treatments with oxidants, salts, or alkaline compounds) and/or physical methods (e.g., heat treatment) seems to be the best choice to improve cell disruption, promote solvent permeation and enhance the accessibility to PHAs granules. Moreover, since the high solvent to biomass ratio necessary for getting a satisfying recovery, a pre-treatment step could be necessary to reduce the amount of solvent itself, and, consequently, the costs.

If a pre-treatment of the microbial biomass with NaClO is performed before the extraction step, the PHA recovery drastically improves, especially in the case of dichloromethane (the polymer recovery is reported to increase up to 90%, López-Abelairas et al., 2015), whereas a pre-treatment with acetone does not substantially improve the results achieved without any pre-treatment (Ramsay et al., 1990). NaClO is claimed to weaken the cell membranes, thus facilitating the subsequent extraction of PHA by solvents (Koller et al., 2013). The combination of a NaClO pre-treatment and the precipitation of PHA using alcohols gives the best extraction yields and purity value (recovery up to 94% and purity up to 99%) (Berger et al., 1989; Ryu et al., 2000; López-Abelairas et al., 2015); this approach exploits the fact that PHA solubility is drastically minimized by adding a "PHA anti-solvent," typically low-molecular alcohols (ethanol or methanol), hexane, ether, acetone, or water (Koller et al., 2013), whereas impurities solubility should not be affected.

Despite being highly performing in terms of recovery yields and polymer properties, halogenated solvents are not eco-friendly, being hazardous for the environment and the operators; chloroform, for example, can affect the central nervous system and the liver, it is highly irritating for mucous membranes, the respiratory tract, and eyes, it is classified as possibly carcinogenic for humans (Group 2B, International Agency for Research on Cancer (IARC), 1999) and as Hazardous Air Pollutant, HAP (EPA Environmental Protection Agency (EPA), 1990). Additionally, halogenated solvents commonly used for PHA extraction are all fossil-based solvents. All these impactful aspects are worsened by the fact that high amounts of solvents are required for the extraction process and the large quantity of energy must be employed for the purification of the solvent and its reuse, making this approach not economically advantageous. Finally, it has been claimed that the natural morphology of the PHA granules can be affected by the use of halogenated solvents, which prevents the use of PHA for certain applications, for example in the production of strong fibers by reducing molecular mass via random and chain-end scission, particularly at a higher temperature and prolonged extraction time (Pérez-Rivero et al., 2019).

A solution to reduce the impact of these solvents on the environment and human health could be the utilization of halogen-free solvents like alkanes, alcohols, linear and cyclic esters and carbonates, ethers, ketones, organosulfur compounds, and amides (**Table 1**). Some of these solvents are not toxic for humans and granted GRAS (Generally Recognized As Safe) by Flavor and Extract Manufacturers Association (FEMA, Flavor and Extract Manufacturers Association), whereas others are bio-based: alcohols (ethanol and butanol) and acetone for example can be produced through biochemical approaches starting from renewable resources (e.g., alcoholic fermentations mediated by *Saccharomyces cerevisiae* or acetone–butanol–ethanol fermentation mediated by Chlostridia), whereas linear and cyclic carbonates (dimethyl carbonate or propylene carbonate) are CO_2-derived solvents with low (eco)toxicity, complete biodegradability, and very low vapor pressure.

Alkanes

Alkanes used in the extraction of PHA from single strains (e.g., hexane) provide polymers with high purity (89 – ≈100%) but in low yields and with low molecular weight (about 50% and 0.2 MDa, respectively). This trend is surely due to the low solubility of PHA into such apolar solvents; on the other hand, this high lipophilicity is beneficial in terms of Gram-negative bacteria's endotoxins abatement, providing polymers useful for medical applications, especially if an anti-solvent is used for recovering PHA (Pérez-Rivero et al., 2019): a decrease in the "endotoxicity" of PHA from 100 endotoxic units (EU $g_{polymer}^{-1}$) (Williams et al., 1994) to 10 EU $g_{polymer}^{-1}$ was achieved by using 2-propanol for precipitating PHA from *n*-hexane (Furrer et al., 2007). Despite the positive effect given by alkanes in terms of endotoxins abatement and thus health issue, alkanes cannot be considered as "green" solvents: hexane and pentane are "hazardous" compounds according to all the solvent selection guidelines published so far (Henderson et al., 2011; Prat et al., 2014, 2016), because of safety (high flammability) and environmental issues (ability to generate harmful Volatile Organic Compounds, VOC, hazardous to the atmospheric ozone layer), the last ones a common feature of longer alkanes like heptane. Therefore, the constraints on scaled-up processes based on these compounds are very strong, and their substitution during process development is a priority (Prat et al., 2016).

Alcohols

Contrary to alkanes, low-medium chain length alcohols are too polar solvents for being reliable candidates for PHA extraction. The fact that the extracted PHA have a low molecular weight (0.2–0.4 MDa) (Nonato et al., 2001; Mohammadi et al., 2012a) can be an indication that these polar solvents are suitable for "peculiar" polymers with short chains, presumably less difficult to be solubilized. A NaClO pre-treatment is often associated

with alcohol extraction to achieve polymer purity up to 99% (Aramvash et al., 2018). As in the case of halogenated solvents, also for alcohols, the temperature affects the PHA recovery yield and purity (Mohammadi et al., 2012a): a combined synergic effect of ethanol and water has been reported by increasing the temperature from 4 to 30°C, since an increasing interaction between water and ethanol improves the cell wall breakage. Water seems to cause the release of cellular proteins and exert an osmotic pressure on the cells that result easily breakable (Mohammadi et al., 2012b). Despite alcohols seem to have a narrow range of applicability in the PHA extraction scenario, their use in scaled-up processes would be strongly encouraged: alcohols, in fact, are generally recognized as "recommended" solvents, benign in terms of acute environmental toxicity and the bio-accumulation (Henderson et al., 2011; Prat et al., 2014; Prat et al., 2016), as well in terms of health issue (butanol, in particular, is granted GRAS by FEMA and synthesizable from renewable resources thus potentially bio-based).

Esters

The extraction protocols based on linear and cyclic esters reported in the literature are generally performed at a quite high temperature (around 100°C); a direct comparison between ethyl and butyl acetate in the extraction of single strain bacteria containing 76 wt% of the copolymer P(HB-HH), indicates the first as the best performing among the two in terms of recovery yields, whereas the purity is excellent with both (Riedel et al., 2013). If wet biomass is extracted, a double effect of water can be observed: the hydrolysis of the esters and the reduction of the solvating power of the esters themselves (Riedel et al., 2013). Both ethyl and butyl acetate are "recommended" solvents, and all the solvent selection guides published so far agree with attributing low health and environmental scores (not problematic compounds) to the entire ester class (Henderson et al., 2011; Prat et al., 2014, 2016).

Carbonates

Linear and cyclic carbonates are probably among the most eco-friendly alternatives to halogenated solvents that could be applied for the extraction of PHA and PHB (the latter usually more difficult to be extracted due to its higher crystallinity and lower solubility in organic solvents), allowing the recovery of biopolymers with good properties. Dimethyl carbonate (DMC) is an example of green (it is fully biodegradable, minimally toxic for the operators and the environment) and well-performing solvent (Samorì et al., 2015a,b): DMC resulted highly efficient in terms of extraction yields, polymer purity, and polymer molecular weight, with the additional benefit of being applied directly on wet biomass. Also, 1,2-propylene carbonate (PC) and ethylene carbonate (EC), two solvents with low toxicity and therefore potentially usable in many human-related applications including cosmetics, give good results (Fiorese et al., 2009; Aramvash et al., 2018); it has been observed a decrease in the molecular weight by increasing the temperature for both solvents, probably due to a collateral reaction between the carbonate and the esters in the polymer. This suggests setting the boiling point as a limit temperature for the extraction. Despite being fewer common solvents used in industrial processes than esters and alcohols, DMC, PC, and EC are also included in the most recent solvent selection guides (Prat et al., 2016): among these polar aprotic solvents, DMC seems the greenest carbonates (ranking "recommended" because it does not have any H3xx statements after full REACh registration) and thus indicated as a potential replacement for various ketones and esters.

Ketones

Among ketones, only the extraction method performed by Koller et al. (2013) with acetone at high temperature and pressure (120°C, 7 bar) gave a polymer with high molecular weight (1.0 MDa) and PDI of 1.5, easily recoverable by cooling the solution at 4°C without the addition of an anti-solvent. All the other conditions/ketones work generally well in terms of recovery and purity but providing short chain length PHA. The temperature at which the extraction is performed results detrimental for recovering PHA from inside the cellular matrix: for example, cyclohexanone recovers only 16% of the polymer at 80°C (even after 20 h) but more than 99% within 3 min if the temperature is increased at 120°C (Jiang et al., 2018). In general, the ketone family has a worse profile than alcohols or esters: despite ranked as "recommended," acetone generates VOCs, but is not toxic and readily biodegradable, whereas cyclohexanone was ranked as "problematic," given that its synthesis via benzene and cyclohexane is not considered as sustainable (Henderson et al., 2011; Prat et al., 2014, 2016).

Other Solvents

Various aprotic solvents like anisole, DMSO, and DMF have been used to extract PHA from single strains. Anisole is probably the most interesting for application in a large-scale plant: PHA extraction and purity yields are over 96% (Rosengart et al., 2015), and it is ranked "recommended" since it does not have any H3xx (health hazard) or H4xx (acute environmental toxicity or bio-accumulation potential) statements (Prat et al., 2016); however, analogously to DMSO and DMF, polymer recovery has to be accomplished using an anti-solvent like ethanol, due to the boiling point above 150°C of all of them. A similar concept must be applied if non-volatile solvents like ionic liquids (IL) are used: these solvents (e.g., 1-ethyl-3-methylimidazolium diethyl- or dimethylphosphate) are capable of interacting with bacterial cell wall components like peptidoglycan and phospholipids via intermolecular H-bonds or electrostatic interactions, dissolving the NPCM and suspending PHA stored inside the cells. But since they are not removable under vacuum due to their negligible vapor pressure, alcohols are required as anti-solvents to precipitate PHA or purifying PHA after filtration (even if filtrating IL's solution needs large porosity filters because of the intrinsic high viscosity of the solution); contrarily to what happens with high-boiling point solvents like anisole, the purity of the extracted PHA is low (from 30 to 86% if filtration or precipitation are applied, respectively) (Kobayashi et al., 2015; Dubey et al., 2018; Koller, 2020). For these reasons (low purity and "PHA-suspension" effect) the use of ILs seems more a pre-treatment than a real extraction, oriented to disaggregate the

cellular components and then facilitate the action of an organic solvent capable of solubilizing PHA.

In summary, the use of solvents for the extraction of PHA from pure cultures is effective as it generally allows the extraction of PHAs with recovery yield up to 90–99% and purity yield up to 89–100%. The factors that influence mostly the extraction yields are:

- the temperature of the process, generally pivotal for increasing the solubility of PHA in the organic solvent and thus increasing the recovery efficiency;
- the nature of the polyester included inside the bacteria, being co-polymers easier to be extracted than homopolymers, especially in the case of a high percentage of monomers longer than hydroxybutyrate due to better solubility in organic solvents and a lower crystallinity,
- the amount of the polyester included inside the bacteria, since a higher number of PHA granules can exert a tensile strength on bacterial cell wall and membranes,
- the presence of water around bacterial cells (extraction of wet biomass), that can both create a barrier around the cells preventing the access of the solvent to the polymer and hydrolyze solvents (e.g., esters).

One of the main limitations in the use of solvents to extract PHA from bacterial cells relies on the need of separating the polymer from the solvent once it has been extracted; this can be done by solvent evaporation or by using an anti-solvent in which PHA is not soluble, but in both cases, a quite relevant amount of energy has to be applied. Another limitation common to both halogenated and non-halogenated solvents is the fact that a high solvent-to-biomass ratio must be used in the extraction process (up to a 20-fold mass of the microbial biomass) (Koller et al., 2013), and this is especially true for some "super-solvents." These solvents (e.g., chloroform, γ-valerolactone, ethyl lactate, DMC) show an excellent affinity for PHA, are capable to give a concentrated liquid solution of PHA at high temperature but they become a jelly-phase (solvent entrapped in the extracted PHA) once they are at rt (Samorì et al., 2015b, 2016; Prati et al., 2019). For keeping the solutions of "super solvents" and high amounts of extracted PHA (necessary for biomass-solvent separation) as liquid, an even higher solvent-to-biomass ratio must be used. It has been claimed that the use of Soxhlet apparatus could contribute to reduce the amount of solvent necessary to achieve a satisfactory PHA recovery on a laboratory scale (Koller et al., 2013), since Soxhlet extraction is operationally a continuous-discrete technique (the solvent acts stepwise but it is also recirculated through the sample, so the system operates both in a continuous manner and as a batch system, De Castro and Priego-Capote, 2010). Some comparisons between Soxhlet extraction and direct extraction in a batch-mode underline that the PHA recovery from bacterial biomass can be higher (1.3–1.8-fold) if the first approach is applied with specific solvents like acetone, hexane, and chlorinated compounds (Jiang et al., 2006; Manangan and Shawaphun, 2010; Lupescu et al., 2016). However, a not uniform effect on the molecular weight of the recovered polymer has been observed: since bacterial samples are extracted at the solvent boiling point over long periods, thermal decomposition of thermolabile target species can occur (De Castro and Priego-Capote, 2010), especially with some solvents like chloroform (Ramsay et al., 1994), and DMC (Samorì et al., 2015a). Other solvents like acetone (Jiang et al., 2006) do not behave in this way, and no significant change in the molecular weight is observed during the Soxhlet extraction process, confirming that the temperature conditions are not in a range in which random chain-end scission can occur (Koller et al., 2013).

To the best of our knowledge, no continuous extraction protocols (e.g., countercurrent extraction or flow processes) have been applied for recovering PHA, nor at a lab-scale neither at higher TRL. However, this approach is described as the best way to obtain requirements of high safety (small volume of solvents being processed at any given time, through the elimination of large reactors), low waste generation, and energy efficiency (improvements in mixing and heating) on a large scale, pillars of the Green Chemistry and Green Engineering philosophy (Newman and Jensen, 2013). The application of continuous extraction with green solvents like carbonates, esters, and ketones capable to recover polymers with high molecular weight (1.1–1.4 MDa) and stable PDI (1.4–3.9) on a lab-scale, could be a suitable and more sustainable alternative to halogenated compounds in a scale-up perspective (Righi et al., 2017; Vogli et al., 2020).

Cellular Lysis Methods Applied to Single Microbial Strains

The solubilization of non-PHA cellular material (NPCM) that surrounds PHA granules is an alternative approach to recover PHAs. The substances used for this purpose are oxidants like sodium hypochlorite (NaClO), alkaline or acid compounds, and surfactants (**Table 2**), or enzymes (**Table 3**).

Oxidants

NaClO is a strong oxidizing chemical able to degrade proteins, lipids, carbohydrates, and nucleic acids that constitute the non-PHA matter, thus enhancing their solubilization in aqueous solutions. Although PHA with a purity up to 99% and a recovery yield over 80% can be obtained (Heinrich et al., 2012; López-Abelairas et al., 2015), the treatment with this salt drastically reduces the molecular weight of the polymer. A high amount of NaClO solution is usually required, making this method appropriate only to enhance recovery yield (Hahn et al., 1993). Besides, the application of NaClO for NCPM digestion always bears the risk of formation of toxic halogenated compounds, and it appears rather difficult to completely remove NaClO traces from the recovered PHA (Koller et al., 2013).

Alkaline and Acid Compounds

Sodium hydroxide (NaOH) and potassium hydroxide (KOH) are milder digestion agents that cause saponification of the lipids present in the cell wall of microorganisms, increasing membrane permeability and helping the release of proteins and non-PHA material (Kosseva and Rusbandi, 2018). This kind of treatment provides a very high polymer recovery and

TABLE 2 | PHAs extraction from single strains through cellular lysis assisted by chemicals.

Chemical compound class	Chemical compound and conditions	PHA type and content (wt%)	Recovery (%)	Purity (%)	Molecular weight (MDa)	Polydispersity index (PDI)	References
Oxidants	NaClO (25°C, 1.9 M)	PHB (65)	87	93	0.5–0.8	2.4	Heinrich et al., 2012
	NaClO (37°C, 1.9 M)	PHB (65)	>80	99	0.25	6.8	López-Abelairas et al., 2015
Alkali	NaOH (30°C, 0.2 M)	PHB (77)	90	91	1.9	2.7	Choi and Lee, 1999
	KOH (30°C, 0.1 M)	PHB (77)	93	92	2.0	2.9	Choi and Lee, 1999
	NH_4OH (30°C, 0.1 M)	PHB (77)	95	85	–	–	Choi and Lee, 1999
	NH_4OH (90°C, 0.1 M)	P(HB-HV)	75	70	0.7	2.2	Samorì et al., 2015b
	NaOH (4°C, 0.05 M)	PHA (30)	97	97	0.15	–	Mohammadi et al., 2012b
	NaOH (30°C, 0.1 M)	P(HB-HH)	90	80	–	–	Anis et al., 2013a
	NaOH (60°C, 1 M)	PHA (70)	45	78	–	–	Yang et al., 2011
	NaOH (37°C, 0.5 M)	PHB (65)	<80	>90	0.3	2.9	López-Abelairas et al., 2015
	NaOH (30°C, NaCl pretreatment)	P(HB-HH) (68)	98	97.5	0.3	2.3–2.5	Anis et al., 2013b
Acids	HCl (30°C)	PHB (77)	95	79	–	–	Choi and Lee, 1999
	H_2SO_4 (30°C)	PHB (77)	96	79	–	–	Choi and Lee, 1999
	H_2SO_4 (80°C)	PHB (65)	80	99	0.4	3.0	López-Abelairas et al., 2015
	H_2SO_4 (121°C, 0.1 M)	PHB (61)	99	98	0.06	3.2	Yu and Chen, 2006
	Acetic acid (100°C, NaClO pretreatment)	PHB (98)	37	97	–	–	Aramvash et al., 2018
Surfactants	Lysol and NaOH	P(3HB-4HB)	98	97	0.4	2.9	Irdahayu et al., 2017
	CTAB (30°C)	PHB (77)	93	84	–	–	Choi and Lee, 1999
	Palmitoyl Carnitine (30°C)		Degree of lysis: 56–78%				Lee et al., 1993
	SDS	PHA (70)	81	90	–	–	Yang et al., 2011
	SDS (30°C)	PHB	90	95	0.5	–	Kim et al., 2003
	SDS (25°C)	PHB (50)	–	87	0.8	3–6	Ramsay et al., 1990)
	SDS (25°C, NaClO pretreatment)	PHB (50)	–	98	0.7	2–7	Ramsay et al., 1990
	SDS (30°C)	PHB (77)	87	98	–	–	Choi and Lee, 1999
	SDS (55°C, NaClO pretreatment)	PHA (75)	87	98	–	–	Dong and Xuenan, 2000
	SDS (50°C, autoclave pretreatment)	PHA (12)	–	95	0.2	–	Strazzullo et al., 2008
	SDS (90°C)	P(HB-HV)	100	99	1.2	2.2	Samorì et al., 2015b
	NH_4-laurate (90°C)	P(HB-HV)	100	98	0.6	1.9	Samorì et al., 2015b
	Sodium polyoxoethylene sulfate (ES702)	PHA (70)	85	90	–	–	Yang et al., 2011
	Sodium alpha olefin sulfonate (AOS-40)	PHA (70)	87	91	–		Yang et al., 2011
	Dioctylsulfosuccinate sodium salt (30°C)	PHB (77)	93	86	–	–	Choi and Lee, 1999
	Linear alkylbenzene sulfonic acid (LAS-99)	PHA (70)	87	86	–	–	Yang et al., 2011
	Tween 20 (30°C)	PHB (77)	92	80	–	–	Choi and Lee, 1999
	Triton X-100 (30°C)	PHB (77)	93	80	–	–	Choi and Lee, 1999
	Triton X-100 (25°C)	PHB (50)	–	87	0.8	3–6	Ramsay et al., 1990
	Triton X-100 (25°C, NaClO pretreatment)	PHB (50)	–	98	0.8	2–7	Ramsay et al., 1990
	Betaine + EDTA (50°C)	PHB (70%)			0.3–0.4	–	Chen et al., 1999

The investigated parameters are the chemicals used for the cellular lysis, the initial content of PHA in bacteria (wt% by dry weight) and the recovery (%), and the properties of the extracted PHA (purity%, molecular weight in MDa and polydispersity index).

purity (above 90%), even under relatively mild conditions (0.1–0.2 M at 30°C); more important, the molecular weight of the extracted PHA seems to be not affected by alkali, being around 1.9–2.0 MDa with a polydispersity of 2.70–2.90 (Choi and Lee, 1999), probably due to the limited digestion time (1 h) performed at low temperature (30°C) (Mannina et al., 2020). In the literature, various acids like H_2SO_4 or HCl were studied for PHAs recovery, providing high recoveries (above 90%), purity (up to 99%) but low molecular weight (around 0.06 MDa) (Yu and Chen, 2006). Furthermore, the

TABLE 3 | PHAs extraction from single strains through cellular lysis assisted by enzymes.

Enzyme class	Extraction conditions	PHA type and content (wt%)	SNPHA (%)	Recovery (%)	Purity (%)	Molecular weight (MDa)	Polydispersity index (PDI)	References
Proteases	Trypsin (50°C, heat pretreatment at 85°C)	PHB (75)[a]	60	88	53	0.63	–	Kapritchkoff et al., 2006
	Bromelain (50°C, heat pretreatment at 85°C)	PHB (75)[a]	67	59	89	0.53	–	Kapritchkoff et al., 2006
	Bromelain and Trypsin (heat pretreatment at 85°C)	PHB (75)[a]	64	89	57	–	–	Kapritchkoff et al., 2006
	Trypsin and Bromelain (heat pretreatment at 85°C)	PHB (75)[a]	73	91	83	0.55	–	Kapritchkoff et al., 2006
	Bromelain (twice) (heat pretreatment at 85°C)	PHB (75)[a]	78	90	88	0.58	–	Kapritchkoff et al., 2006
	Pancreatin (50°C, heat pretreatment at 85°C)	PHB (75)[a]	–	90	62	–	–	Kapritchkoff et al., 2006
	Enzymes from *A. oryzae* (48°C, heat pretreatment at 85°C)	P(HB-HV) (79)[b]	89	–	99	0.13	2.1	Kachrimanidou et al., 2016
	Protease (37°C)	PHB (90)[b]	–	45	97	1.4	–	Divyashree et al., 2009
	Microbispora culture filtrate (50°C, heat pretreatment at 85°C)	P(HB-HV) (50)[a]	–	21	90	–	–	Lakshman and Shamala, 2006
	Microbispora culture filtrate (50°C, heat pretreatment at 85°C)	P(HB-HV) (50)[b]	–	49	90	–	–	Lakshman and Shamala, 2006
	Alcalase (70–50°C, heat pretreatment at 135°C)	P(HB-HV) (71)[b]	53	84	–	–	–	Holmes and Lim, 1990
	Alcalase (twice, 55°C, heat pretreatment at 135°C)	P(HB-HV) (71)[b]	79	92	–	–	–	Holmes and Lim, 1990
	Alcalase and SDS (heat pretreatment at 135°C)	P(HB-HV) (71)[b]	63	87	–	–	–	Holmes and Lim, 1990
	Alcalase, SDS and H_2O_2 (80°C, heat pretreatment at 135°C)	P(HB-HV) (71)[b]	90	96	–	–	–	Holmes and Lim, 1990
	Alcalase, SDS and H_2O_2 (80°C, heat pretreatment at 135°C)	P(HB-HV) (71)[b]	95	98	–	–	–	Holmes and Lim, 1990
	Alcalase and Neutrase (70°C), Protease L330 (75°C), then SDS (100°C) (heat pretreatment at 150°C)	P(HB-HV) (75)[a]	77	93	–	–	–	Holmes and Lim, 1990
	Esperase and SDS (heat pretreatment at 85°C)	PHB[c]	82	–	71	–	–	Suzuki et al., 2008
	Alcalase and SDS (55°C), then EDTA and lysozyme (30°C) (heat pretreatment at 120°C)	PHA (34)[c]	–	–	93	–	–	Yasotha et al., 2006
	Alcalase and SDS (55°C), then EDTA and lysozyme (30°C) (heat pretreatment at 120°C)	PHA[a]	–	–	97	0.06	1.87	Kathiraser et al., 2007
	Alcalase (55°C), SDS (60°C), heat pretreatment at 120°C)	PHA (30-60)[a]	–	98	92	–	–	De Koning and Witholt, 1997
	Alcalase (55°C) and Lecitase 100S (40°C), heat pretreatment at 100°C)	PHB (52)[b]	75	88	–	–	–	Holmes and Lim, 1990
Phospholipases	Lecitase 100S (40°C, heat pretreatment at 100°C)	PHB (52)[b]	27	65	–	–	–	Holmes and Lim, 1990
Glycosidases	Lysozyme (37°C, heat pretreatment at 85°C)	PHB (75)[a]	26	–	20	0.55	–	Kapritchkoff et al., 2006
	Celumax (70°C, heat pretreatment at 120°C)	P(HB-HV) (75)[a]	–	93	94	–	–	Neves and Müller, 2012
	Celumax (60°C, heat pretreatment at 120°C)	P(HB-HV) (75)[a]	–	86	–	–	–	Neves and Müller, 2012

The investigated parameters are the enzymes used for the cellular lysis, the initial content of PHA in bacteria (wt% by dry weight), the non-PHA biomass solubilization (SNPHA) and the recovery (%), and the properties of the extracted PHA (purity%, molecular weight in MDa and polydispersity index).
[a] *PHA recovered by solvent extraction.*
[b] *PHA recovered by centrifugation.*
[c] *PHA recovered by ultrafiltration/diafiltration.*

potential hazard and corrosivity of these chemicals cause risks for the operators when they are used in high concentrations, making their use inappropriate for industrial applications (Yu and Chen, 2006).

Surfactants

Surfactants' mode of action is based on first incorporation into the lipidic bilayer of cell membranes, followed by an increase of the cellular volume until the membrane breaks to produce large micelles of surfactant and phospholipids. This finally causes the PHA release (Pérez-Rivero et al., 2019). Furthermore, surfactants can solubilize proteins and other non-PHA cellular material facilitating the disruption of cell membranes (Mannina et al., 2020). Anionic surfactants like sodium dodecyl sulfate (SDS) or alkylbenzene sulfonates (LAS) are the most used surfactants, tested alone or in combination with chemical or thermal pretreatments; however, SDS seems to give random results in terms of recovery, purity, molecular weight, and polydispersity index, and it is not easy to attribute its real effect on all of these parameters. Cationic surfactants like benzalkonium chlorides (BAC), palmitoyl carnitine, or hexadecyltrimethylammonium bromide (CTAB) are less used compounds than anionic ones and seem to decrease the molecular weight of the recovered polymer (despite the purity and the recovery are similar). Non-ionic surfactants like Triton X-100 or Tween-20 perform well in terms of polymer recovery, polymer purity, and molecular weight of the recovered polymer.

Surfactants are often combined with NaOH, NaClO, and chelating agents to increase the purity and recovery yield of the extracted polymer (Ramsay et al., 1990; Dong and Xuenan, 2000). Degradation of polymer granules and therefore reduction of molecular weight can generally occur only if high temperatures or pH are used (Kunasundari and Sudesh, 2011). The main limitation of this approach is the high dosage of highly water-soluble surfactants that have to be used for achieving satisfactory PHA recovery; this results in high costs of recovery and treatment of wastewater (Kapritchkoff et al., 2006). To overcome this drawback, the application of recoverable and recyclable surfactants can bring an overall improvement of the process, both in terms of economics and environmental footprint; for example, Switchable Anionic Surfactants (SAS) are compounds whose water solubility can be tuned by the addition and removal of CO_2 (Samorì et al., 2015b): cellular lysis is accomplished by using the water-soluble (ionic) form of the surfactant, whereas the recovery (more than 90%) of the surfactant in its non-ionic form is achievable by changing the pH of the aqueous solution using CO_2. In this way, even if high doses of surfactants are necessary to achieve an excellent polymer recovery, the surfactant can be completely recycled.

Enzymes

Specific enzymes like proteases and glycosidases can be applied to PHA-producing bacteria to hydrolyze peptide or glycosidic bonds of microbial proteins and carbohydrates/complex carbohydrates (e.g., glycoproteins and glycolipids), respectively. Alcalase (subtilisin A) is one of the most used proteases applied for PHA recovery, thanks to very broad substrate specificity and a good tolerance toward a quite large working temperature range (45–65°C); it usually provides recoveries above 90%. Other proteases like Trypsin and Bromelain work well in terms of polymer recovery (around 90%) and molecular weight (0.5–0.6 MDa); the non-PHA biomass solubilization (SNPHA) is generally higher with this class of enzymes than with glycosidases or phospholipases, ranging between 53–95% vs. about 26%. To increase the extraction efficiency, cocktails of proteases, nucleases, phospholipases, lysozymes, and other enzymes, in combination with surfactants and chelating agents can be applied. Centrifugation and use of solvents are the most employed extraction method but ultrafiltration and diafiltration are also applied (Kapritchkoff et al., 2006; Kathiraser et al., 2007); if the purity of the recovered polymer is similar, simple centrifugation seems to provide higher recovery than the use of organic solvents (Lakshman and Shamala, 2006).

In summary, enzymatic processes allow extraction of PHA with a wide range of purity (up to 88.8–97%) because the enzyme lysis effect is strictly related to reactions they catalyze. However, the process is expensive due to the high cost of enzymes and the complexity of the extraction process (Patel et al., 2009).

An alternative cell lysis approach recently described to recover PHA from single strains exploits whole microorganisms as cell-lytic agents (namely predatory bacteria of other Gram-negative bacteria), instead of isolated enzymes (Koller, 2020); these predatory bacteria (e.g., the species *Bdellovibrio bacteriovorus*) have a hydrolytic arsenal (e.g., extracellular-like PHA depolymerase) capable of breaking the cell walls of the prey (the culture of PHA-producing bacteria) in a relatively short time frame (24 h of "predation"), causing the release of the intracellular PHA in the culture medium (Martínez et al., 2016). To avoid the further hydrolysis of the released PHA granules by extracellular-like PHA depolymerase (that can hydrolyze up to 80% of the PHA produced by the prey), engineering of the predator seems to be the method of choice (Martínez et al., 2016). This process has been proposed as robust and generalizable since applicable to prey upon a wide range of Gram-negative bacteria, however, it is worth mentioning that high PHA purity values can be achieved just after extraction of the co-culture's sediments with chlorinated solvents. A strictly related approach (even if on a longer time frame) is the use of some animals like the mealworm *Tenebrio molitor* (Murugan et al., 2016) or rats (Kunasundari et al., 2013; Ong et al., 2018) to digest the NPCM of PHA-producing bacteria and excrete PHA granules with a high purity level, intact molecular weight and native morphology. Even in this case, PHA must be separated from the fecal pellets with chemicals (e.g., alkaline water), thus these approaches could be considered as low-cost and sustainable pre-treatments instead of self-sustaining recovery methods.

EXTRACTION OF PHA FROM MIXED MICROBIAL CULTURES

Mixed microbial cultures (MMC), able to accumulate intracellular PHA similarly to what occurs with single strains

and with comparable production rates, are probably the new frontier in the field of marketable PHA production, especially if this activity is coupled with wastewater treatment for reducing the cost of raw material and energy consumption aspects (Dias et al., 2006; Jiang et al., 2018). However, MMC downstream costs can be even more impacting on the overall process than single strain downstream costs especially because of a more challenging PHA extraction phase (Patel et al., 2009; Samorì et al., 2015a) and the difficulty in extracting PHA from MMC can be reflected both in terms of polymer recovery and purity (Mannina et al., 2019).

Solvent Extraction Methods Applied to Mixed Microbial Cultures

Halogenated Solvents

Chloroform and dichloromethane are the most used solvents applied so far in the extraction of PHA from MMC (**Table 4**). In some cases, an acetone pre-treatment was used to enhance cell breakage and improve the recovery of PHA (Serafim et al., 2008b); PHA recovery is usually achieved by solvent evaporation or by polymer precipitation with water, alcohols (methanol and ethanol) or cold petroleum ether. Occasionally a subsequent purification step, which consists of a washing step with acetone and diethyl ether, has been applied.

Patel et al. (2009) obtained an 'ultra-high molecular weight PHAs' (2–3 MDa) with a very narrow size distribution (PDI of 1.3) by applying dichloromethane; however, the recovery was very low (18–30%), suggesting that such a low result could be due to the presence of a PHA "difficult to be extracted" or to the presence of extracellular biomass matrix in which the PHA containing cells are embedded (Patel et al., 2009). This observation was confirmed by Samorì et al. (2015a), with a polymer recovery of 52%, a polymer molecular weight of 1.4 MDa, and a polydispersity of 2.0. Generally, chloroform allows the recovery of polymers with a lower molecular weight if compared to dichloromethane (Serafim et al., 2008b) (**Table 4**).

Halogen-Free Solvents

The use of non-conventional solvents for extracting PHA from MMC is not so well documented as in the case of single strains; in particular, the presence of longer co-monomers than 3HB can influence the extraction thanks to a change in the polarity and the crystallinity of the polymer (Laycock et al., 2014).

Alcohols like butanol and ketones like acetone allowed the recovery of PHAs with a purity up to 98% but the molecular weight dropped to 0.2–0.6 MDa with a polydispersity of 1.7–3.4 (Laycock et al., 2014).

Carbonates like dimethyl carbonate provide a recovery percentage of about 60% over two cycles, with a molecular weight above 1 MDa and a high purity level (98%) (Samorì et al., 2015a). If a NaClO pre-treatment is coupled to DMC extraction, the overall recovery process increases up to 82% but the molecular weight drops down by five times (0.2 MDa): reaction time and temperature of this chemical pre-treatment strongly influence the molecular weight of the recovered polymers because of the oxidizing properties and nucleophilicity of NaClO. The degrading effect on the polyesters is also emphasized by the increase in the PDI values, a sign of the polymer chains shortening due to some random chain scission.

Cellular Lysis Methods Applied to Mixed Microbial Cultures

The chemicals mainly used for the extraction of PHA from MMC through cellular lysis are oxidants, alkali, and surfactants, similarly to what is usually applied to single strains (**Table 5**). On the other hand, the use of enzymes to solubilize the non-PHA cellular matrix is not reported, presumably because the enzymatic digestion is "too mild" for the high cellular resistance of MMC.

Oxidants

Analogously to single strains (see section "Cellular lysis methods applied to single microbial strains"), NaClO can degrade most of the NPCM (it becomes water-soluble when oxidized) of MMC, while the PHA granules can be easily separated by precipitation (Mannina et al., 2020). However, also in this case, the treatment with NaClO can affect the quality of the obtained PHA (in terms of purity, polydispersity index, and molecular weight), compromising the final polymer applications (Kunasundari and Sudesh, 2011; Samorì et al., 2015a; Mannina et al., 2019). Villano et al. (2014) treated MMC with NaClO for 3 and 24 h at rt and noticed that the effect of NaClO on the purity and the molecular weight of the extracted polymer is similar regardless of the reaction time; on the other hand, the temperature of the NaClO treatment inversely impacts both the recovery and the polydispersity index: the PHA recovery decreases from rt (100%) to 85°C (50%), whereas the polydispersity index increases (giving more heterogeneous polymers) if the treatment is performed at high temperature (values around 3) or rt (values of 4–10) (**Table 5**). Recently, Lorini et al. (2020) investigated the impact of two stabilization protocols applied at the end of the accumulation phase (thermal drying at 145°C for 30 min and at 70°C overnight, and wet acidification with H_2SO_4) in combination with NaClO digestion (at rt overnight) for preserving the amount and the length of PHA produced by MMC. A wet-acidification pretreatment provides three-times higher molecular weights (0.4 MDa) than a thermal pre-treatment, a recovery of 96%, and a polymer purity of 90%.

Alkaline Compounds

According to Jiang et al. (2015), alkaline treatments cause PHA hydrolysis and the consequent decline of the molecular weight (generally between 0.3 and 0.5 MDa) and increase of the polydispersity index, but if a pretreatment like freeze-drying has applied this phenomenon is mitigated. A possible explanation relies on the fact that PHA crystallization can increase the resistance toward chemical treatments induced either by complete removal of water or by damaging the cell membrane through surfactants (Jiang et al., 2015). Among the tested alkaline compounds, NaOH gives higher PHA recovery, purity, and molecular weight than NH_4OH, but also higher polydispersity index values (4–10 *vs.* 2–3) (Jiang et al., 2015; Mannina et al., 2019). However, NH_4OH could be a better

TABLE 4 | PHAs extraction from mixed microbial cultures with solvents.

Solvent class	Solvent type and extraction conditions	PHA type and content (wt%)	Recovery (%)	Purity (%)	Molecular weight (MDa)	Polydispersity index (PDI)	References
Halogenated solvents	CH_2Cl_2 (50°)	P(HB-HV) (40)	52	94	1.4	2.0	Samorì et al., 2015a
	CH_2Cl_2 (acetone pretreatment)	P(HB-HV) (25)	18–30	–	2-3	1.3	Patel et al., 2009
	CH_2Cl_2	PHB (72)	56	98	1.8	–	Jiang et al., 2015
	$CHCl_3$ (37°C, HCl pretreatment)	P(HB-HV) (40–65)	–	–	0.2–0.4	1.3–1.7	Duque et al., 2014
	$CHCl_3$ (100°C)	PHA	–	–	0.3–0.9	1.7–3.9	Bengtsson et al., 2010
	$CHCl_3$ (100°C)	P(HB-HV) (25)	–	–	0.3–1.0	1.5–2.5	Morgan-Sagastume et al., 2010
	$CHCl_3$ (37°C)	P(HB-HV) (65–72)	–	–	0.2–0.6	2.3–2.7	Albuquerque et al., 2011
	$CHCl_3$	P(HB-HV) (28)	–	–	0.1–0.4	2.2–3.7	Dai et al., 2007
	$CHCl_3$ (4°C)	PHA	–	–	0.4–0.6	1.4–1.7	Dai et al., 2008
	$CHCl_3$ (reflux)	PHB (60)	–	–	0.5	–	Dobroth et al., 2011
	$CHCl_3$ (100°C, acidic MeOH and benzoic acid pre-treatment)	P(HB-HV)	–	–	0.1–0.9	1–6	Lemos et al., 1998
	$CHCl_3$	PHB (66)	–	–	2.1–3.4	1.3–2.2	Serafim et al., 2008b
	$CHCl_3$ (reflux, acetone pretreatment)	PHB	–	–	0.2–0.4	1.2–2.7	Hu et al., 2013
	$C_2H_4Cl_2$ (100°C, HCl and PrOH)	PHB (89)	–	–	–	–	Johnson et al., 2009
Alcohols	Butanol (125°C)	P(HB-HV) (15)	–	95–98	0.4–0.6	1.8–3.4	Laycock et al., 2014
	2-butanol (125°C)	PHB (44)	83	–	0.7	–	Werker et al., 2015
Carbonates	Dimethyl carbonate (90°C)	P(HB-HV) (40)	49	98	1.3	1.9	Samorì et al., 2015a
	Dimethyl carbonate (soxhlet)	P(HB-HV) (40)	41	87	0.5	2.9	Samorì et al., 2015a
	Dimethyl carbonate (90°C, NaClO pretreatment for 5 min at rt)	P(HB-HV) (40)	40	92	0.6	2.6	Samorì et al., 2015a
	Dimethyl carbonate (90°C, NaClO pretreatment for 15 min at rt)	P(HB-HV) (40)	62	98	0.8	2.4	Samorì et al., 2015a
	Dimethyl carbonate (90°C, NaClO pretreatment for 1h at rt)	P(HB-HV) (40)	76	88	0.6	2.3	Samorì et al., 2015a
	Dimethyl carbonate (90°C, NaClO pretreatment for 5 min at 100°C)	P(HB-HV) (40)	72	89	0.5	2.8	Samorì et al., 2015a
	Dimethyl carbonate (90°C, NaClO pretreatment for 15 min at 100°C)	P(HB-HV) (40)	72	92	0.5	2.4	Samorì et al., 2015b
	Dimethyl carbonate (90°C, NaClO pretreatment for 1h at 100°C)	P(HB-HV) (40)	82	93	0.2	2.5	Samorì et al., 2015a
	Dimethyl carbonate (90°C, twice)	P(HB-HV) (40)	12	95	1.2	2.7	Samorì et al., 2015a
Ketones	Acetone (125°C)	P(HB-HV) (15)	–	95–98	0.4–0.6	1.9–3.4	Arcos-Hernández et al., 2013
	Acetone (125°C)	P(HB-HV) (15)	–	95–99	0.5–0.6	1.9–3.0	Laycock et al., 2014
	Acetone (125°C)	P(HB-HV)	51	75–98	0.2–0.6	1.7 ± 0.1	Chan et al., 2017

The investigated parameters are the solvents used for the extraction, the initial content of PHA in bacteria (wt% by dry weight) and the recovery (%), and the properties of the extracted PHA (purity%, molecular weight in MDa and polydispersity index).

TABLE 5 | PHAs extraction from mixed microbial cultures through cellular lysis assisted by chemicals.

Chemical compound class	Chemical compound and conditions	PHA type and content (wt%)	Recovery (%)	Purity (%)	Molecular weight (MDa)	Polydispersity index (PDI)	References
Oxidants	NaClO (85°C)	P(HB-HV) (54)	50	86	0.1	2.2–3.2	Mannina et al., 2019
	NaClO (100°C)	P(HB-HV) (40)	79	77	0.3	2.7	Samorì et al., 2015a
	NaClO (rt, 3 h)	P(HB-HV) (46)	100	90	0.3–0.5	4–10	Villano et al., 2014
	NaClO (rt, 24 h)	P(HB-HV) (46)	100	90	0.3–0.5	4–10	Villano et al., 2014
Surfactants	Ammonium Laurate (75°C, NaClO pretreatment)	P(HB-HV) (54)	74?	>99	0.1	2.2–3.2	Mannina et al., 2019
	Ammonium Laurate (75°C)	P(HB-HV) (54)	80	48	0.1	2.2–3.2	Mannina et al., 2019
	SDS (75°C, NaClO pretreatment)	P(HB-HV) (54)	59	92	0.1	2.2–3.2	Mannina et al., 2019
	SDS (75°C)	P(HB-HV) (54)	83	42	0.1	2.2–3.2	Mannina et al., 2019
	SDS (30°C)	PHB (68)	63	79	–	–	Jiang et al., 2015
	SDS (30°C, freeze-dried pretreatment)	PHB (70)	94	93	–	–	Jiang et al., 2015
	SDS (rt)	P(HB-HV) (50)	67	56	–	–	Samorì et al., 2015a Jiang et al., 2015
Alkali	NaOH (30°C, 1 h)	PHB (69)	97	87	0.3	–	Jiang et al., 2015
	NaOH (30°C, 0.5 h)	PHB (69)	98	89	0.3	–	Jiang et al., 2015
	NaOH (30°C, 1 h, lyophilization and freezing pretreatment)	PHB (70)	95	96	0.3	–	Jiang et al., 2015
	NaOH (rt, 3 h)	P(HB-HV) (46)	87	90	0.3–0.5	4–10	Villano et al., 2014
	NaOH (rt, 24 h)	P(HB-HV) (46)	80	91	0.3–0.5	4–10	Villano et al., 2014
	NaOH (30°C, 1 h, SDS)	PHB (66)	91	99	0.5	–	Jiang et al., 2015
	NH_4OH (30°C, 1 h)	PHB (69)	63	63	–	–	Jiang et al., 2015
	NH_4OH (30°C, 1 h, lyophilization and freezing pretreatment)	PHB (70)	95	87	–	–	Jiang et al., 2015
	NH_4OH (75°C, 3 h, NaClO pretreatment)	P(HB-HV) (54)	59	97	0.1	2.2–3.2	Mannina et al., 2019
	NH_4OH (75°C, 3 h)	P(HB-HV) (54)	73	47	0.1	2.2–3.2	Mannina et al., 2019

The investigated parameters are the chemicals used for the cellular lysis, the initial content of PHA in bacteria (wt% by dry weight) and the recovery (%), and the properties of the extracted PHA (purity%, molecular weight in MDa and polydispersity index).

alternative than NaOH since it is claimed to be potentially easier to be recycled (Mannina et al., 2019); additionally, NH_4OH performances can be improved by applying specific pretreatments (Jiang et al., 2015): if a NaClO pretreatment is applied, the polymer purity increases, whereas if a freeze-drying pretreatment is applied the polymer recovery increases (**Table 5**). Recently Burniol-Figols et al. (2020) investigated the effect of NH_4OH on PHA purity, recovery, and molecular weight: long incubation times and high NH_4OH concentrations negatively impact the polymer recovery but have no influence on its purity, whereas increasing the temperature of the digestion both purity and recovery increase. However, even if at 140°C the highest recovery and purity is achieved (90 and 83%, respectively), a drastic reduction of the molecular weight is also observed (from 0.6 MDa at 30°C to 0.08 MDa at 140°C), suggesting an optimal temperature range of 75–115°C to get high recovery of PHA (above 90%) and maintaining the molecular weight of PHA at reasonable values (0.2 MDa).

A direct comparison between NaOH and NaClO treatments underlines that NaClO works better under the same conditions (Villano et al., 2014) and the PHA recovered using NaClO is more thermally stable (up to 200°C) than that recovered by NaOH (between rt and 100°C) (Laycock et al., 2014).

Surfactants

Surfactants seem to be an effective way to extract PHA with good purity and in a satisfactory amount just in combination with specific pre-treatments (**Table 5**). For example, if SDS or ammonium laurate are applied alone, purity values of 40–60% are obtained, whereas if NaClO or freeze-drying are applied as pre-treatments before the use of the surfactant, this parameter increases up to 90%. However, surfactants have an impact on PHAs molecular weight (around 0.1 MDa), independently by

the pre-treatment (Mannina et al., 2019). Furthermore, a high surfactant to biomass ratio is usually required, and consequently, the surfactant recovery generates a large quantity of wastewater that has to be treated (Jacquel et al., 2008); for overcoming this issue, as for single strains, recyclable surfactants like Switchable Anionic Surfactant (SAS) have been applied on MMC by Mannina et al. (2019): ammonium laurate gives a polymer purity of 48%, improvable by a pre-treatment with NaClO (100%). Also in this case, the molecular weight of extracted polymers is quite low (0.1 MDa) and the polydispersity is 2.2–3.2 (Mannina et al., 2019) (**Table 5**), but the surfactant proved to be completely recyclable.

OVERALL COMPARISON BETWEEN THE TWO EXTRACTION APPROACHES

The overview of the available extraction methods reported in **Tables 1–5** allows a comparison of the performance on single strains and mixed microbial cultures in terms of the overall efficiency of the process of PHA recovery.

Recovery

Mean PHB or PHA recoveries reported in the literature through solvent extraction or cellular lysis from single strains or MMC have been reported in **Figures 4**, **5**.

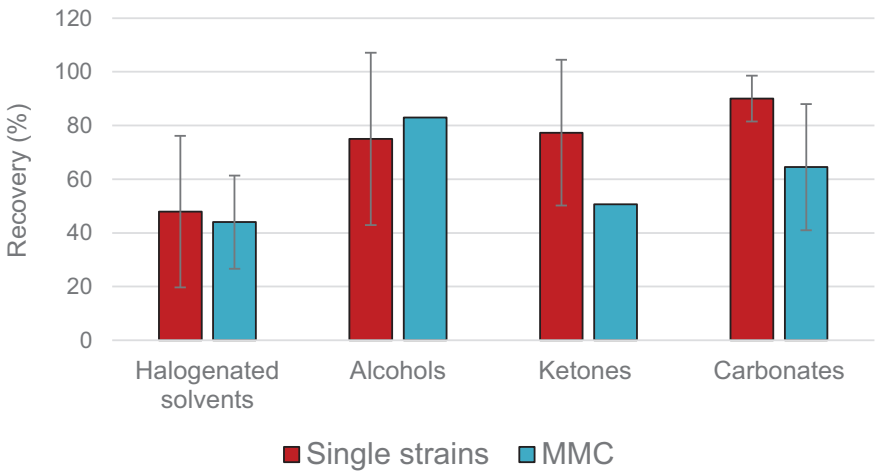

FIGURE 4 | Mean recovery (%) of PHA extracted with solvents (halogenated solvents, alcohols, ketones, and carbonates) from single strains or MMC.

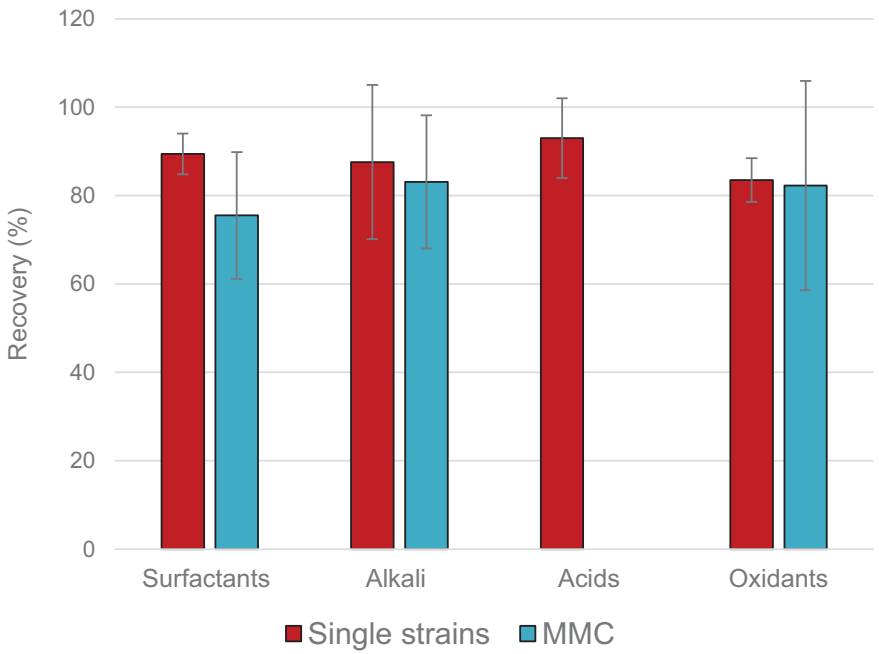

FIGURE 5 | Mean recovery (%) of PHA through cellular lysis (with surfactants, alkali, acids, and oxidants) from single strains or MMC.

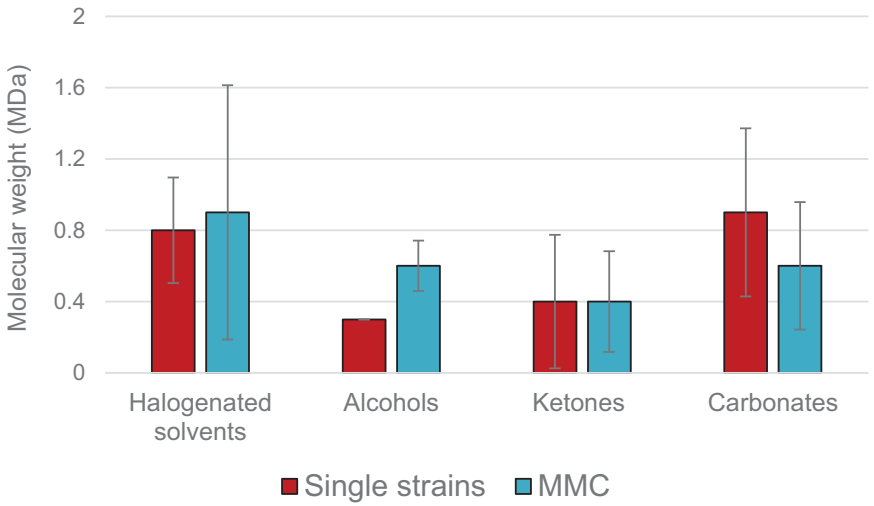

FIGURE 6 | Mean molecular weight (MDa) of PHA extracted with solvents (halogenated solvents, alcohols, ketones, and carbonates) from single strains or MMC.

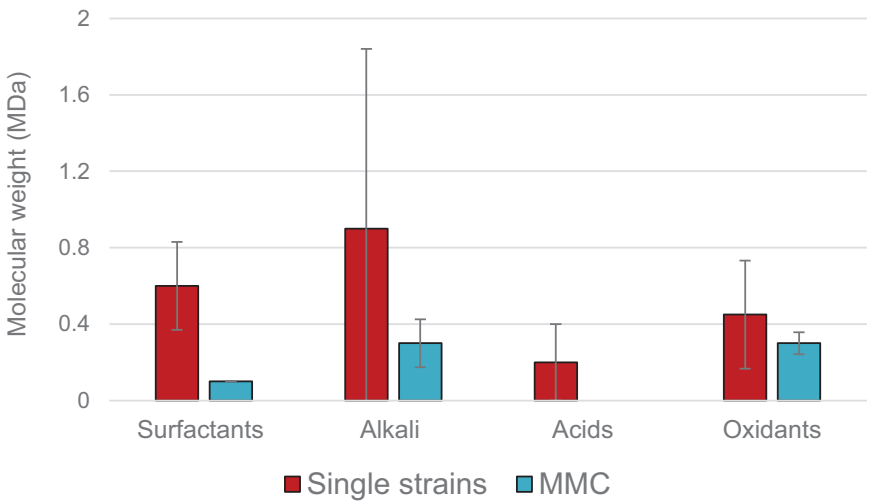

FIGURE 7 | Mean molecular weight (MDa) of PHA extracted through cellular lysis (with surfactants, alkali, acids, and oxidants) from single strains or MMC.

The variability of results expressed here as standard deviations (SD) of all the values reported in the literature (**Table 1**) is very high, mainly due to the small amount of data related to solvent extraction on MMCs. For almost all solvent classes reported in the literature, PHA recovery is lower when MMC are extracted, confirming that the extraction process is more difficult for MMC than for single strain cultures. MMC are claimed to be more resistant to cell hydrolysis, probably because of a strong extracellular biomass matrix that contains the PHA accumulating cells. Furthermore, Patel et al. (2009) hypothesized a decrease in cellular fragility and therefore a minor amount of solvent able to access polymer granules because of a stronger non-PHA cellular materials (NPCM) and lower initial PHA content which results in a lower cell constrain.

The recovery data achieved from single strains are considerably higher (about 25%) when ketones or carbonates are applied; however, the literature does not report plenty of data about the extraction of MMC with ketones, therefore more data are needed for a more accurate analysis. The results obtained with halogenated solvents from single strains and mixed cultures are more homogenous and, in both cases, an average recovery below 50% is achieved. Alcohol extraction seems to be more efficient for MMC than for single strains (recovery of 83% vs. 75%), however, the result is not robust as there is just one study about the extraction of MMC with this kind of solvents that cannot be considered as a real benchmark (the same holds true for ketones applied to MMC, Werker et al., 2015).

The recovery of PHA from both single strains and MMC by applying a cellular lysis approach (**Figure 5**) is higher

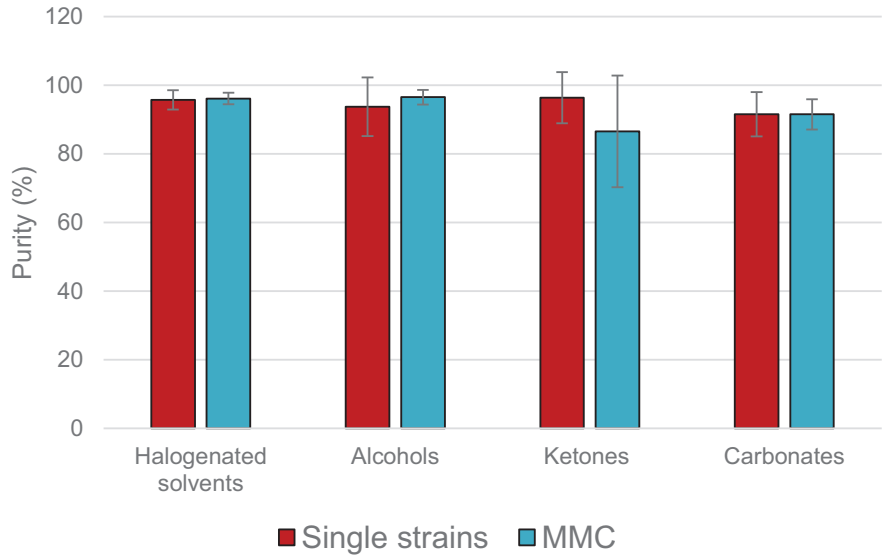

FIGURE 8 | Mean PHA purity (%) achieved through extraction with solvents (halogenated solvents, alcohols, ketones, and carbonates) from single strains or MMC.

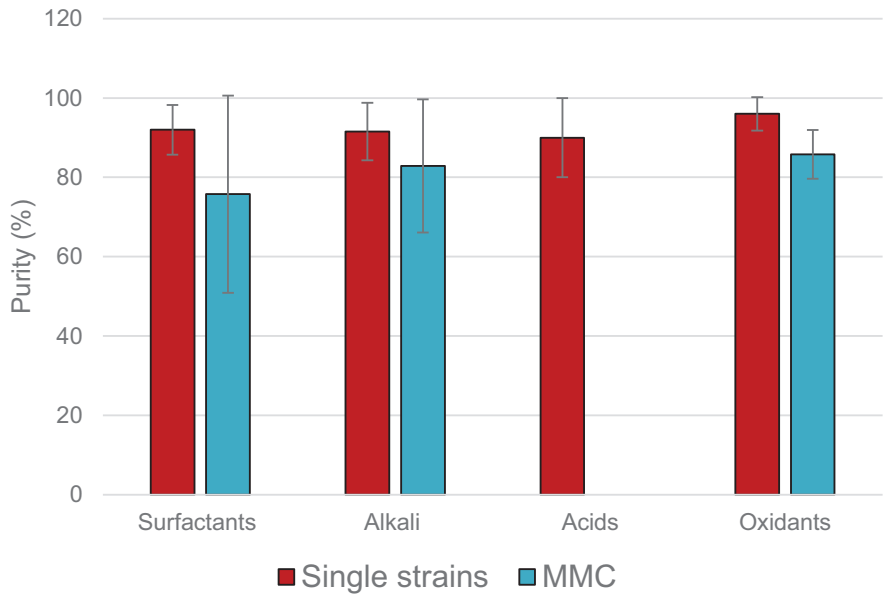

FIGURE 9 | Mean PHA purity (%) achieved through cellular lysis (with surfactants, alkali, acids, and oxidants) from single strains or MMC.

than what achievable by applying a solvent extraction; the average recoveries with surfactants, alkali, acids or oxidants are typically above 80%, whereas few solvents perform better than this threshold (**Figure 4**). The major differences between single strains and MMC are observed when cellular lysis is performed with surfactants (**Figure 5**), being the average recovery of PHA of 89 and 75%, respectively, whereas both the treatments with alkali and NaClO provide very similar results. By considering just the recovery percentage, surfactants, alkali, acids and oxidants perform well and similarly.

It is important to highlight that method that involve surfactants and alkali often include some pretreatments, especially with NaClO (**Tables 2**, **5**) which has a strong oxidizing behavior capable of breaking cell membranes and enhancing the recovery.

Molecular Weight

The molecular weights of polymers extracted with halogenated solvents are the highest among the ones achievable from both single strains and MMCs, ranging between 0.8 and 0.9 MDa, even if a large variability of the literature results is observed (**Figure 6**).

Carbonates behave similarly, especially on single strains, and even in this case, a large variability is observed. Both carbonates and halogenated solvents seem to not shorten the length of polymer chains by side reactions (e.g., transesterification), and, at the same time, seem to be the best solvents for solubilizing high molecular weight PHA; this confirms alkyl carbonates as an effective and environmentally alternative for PHA extraction instead of other solvents.

In the literature there are few reports about the molecular weight of polymers extracted with ketones and alcohols, especially for MMCs, therefore the results are not very robust. The average PHA's molecular weight extracted with ketones or alcohols from mixed and single strains is around 0.5 MDa but with high standard deviation in both cases (**Figure 6**); however, these lower values can be a confirmation of the suitability of ketones and alcohols for recovering "shorter" polymers than those extractable with carbonates and halogenated solvents.

The molecular weights of polymers extracted through cellular lysis (**Figure 7**) are largely variable. However, it seems quite clear that even if alkaline compounds do not compromise the length of PHA extracted from single strains (about 0.9 MDa), the same does not hold true for acids (0.2 MDa), oxidants (0.4 MDa) and surfactants (0.6 MDa). If the results got with NaClO are not surprising given its oxidizing nature, surfactants action is more unexpected; however, many data used here for creating the average molecular weight data achieved with surfactants come from a cellular lysis process composed by a pre-treatment with NaClO, followed by treatment with the surfactant itself. Another common pretreatment is a drying step prior to the polymer extraction (freeze-drying or thermal treatment); according to Koller et al. (2013), these pretreatments enhanced the polymer extraction causing the hydrolytic shortage of the polyester chains, more intrusive in the case of heat treatments than freeze-drying.

PHA recovered from MMC have much lower molecular weight averages than single strains, especially in the case of alkali and surfactants; this finding can be a confirmation of the higher resistance of MMC membranes than single strain membranes toward a "chemical" agent. Such resistance decreases when a strong oxidant is used, giving similar average molecular weights between single and mixed bacteria.

Purity

The purity level of PHA extracted with solvents is very high (above 90%, **Figure 8**), independent by the type of solvent and the bacteria (single strains or MMC), the variability of this parameter is the lowest among those of all the parameters here used for comparing the extraction methods found in the literature (recovery and molecular weight). The PHA purity values obtained from cellular lysis approaches are lower, especially when MMCs are used (values below 90% with all the chemicals used, **Figure 9**). Larger variabilities can be noticed with this approach in comparison to solvent-based protocols, especially for less harsh treatments like the ones with surfactants and alkaline compounds.

CONCLUSION

Considering the whole literature about PHA recovery from single strains and MMC it appears clear that a large variability of the results can be found. However, this variegate scenario strictly reflects the variability of bacteria, PHA initial content (that can influence the strength of the membranes), PHA type (homopolymers or co-polymers, and the ratio between short and long monomers), and recovery methods (temperature, pressure, need of pretreatments, concentrations of chemicals,. . .). Such variability is especially relevant for MMC, and this can be attributed to the variability of PHA content of MMC and the chemical features of MMC-derived PHA.

A focus on two of the most crucial parameters (purity and molecular weight) that can influence the applicability of the recovered PHA reveals that the solvent extraction approach generally gives purity above 98%, and molecular weight values above 0.8 MDa in the case of halogenated solvents and carbonates; lower values are got with ketones and alcohols, reasonably for a lower affinity of these class of solvents for PHA and not for a real shortening-chain effect.

The cellular lysis approach provides higher recoveries than the solvent-based approach, mainly due to the definition of "extraction yields" within such procedure. Given that the "extraction yields" are defined as the amount of PHA that stays in the solid phase, the PHA losses during cellular lysis are mainly due to severe PHA degradation. It follows that the two issues that affect the cellular lysis approach are the PHA purity and quality (e.g., molecular weight and PDI), and both fall in a quite wide range independent of single strains or MMC. The lower average purity obtained in some studies presented in the literature (70–80%) could be considered closer to a "refined microbial biomass" than "extracted PHA."

AUTHOR CONTRIBUTIONS

GP developed the whole manuscript preparing and integrating the different parts. PG participated in the manuscript design and revised the manuscript. CS conceived the study and contributed to write the chemical aspects of the work and revised the manuscript. AZ contributed to collect and study the references cited. CT contributed to write the aspects of efficiency issues of PHA extraction and revised the manuscript. All the authors read and approved the final manuscript.

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Characterization of Polyhydroxyalkanoates Produced at Pilot Scale From Different Organic Wastes

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Polyhydroxyalkanoates (PHAs) production at pilot scale has been recently investigated and carried out exploiting different process configurations and organic wastes. More in detail, three pilot platforms, in Treviso (North-East of Italy), Carbonera (North-East of Italy) and Lisbon, produced PHAs by open mixed microbial cultures (MMCs) and different organic waste streams: organic fraction of municipal solid waste and sewage sludge (OFMSW-WAS), cellulosic primary sludge (CPS), and fruit waste (FW), respectively. In this context, two stabilization methods have been applied, and compared, for preserving the amount of PHA inside the cells: thermal drying and wet acidification of the biomass at the end of PHA accumulation process. Afterward, polymer has been extracted following an optimized method based on aqueous-phase inorganic reagents. Several PHA samples were then characterized to determine PHA purity, chemical composition, molecular weight, and thermal properties. The polymer contained two types of monomers, namely 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) at a relative percentage of 92.6–79.8 and 7.4–20.2 w/w, respectively, for Treviso and Lisbon plants. On the other hand, an opposite range was found for 3HB and 3HV monomers of PHA from Carbonera, which is 44.0–13.0 and 56.0–87.0 w/w, respectively. PHA extracted from wet-acidified biomass had generally higher viscosity average molecular weights (M_V) (on average 424.8 ± 20.6 and 224.9 ± 21.9 KDa, respectively, for Treviso and Lisbon) while PHA recovered from thermally stabilized dried biomass had a three-fold lower M_V.

Keywords: polyhydroxyalakanoates, mixed microbial culture (MMC), thermal properties, molecular weight, urban waste

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INTRODUCTION

Among different kinds of biopolymers, polyhydroxyalkanoates (PHAs) are one of the most interesting and promising thanks to their mechanical properties and biodegradability. As an alternative to industrial scale production based on pure culture (Gholami et al., 2016), the application of low-cost substrates based on organic wastes and by-products is of an extreme interest, specially coupled with the use of mixed microbial cultures (MMCs) that do not require

sterile conditions or excessive operation control (Serafim et al., 2008a; Nikodinovic-Runic et al., 2013; Echegoyen et al., 2017). In fact, it is possible to combine the use of both MMCs (Beccari et al., 1998; Dionisi et al., 2004) and economic feedstocks (Dionisi et al., 2005; Bengtsson et al., 2008; Albuquerque et al., 2010; Bengtsson et al., 2010b; Campanari et al., 2014). Recently, the conversion of both wastewaters and volatile fatty acids (VFAs) mixtures obtained from the fermentation of different organic feedstocks, by MMC, have been investigated for the implementation of such a three-step process at pilot scale, by the integration of MMC-PHA production to wastewater treatment (Valentino et al., 2019; Conca et al., 2020; Moretto et al., 2020). However, downstream processing, including PHA-rich biomass stabilization and polymer extraction, are among the most important factors affecting the overall PHA production cost and need to be optimized for pilot scale. The most studied methods for recovering PHAs have been summarized in recent reviews (Kosseva and Rusbandi, 2018; Pérez-Rivero et al., 2019) and can be grouped into two main categories: solvent extraction and digestion of the NPCM (non-PHA cellular material). Up to now, regarding the MMC PHA-rich biomass, the use of several non-chlorinated solvents (Werker et al., 2014; Samorì et al., 2015) and, more recently, the disruption of NPCM through chemical agents (Burniol-Figols et al., 2020) and surfactants (Colombo et al., 2020) have been investigated. In the view of MMC-PHA production at industrial scale and a subsequent market scenario, specific chemo-mechanical and thermal properties of the polymer need to be controlled. In a recent study, two types of stabilization method have been applied at pilot scale on PHA obtained from organic wastes, in order to preserve the amount of PHA produced after the accumulation step and its properties (Lorini et al., 2020). The recovered polymer was extensively characterized and resulted in good thermal and chemical properties, while it showed variable molecular weight (between 100 and 450 kDa) dependent on the applied stabilization method. Indeed, molecular weight should be higher than 200 kDa and it is influenced by the PHA production process and downstream, including the extraction step (Laycock et al., 2014b). The availability in literature regarding the chemo-mechanical and thermal characterization of MMC-PHAs is very limited, however, the published studies highlighted an increase in melting temperature (T_m) for MMC-PHA respect to pure culture derived PHA (Bengtsson et al., 2010b) and a similar decomposition temperatures (270°C) to the commercial poly-3-hydroxybutyrate-co-3-hydroxyvalerate [P(3HB-co-3HV)] (Carrasco et al., 2006). Furthermore, monomeric composition, i.e., 3HV content, showed to have effects on different polymer properties (Arcos-Hernández et al., 2013; Lorini et al., 2020), such as crystallinity.

Thus, the evaluation of the quality of waste-derived PHA and the comparison with the properties of traditional plastics are necessary for commercial purposes and they must meet the standards required for various applications. Furthermore, for the scaling up of MMC-PHA production it is important to optimize an extractive protocol in order to minimize the overall production costs. On the other hand, the concept of novel biorefineries for urban waste valorization allows to integrate MMC–PHA production with wastewater treatment and other biowaste collection, reducing the production costs and the environmental impact. In this regard, the present study shows the results of a wide characterization, including thermal and chemical properties, of several samples of three grades of PHAs, produced by three pilot plants for PHA production from biowaste and recovered with the same extractive method (based on the use of aqueous-phase inorganic reagents). Specifically, the three pilot units produced PHA from different organic feedstocks: the organic fraction of municipal solid waste (OFMSW) and waste activated sludge (WAS) in Treviso plant; fruit waste in Lisbon; cellulosic primary sludge (CPS) in Carbonera.

MATERIALS AND METHODS

The effects of an extraction method based on aqueous-phase inorganic reagents were evaluated on PHAs recovered from different biomasses, coming from three pilot plants using three types of feedstock, named TV-samples (from Treviso, OFMSW-WAS), L-samples (from Lisbon, FW), and C-samples (from Carbonera, CPS).

PHA From Treviso Pilot Plant

Within the pilot platform of Treviso (northeast Italy), the PHA was produced from a feedstock composed by a mixture of the liquid slurry coming from squeezed OFMSW and WAS from the treatment of municipal wastewater. For the sake of simplicity, this feedstock mixture is indicated as OFMSW-WAS. The latter was characterized by a volatile/total solids ratio of 80 ± 1% (VS/TS); a soluble chemical oxygen demand (COD_{SOL}) of 22 ± 1 (gCOD L^{-1}); total Kjeldahl nitrogen (TKN) equal to 29 ± 3 (g N kg^{-1} TS) and total phosphorus (P) equal to 2.3 ± 0.1 (g P kg^{-1} TS) (Moretto et al., 2020). The main process setup and operative conditions are extensively described in Valentino et al. (2019) and Moretto et al. (2020). The pilot plant consisted in a first anaerobic fermentation reactor (380 L) for PHA-precursors production (VFA), a second aerobic reactor (sequencing batch reactor, SBR; 100 L) for biomass cultivation, and a third fed-batch aerobic reactor (70–90 L) for PHA accumulation inside the cells (40–50% wt). This PHA-rich biomass (raw biomass) was collected following two different protocols of biomass stabilization, both addressed to the long-term PHA conservation inside the cells before the extraction/purification steps.

Following the first protocol, at the end of each accumulation, the PHA-rich biomass was left to settle under gravity and then the thickened slurry was centrifuged in a Heraeus Megafuge 40 with a Swinging Bucket Rotor (maximum radius: 195 mm; minimum radius: 83 mm) from Thermo Fisher Scientific (Waltham, MA, United States) for 15 min at 4,500 rpm. The wet pellet was thermally pre-treated at 145°C for 15 min, and then dried overnight in the same oven at 60°C (sample TV20).

In the second protocol, at the end of each accumulation, the PHA-rich biomass was acidified with H_2SO_4 down to pH 2.0, and left to settle under gravity overnight. The thickened slurry was then centrifuged in a Heraeus Megafuge 40 with a Swinging Bucket Rotor (maximum radius: 195 mm; minimum

radius: 83 mm) from Thermo Fisher Scientific (Waltham, MA, United States) for 15 min at 4,500 rpm. Finally, the wet pellet was stored in the fridge (4°C) (samples TV1-12).

PHA From Lisbon Pilot Plant

The concept of PHA production process is similar to that previously described. The main difference is the kind of the feedstock, which in this case was organic waste coming from fruit processing. The FW was characterized by a high VS/TS ratio (98.0 ± 2.27%); a COD_{SOL} equal to 3.76 gCOD/L; very low ammonia ($N\text{-}NH_4^+$) and phosphate ($P\text{-}PO_4^{3-}$) concentration, 1.26 ± 0.07 mg N/L and 2.39 ± 0.67 mg P/L, respectively. The pilot units in Lisbon's facility consists in three reactors: an upflow anaerobic sludge bioreactor (UASB) with a volume of 60 L, a sequential batch reactor (SBR) with a volume of 100 L and a stirring tank reactor operating in fed-batch mode with 60 L of volume. Fermented FW was the feedstock for both biomass cultivation and PHA accumulation steps. The selected biomass was able to accumulate the polymer up to 70% of its cell dry weight. At the end of accumulation operation, the second stabilization protocol (described in section "PHA From Treviso Pilot Plant") was applied: the reactor slurry was settled to remove as much water as possible and the biomass was acidified down to pH of 2.0–2.5, in order to prevent polymer degradation. The PHA-rich biomass was then stored in a fridge (4°C) before extraction.

PHA From Carbonera Pilot Plant

The pilot plant located in Carbonera wastewater treatment plant (WWTP) (Treviso, Italy) integrated the via-nitrite nitrogen removal with PHAs production from the sidestream of Carbonera WWTP (Treviso, Italy). A novel substrate derived from fermentation of CPS was used as carbon source to evaluate the impacts on PHAs production and it was characterized by a VS/TS ratio equal to 92%; a relatively high content in terms of COD (1.5 g O_2/g TS) and a low content of TKN (16/22 mg N/g TS) and P (1.7/4.7 mg P/g TS) (Da Ros et al., 2020). Process setup and operative conditions are extensively described in Conca et al. (2020). The pilot plant comprised the following units: i) rotating belt dynamic filter (RBDF) for the recovery of CPS; ii) fermentation unit for the production of VFAs; iii) ultrafiltration unit (UF) for solid/liquid separation of the fermented sludge; iv) nitritation sequencing batch reactor (N-SBR) for the oxidation of ammonia to nitrite; v) selection SBR (S-SBR) where aerobic-feast and anoxic-famine conditions were established to select PHA-accumulating biomass; and vi) an accumulation SBR (A-SBR) were intracellular PHA content was maximized through the feed-on-demand strategy. The S-SBR consisted in a stainless-steel SBR with a total volume of 2.8 m^3 and operated under (aerobic)-feast and (anoxic)-famine conditions. The accumulation reactor (A-SBR) consisted in a stainless-steel tank with a volume of 1 m^3 which operated as a fed-batch reactor. At the end of accumulation operation, the second stabilization protocol (described in section "PHA From Treviso Pilot Plant") was applied: the reactor slurry was settled to remove as much water as possible and the biomass was acidified down to pH of 2.0–2.5, in order to prevent polymer degradation. The PHA-rich biomass was then stored in a fridge (4°C) before extraction.

PHA Extraction

Aqueous Phase Inorganic Reagents (Reserved Protocol)

An extraction method involving a mixture of aqueous phase inorganic reagents has been optimized by Biotrend S. A. for PHA recovery from the cellular material of enriched biomass. The optimized method was carried out on PHA-rich biomasses coming from the three previously described pilot plants, stabilized at the end of the accumulation step following the thermal (sample TV20) and the acidification protocol (samples TV 1-12; L-samples; C-samples). At the end of the extraction, the polymer was dried obtaining a white powder. The optimized extraction method is reserved by Biotrend S. A. and under patent application procedure.

PHA Characterization

PHA Content and Composition Determination (GC-FID)

Approximately 3.5 mg of dried biomass or 5.0 mL of re-suspended samples (wet and dried thermally treated biomasses) were suspended in 2 mL of acidified methanol solution (at 3% v/v H_2SO_4) containing benzoic acid (at 0.005% w/v) as internal standard and 1 mL of chloroform in a screw-capped test tube. Then, an acid-catalyzed methanolysis of the PHA occurred and the released methyl esters were quantified by gas-chromatography (GC-FID Perkin Elmer 8410) according to the method described in Braunegg et al. (1978). The relative abundance of 3HB and 3HV monomers was determined using a commercial P(3HB-co-3HV) copolymer with a 3HV content of 5 wt. % (Sigma–Aldrich, Milan, Italy) as reference standard. The 3HV content in PHA was calculated as the ratio of 3HV and (3HB + 3HV) monomers (as wt. %).

Capillary Viscosimetry

A volume of 25 mL of PHA solution at the concentration of 0.5% w/v in chloroform were prepared for all the samples of extracted polymer. The equipment for the measure of the gravimetric flow time of each solution was composed by a SCHOTT AVS 350 viscosimeter with AVS/SHT sensor, a LAUDA CD15 thermostatic bath (working at 30°C) and a SCHOTT GERÄTE Ubbelohde capillary viscosimeter (ID = 0.46 mm). For each sample, 15 mL of solution 0.5% w/v were transferred in the Ubbelohde capillary viscosimeter and the flow time was measured through the optical sensor. Then, at least four dilutions were made directly in the viscosimeter by adding predetermined aliquots of solvent. From the flow time of each solution the intrinsic viscosity [η] of polymer samples was determined. The viscosity average molecular weight (M_v) was calculated according to the Mark–Houwink equation:

$$M_v \rightarrow [\eta] = K \cdot M_v^{\alpha} \quad (1)$$

with $K = 7.7 \times 10^{-5}$ e $\alpha = 0.82$ (Marchessault et al., 1970)

Thermogravimetric Analysis (TGA)

The thermal stability of the samples was evaluated by TGA using a Mettler TG50 thermobalance. Approximately 5 mg of dried samples were weighted on the balance. The analysis was conducted in nitrogen flow (20 mL min^{-1}) by heating the samples from 30 to 500°C at 10°C min^{-1}.

Differential Scanning Calorimetry (DSC)

The thermal properties of the PHA samples were characterized by a differential scanning calorimeter Mettler Toledo DSC 822e. All the experiments were carried out under N_2 flux (30 mL min^{-1}) on about 3–6 mg of polymer, weighted in aluminum pans. TV-samples were analyzed applying a single heating scan at 10°C min^{-1} from room temperature to 190°C, in order to evaluate the real thermal behavior of the final product.

The sample crystallinity (X_c) was evaluated from the equation:

$$X_c = 100 \cdot \Delta H_m / (\Delta H_m^0 \cdot \text{purity}) \quad (2)$$

where ΔH_m is the melting enthalpy obtained from the single DSC heating scan and $\Delta H_m{}^0 = 146$ J g^{-1} is the enthalpy of fusion of 100% crystalline PHB sample (Barham et al., 1984).

On the contrary, in order to evaluate the intrinsic thermal properties, the following temperature program was selected for the analysis of C-samples:

1. a heating scan at 10°C min^{-1} from RT to 190°C;
2. a rapid cooling at 30°C min^{-1} from 190 to −70°C;
3. a second heating up to 190°C at 10°C min^{-1}.

The first heating is necessary to erase all the previous sample history.

PCA Analysis

Principal component analysis (PCA) was conducted using the software The Unscrambler ver. 10.5 (CAMO Software AS, Oslo, Norway).

RESULTS

Three grades PHA produced from three different organic wastes have been extracted and characterized. For the sake of a deeper discussion, the characteristics of fermented streams and the results in terms of overall PHA production yield of the three pilot plants are here reported. Within the pilot plant located in Treviso, the fermented mixture of OFMSW – WAS showed a predominance of acetic acid (24–34%) and propionic acid (10–20%), along with butyric acid (30–40%) and caproic acid (25–35%) fed to both selection and accumulation steps for PHA production. The overall process yield was calculated and resulted of 110 g PHA/kg VS (Moretto et al., 2020). On the other hand, pilot plant in Lisbon produced a fermented stream, starting from FW, characterized by a predominance of butyric and acetic acid, along with propionic acid, valeric acid, lactic acid and ethanol, with a resulting fermentative products concentration of 21.2 ± 1.6 gCOD L^{-1}. As for Treviso process, the fermented stream fed both the selection and accumulation steps. It was estimated that 37.4 L of FW are necessary to produce 1.0 kg of PHA and then the resulted global yield of conversion was 26% on COD basis (*data under submission*). Finally, the fermentation of CPS in Carbonera pilot plant led to a fermented stream characterized by the presence of acetic, propionic, butyric and valeric acid and a quite high molar ratio between even and odd VFAs [0.49–0.52% mol/mol $C_3/(C_2 + C_3)$]. The fermented stream CPS fed both the selection and the accumulation steps. As a result, the PHA production represented up to 17.5% of the COD conversion and a potential PHA production of 1.2 kg PHA/PE year was calculated (Conca et al., 2020).

Purity and Monomeric Composition

Results obtained by GC-FID analysis conducted on extracted samples (**Table 1**) showed that the optimized extraction method allowed to reach very high purity, with an average value of 103.4 ± 2.1% w/w. The high PHA content in the recovered material was confirmed through TGA analysis. In fact the PHA content (% w/w), that indicates the purity of the sample, showed an average value of 98.9 ± 0.7% w/w. Sample TV20, extracted from a thermal stabilized biomass, had the lowest purity (85.5 ± 3.6% w/w). In **Table 1**, the 3HV content quantified from GC-FID analysis is also reported. It is possible to notice that samples from OFMSW-WAS and from FW are characterized by a 3HV content ranging between 16.3 and 19.1% w/w, with some exceptions. More in detail, samples TV20, L1, L2, and L3 (having 3HV content between 7.4 and 7.7% w/w) have been produced during the first operative year, while the remaining samples have been produced during the following operative years. In both cases, the two pilot platforms in Treviso and in Lisbon were operated in parallel, in order to obtain polymers with the same monomeric composition. Indeed, VFAs composition of the fermented mixtures, characterized by the predominance of even VFAs, led to a high 3HB percentage. On the other hand, a higher variability and an opposite trend were found for C-samples, in fact 3HV content is higher than 3HB, varying from 56 to 87% w/w, as a direct consequence of the high odd VFAs content in the feedstock (Albuquerque et al., 2011).

Thermal Characterization

Thermal characteristics of the extracted polymers, such as decomposition temperatures and related thermal stability, melting temperature and melting enthalpy have been determined through TGA and DSC. The results of the TGA experiments, including the PHA content (discussed in the previous section), the temperature at 10% decomposition ($T_d{}^{10\%}$) and the temperature at maximum decomposition rate ($T_d{}^{max}$), are reported in **Table 1**, while melting temperature (T_m) and melting enthalpy (ΔH_m) from DSC are reported in **Table 2**. The comparison of the results showed that PHA thermal stability seems not to be affected by the feedstock used for PHA production. In fact, the average $T_d{}^{10\%}$ and $T_d{}^{max}$ of extracted TV-samples were 265 ± 3°C and 288 ± 3°C while for L-samples the corresponding values were 257 ± 5°C ($T_d{}^{10\%}$) and 276 ± 5°C ($T_d{}^{max}$) respectively. On the contrary, C-samples showed a lower thermal stability, with an average $T_d{}^{10\%}$ and $T_d{}^{max}$ at 238 ± 4°C and 257 ± 3°C, respectively. The DSC profiles of sample TV1

TABLE 1 | Chemical and thermal properties of PHA samples produced in Treviso, Lisbon, and Carbonera pilot plants.

Sample	Feedstock	Pretreatment	Extraction	GC Purity (PHA % w/w)	TGA Purity (PHA % w/w)	T^d_{max} (°C)	$T^d_{10\%}$ (°C)	HV (% w/w)	References
*TV20	Treviso (OFMSW-WAS)	Wet acidification	Optimized aqueous-phase inorganic extraction method	85.5 ± 3.6	86	307	289	7.67	This study
TV1				92.8 ± 1.9	100	274	249	18.9	
TV2				95.7 ± 0.6	100	299	274	18.3	
TV3				98.5 ± 3.8	100	295	271	18.6	
TV4				99.2 ± 2.4	100	284	260	18.4	
TV5				92.5 ± 1.3	98	278	253	18.1	
TV6				105.1 ± 1.2	100	276	251	18.9	
TV7				97.4 ± 1.6	100	293	267	18.2	
TV8				108.4 ± 1.3	100	290	264	19.1	
TV9				95.8 ± 3.9	100	289	270	16.3	
TV10				99.3 ± 0.1	100	285	264	16.3	
TV11				99.4 ± 1.1	100	285	263	18.8	
TV12				99.7 ± 3.1	99	290	268	18.6	
L1	Lisbon (FW)			100.3 ± 3.1	94.6	270	247	7.7	
L2				100.9 ± 1.3	98.5	302	286	7.4	
L3				92.0 ± 0.3	93.7	272	254	7.4	
L4				116.1 ± 4.0	94.1	272	258	17.7	
L5				104.7 ± 3.1	97.1	279	259	17.7	
L6				97.7 ± 1.8	99.4	290	273	17.2	
L7				94.4 ± 0.8	97.1	266	243	17.7	
L8				117.8 ± 3.3	99.6	257	236	16.8	
L9				114.4 ± 2.5	99.4	279	259	16.3	
C1	Carbonera (CPS)			119.4 ± 0.4	96.9	257	241	87	
C2				122.8 ± 0.7	100	265	244	79	
C3				128.9 ± 5.9	100	252	229	85	
C4				111.1 ± 3.8	97.6	253	238	56	
MMC-PHA	OFMSW-WAS	Thermal drying	$CHCl_3$ NaClO	75.2–100.9	75–100	269–303	252–278	7.0–20.2	Lorini et al. (2020)
MMC-PHA	OFMSW-WAS	Wet acidification	NaClO	95.8–97.5	99–100	289–293	268–270	13–13.6	
MMC-PHA	WAS	Freeze-drying	$CHCl_3$	–	–	275	–	42	Frison et al. (2015)
MMC-PHA	WAS	Drying	$CH_3CH_2CH(OH)CH_3$	–	–	291	–	26–34	Morgan-Sagastume et al. (2015)
MMC-PHA	Molasses	–	$CHCl_3$	–	–	277–291	–	13–53	Bengtsson et al. (2010a)
MMC-PHA	Crude glycerol	Freeze-drying	Sonication + NH_3	86	89	302–307	–	13	Burniol-Figols et al. (2020)
MMC-PHA	Dairy wastes	Freeze-drying	Non-ionic surfactants + DMC	57–92	78–90	242–255	–	9	Colombo et al. (2020)

**Thermal stabilized sample.*

TABLE 2 | Thermal properties and crystallinity of PHA samples produced in Treviso and Carbonera pilot plants.

Sample	Feedstock	T_m (°C)	ΔH_m (J g^{-1})	χ_c (%)	T_g (°C)	References
TV1	Treviso (OFMSW-WAS)	167	41.8	28.6	–	This study
TV2		168	37.3	25.5	–	
TV3		171	47.7	32.7	–	
TV4		168	28.1	19.2	–	
TV5		168	38.2	26.1	–	
TV6		169	39.9	27.4	–	
TV7		170	38.8	26.6	–	
TV8		168	37.9	25.9	–	
TV9		170	48.5	33.2	–	
TV10		168	44.5	30.5	–	
TV11		167	36.2	24.8	–	
TV12		168	43.1	29.5	–	
C1	Carbonera (CPS)	104.5	102	–	–19	
C2		94	64	–	–14.5	
C3		104.7	96	–	–22	
C4		83	36.3	–	–12.5	
MMC-PHA	OFMSW-WAS	157–169	26–45	30–42	–	Lorini et al. (2020)
MMC-PHA	WAS	144	24	–	–0.5	Frison et al. (2015)
MMC-PHA	Cheese Whey	144–163	–	–	–3; 1	Hilliou et al. (2016)
MMC-PHA	Olive Mill Wastewater	150	–	–	0	
MMC-PHA	Molasses	144–165	5–17	–	–10.3; –3.3	Bengtsson et al. (2010a)
MMC-PHA	Synthetic VFA mix	150–172	20–82	–	–14; 4.8	
MMC-PHA	Synthetic VFA mix	153	35	–	–12.8	Laycock et al. (2014a)
MMC-PHA	Crude glycerol	154	–	–	–6.8	Burniol-Figols et al. (2020)
MMC-PHA	Dairy wastes	146–147	13–51	9–34	–7.3; –5	Colombo et al. (2020)

is reported in **Figure 1**. The thermograms of the other TV samples did not remarkably change from that of TV1. In all the thermograms the main transition is the melting process occurring in the 160–175°C temperature range, but also a low temperature transition at about 60°C occurred.

On the other hand, C-samples showed a different thermal behaviour, indeed the melting process occurred in a wider range shifted at lower temperature (80–105°C). Moreover, for C-samples, the glass transition occurred after the cooling step and it ranges between −22 and −12.5°C.

From the integrated melting enthalpy, the sample crystallinity was evaluated by Eq. 2 for TV-samples and then reported in **Table 2**. On the contrary, while there is adequate agreement between the results obtained from DSC for the P(3HB) homopolymer, in PHBV copolymers, it is quite difficult to measure a true crystallinity, particularly for copolymers with 3HV content higher than 20% w/w (Laycock et al., 2014b). Thus, in order to compare the melting behavior, the melting enthalpies (ΔH_m) of TV and C-samples, determined by DSC, are reported as a function of 3HV content in **Figure 2**. Moreover, for a more complete evaluation of the 3HV content effects, results from a previous work (Lorini et al., 2020) are reported, too. In this way, it was possible to have a wider range of 3HV percentage, specifically between 7.7 and 87% w/w. The results clearly show that ΔH_m linearly increased with the 3HV content in the C-samples copolymer (3HV % w/w = 56-87), while it linearly decreased for the whole TV-samples (3HV % w/w = 7.7-20.2).

Viscosity Average Molecular Weight

The determination of viscosity average molecular weight (M_v) of each extracted sample gave different results for the three grades of collected material.

The results, reported in **Figure 3**, clearly appeared divided in two main groups: TV-samples and L and C-samples, with average values of 430 ± 24 and 208 ± 20 kDa, respectively, and one outlier for each group (102 and 508 kDa, respectively, for TV20 and C2).

PCA Analysis

Principal component analysis was defined as an effective statistical method used to establish relationships between different observations, such as similarities and inequalities.

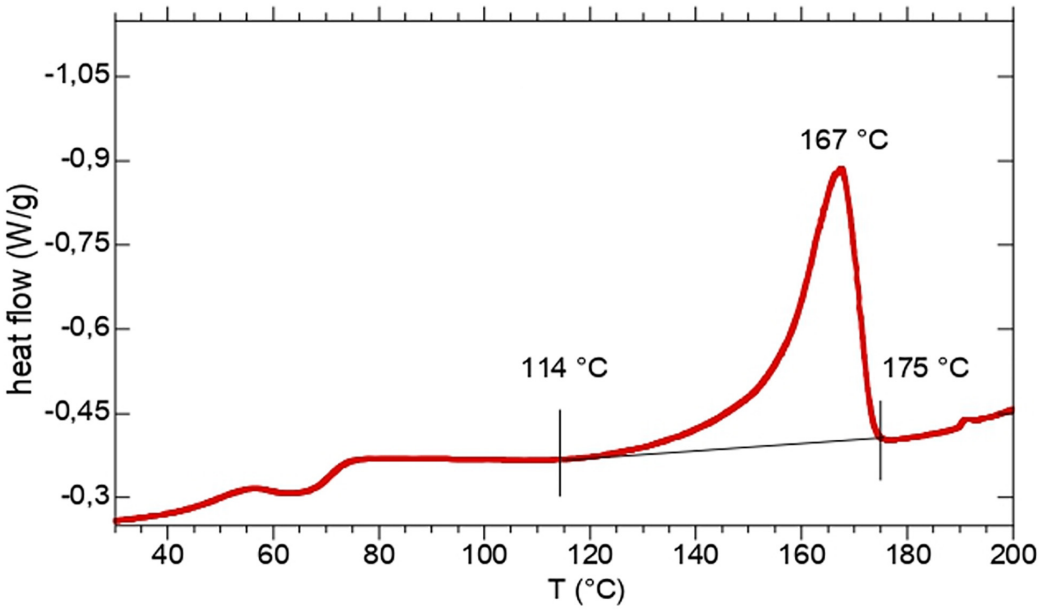

FIGURE 1 | Single heating scan from DSC thermogram of sample TV-1.

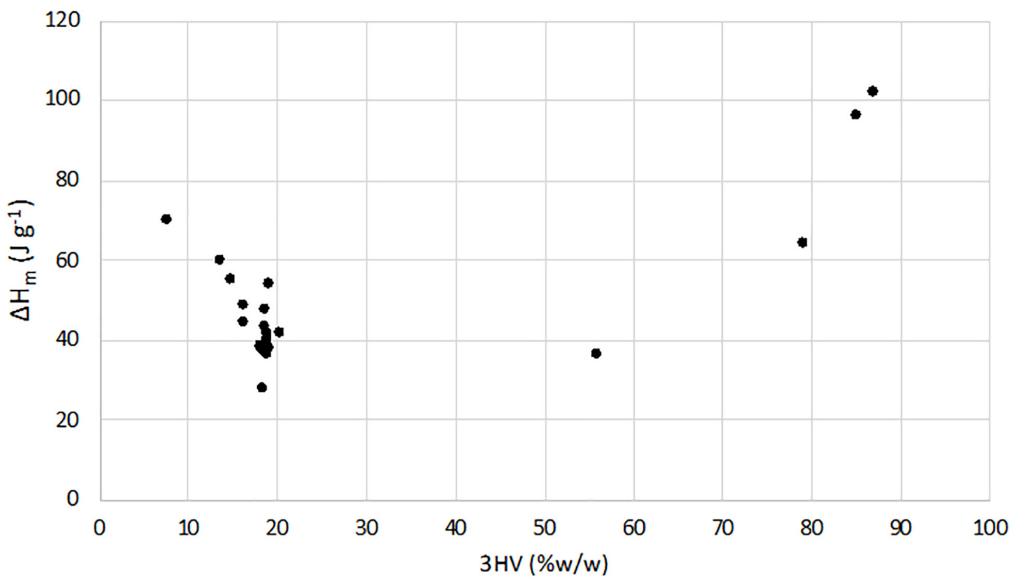

FIGURE 2 | Relationship between ΔH_m (J g^{-1}) and monomeric composition (3HV content).

According to the PCA results, the first principal component (PC1) accounted for 48% of the total variance, 31% for the 2nd component (PC2). **Figure 4A** shows the score plot graphic and **Figure 4B** shows the loading plot graphic for PC1 and PC2 in PHA samples. When **Figures 4A** and **B** are evaluated together, analysis of the data obtained from the complete characterization of the PHA samples with the PCA allowed grouping the PHA samples into three groups to express and demonstrate their similarity and differences.

DISCUSSION

Purity and Monomeric Composition

Recovery procedures for PHA from MMC, including non-chlorinated solvents extraction and disruption of NPCM through chemical or oxidizing agents, were widely investigated in the last decade as alternatives to traditional chloroform extraction (Werker et al., 2012; Villano et al., 2014; Samorì et al., 2015).

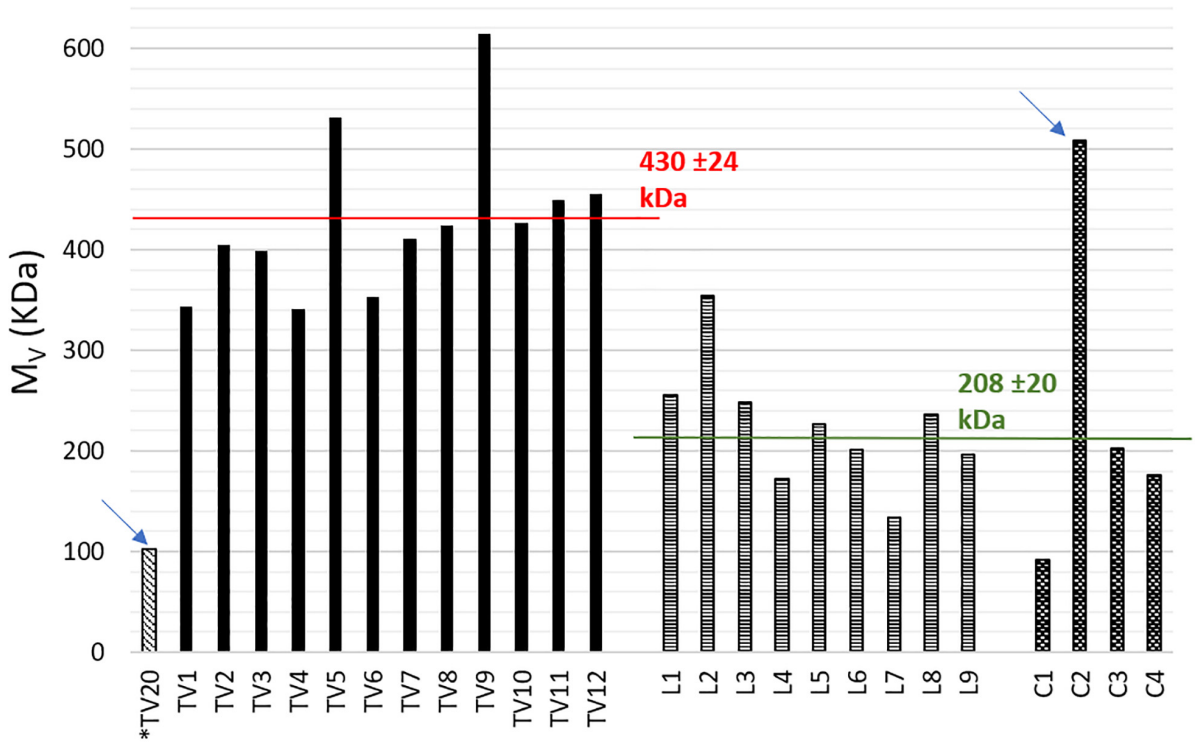

FIGURE 3 | Comparison of viscosity average molecular weights (M_v). Starred sample TV20 is thermal stabilized.

More recently, disruption of NPCM was obtained by using non-ionic surfactants as pretreatment before PHA extraction with DMC (Colombo et al., 2020) and by treating the biomass with ammonia solutions at high temperatures (Burniol-Figols et al., 2020). In a previous study (Lorini et al., 2020), NaClO digestion was investigated for PHA recovery from MMC produced at pilot scale. More in detail, PHA was produced in the pilot plant operating in Treviso, exploiting the same feedstock (OFMSW-WAS) and operative conditions and applying the same biomass stabilization methods described in this work. In this study, the effects of the extraction method optimized by Biotrend S. A. were evaluated on PHAs recovered from different biomasses. The extraction has been conducted on the wet acid stabilized centrifuged biomass for the whole set of PHA sample, except for sample TV20. Indeed, the latter was extracted from a thermal-stabilized dry biomass, produced during the first operative year of the pilot platform together with the samples analyzed elsewhere (Lorini et al., 2020). Results of the latter study are reported in **Table 1** for a comparison. As explained in section "Purity and Monomeric Composition of Results," sample TV20 had a lower purity, comparable with those thermal dried determined in the reference work by Lorini et al. (2020), than the rest of the whole set. Compared to other reference studies, in which NPCM disruption was conducted, the extraction method discussed in this work shows better performances in terms of polymer purity (higher than 92.0% w/w), in fact using NH_3 (Burniol-Figols et al., 2020) or a combination of non-ionic surfactants and DMC (as PHA solvent) (Colombo et al., 2020) allowed to reach a PHA purity of 86 and 57 − 92% w/w, respectively. This evidence suggests that the optimized extraction method can be considered effective on all the types of biomass, in fact the same results were obtained independently from the PHA origin, that is kind of feedstock and operative conditions.

Thermal Characterization

The purity obtained by GC-FID was confirmed through TGA analysis, in fact the polymer content in the samples was estimated from the main weight loss occurring between 250 and 320°C, as reported in literature (Hahn and Chang, 1995).

Moreover, in terms of thermal stability, the C-samples, with the highest 3HV content (56-87% w/w), showed the lowest degradation temperatures (on average $T_d{}^{10\%}$ and $T_d{}^{max}$ at 238 ± 4°C and 257 ± 3°C, respectively). On the other hand, samples TV20 and L1, L2, L3, L4, with the lowest 3HV content (7.6% w/w) gave non-homogeneous results, indeed TV20 exhibited the highest $T_d{}^{10\%}$ (289°C) and $T_d{}^{max}$ (307°C), while L-samples had lower values. Hence, the variability of the reported available data (**Table 1**) did not allow to enunciate a statement on the possible effect of monomeric composition on thermal stability.

Overall, such values indicated the high thermal stability of the polymer, comparable to those observed in other studies on MMC-PHA (**Table 1**) and on commercial P(3HB-co-3HV) ($T_d{}^{max}$ = 256–290°C; Morgan-Sagastume et al., 2015). DSC analysis allowed to evaluate the thermal behaviour of the three

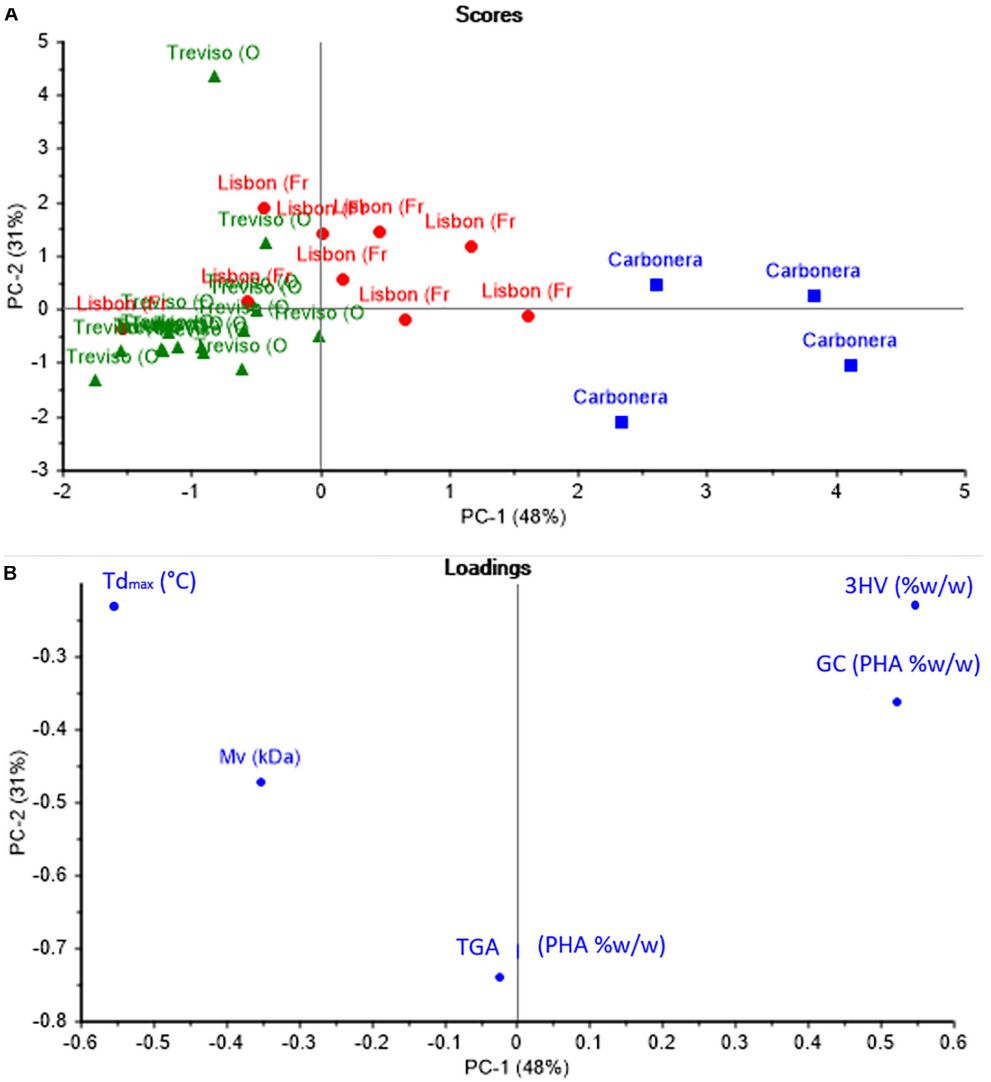

FIGURE 4 | Complete PCA. **(A)** Scores plot. **(B)** Loadings plot.

grades PHA. The melting temperature determined for TV-samples, ranging in 130–175°C, resulted comparable or higher than those reported in literature (**Table 2**). The low temperature exothermic transition at about 60°C could be considered as a cold crystallization (**Figure 1**), probably due to a partial crystallization started at low temperature during the extraction process (Wellen et al., 2015). Different thermal properties were found for PHA from Carbonera, in fact C-samples presented a lower T_m than TV and L-samples, and a T_g comparable or even lower than those reported in previous studies (**Table 2**). In fact, Bengtsson et al. (2010b) reported a T_g of −10.3°C and −14°C for PHA with 53 and 63% mol 3HV, produced from molasses and synthetic VFA solution, respectively. On the other hand, Serafim et al. (2008b) characterized a PHA, produced with a synthetic feedstock and having a 3HV content of 72% mol, which presented a T_m of 99.2°C and a T_g of −29.6°C, more similar to those of C-samples. For TV-samples, the determination of melting enthalpy allowed to calculate the crystallinity and to confirm the semicrystalline behavior of copolymer P(3HB-co-3HV) (Bengtsson et al., 2010a). Indeed, TV-samples are characterized by a χ_c (%) comparable to that reported in the previous work of Lorini et al. (2020) and in a study from Colombo et al. (2020) (**Table 2**). As reported in literature, it is quite difficult to determine the crystallinity of PHBV copolymers having 3HV content higher than 20%, since the melting process is affected by the presence of amorphous regions (Laycock et al., 2014b). Thus, **Figure 2** shows a typical trend of ΔH_m as a function of 3HV content, indeed, as reported in Laycock et al. (2014b), a minimum in the melting enthalpy at 30–55% HV occurs.

Overall, these results highlight that the PHA samples, obtained with different process configuration and different feedstock, did not differ in thermal behaviour from other MMC-PHAs, independently from the kind of substrate exploited.

Viscosity Average Molecular Weight

As mentioned in section "Viscosity Average Molecular Weight" of Results, **Figure 3** shows the results of M_v determination. They are divided in two main groups having average values of 430 ± 24 and 208 ± 20 kDa.

To better understand the reason of this marked difference, it is useful to make a comparison between TV-samples of the present work and those of Lorini et al. (2020) (same pilot plant, feedstock, and stabilization methods).

As a main result of the previous work, the average M_v values of thermal dried samples were 127 ± 16 and 132 ± 10 kDa for NaClO- and $CHCl_3$-extracted samples, respectively. Hence, no effects of the extraction step were found. Indeed, chloroform extraction was considered as a benchmark and it was concluded that NPCM disruption had not damaged the polymer. In this sense, we can consider NaClO extraction of the previous work as a benchmark, because it was applied on biomasses obtained in the same operative periods and with the same operative conditions. More in detail, sample TV-20 was thermally stabilized and obtained in parallel with thermal dried samples of the previous work, while samples TV-1-12 were acid stabilized and obtained in parallel with two acid stabilized samples of the previous work. These latter had a significantly higher M_v (370 and 424 kDa) than that obtained from thermal dried samples.

Hence, the M_v determination has allowed to evidence any possible effects of the biomass pre-treatment on the PHA and the effective capacity of acidification to stabilize PHA for downstream processing without affecting its molecular weight was confirmed (Werker et al., 2012). This evidence can be further confirmed by the results obtained in the present study. In fact, the same trend was found for sample TV-20 (M_v = 102 kDa) and TV-1-12 (341-615 kDa). Moreover, a study on PHB produced from crude glycerol and MMC reports significantly different M_w values for PHB after drying at 65°C and after lyophilization, respectively, 84-195 and 200-380 kDa. The authors justified the M_w decrease after drying with the occurrence of a partial hydrolysis due to heating (Hu et al., 2013). Besides, it can be concluded that the extraction method applied by Biotrend S. A. had no negative effects on PHAs chains, since the results of TV-samples are similar those obtained from the benchmark NaClO extraction (Lorini et al., 2020). On the other hand, L and C-samples showed a significantly lower M_v, ranging between 134-354 and 92-203 kDa with C2-sample as an outlier (508 kDa). Taking into account that the stabilization method applied on PHA-rich biomass at the end of the accumulation and the extraction method are the same for all the three grades PHA (TV, L, and C-samples), it is possible to infer that the marked difference in M_v is probably due to the different feedstocks and operative conditions applied in the pilot plants. According to what has been reported in recent studies of MMC-PHA produced from wastes and/or synthetic VFA mixture there is no correlation between the M_w and the 3HV content in the copolymer (Frison et al., 2015; Hilliou et al., 2016). Literature data on commercial polymers from pure culture report that molecular weight (M_w) ranges in 200–660 kDa (Morgan-Sagastume et al., 2015; Colombo et al., 2017). On the contrary, it is often reported that MMC are able to produce PHA with even higher M_w, comparable to the highest M_v determined for TV-samples. Previous studies reported M_w of 340–540 kDa (Villano et al., 2014), 440–630 kDa (Laycock et al., 2014a) for PHA produced from synthetic VFA mixture; 400–600 kDa (Burniol-Figols et al., 2020) from crude glycerol fermentation liquid; 650 kDa (Frison et al., 2015) from WAS; 800 kDa (Colombo et al., 2017) from percolates of the OFMSW; 800 kDa (Colombo et al., 2020) from dairy wastes. The cited works have been conducted at laboratory scale, while a study from full-scale is noteworthy and it reported a PHA from activated sludge with M_w of 400–500 kDa (Arcos-Hernández et al., 2015; Werker et al., 2018).

PCA Analysis

Considering **Figures 4A** and **B** together, analysis of the data obtained from the complete characterization of the PHA samples with the PCA allowed grouping the PHA samples into three groups to express and demonstrate their similarity and differences. According to that discussed in the previous sections, it is possible to conclude that the main differences among the three grades PHA are due to the different feedstocks and pilot plants, with more remarkable evidence in C-samples. Indeed, PC1 presents a direct correlation with 3HV content that is determining for separate C-samples as a single group, since 3HV is even higher than 80% w/w, namely the opposite composition of TV and L-copolymer. Loadings plot (**Figure 4B**) shows that M_v is also a relevant parameter, in fact TV and L-samples are separated mainly because of the marked difference in M_v values and the two groups are shown in scores plot (**Figure 4A**).

Potential Applications and Further Scenario

Polyhydroxyalkanoates produced in Treviso showed M_v in line with values for good mechanical properties, required for thermoplastic applications (Laycock et al., 2014b). Hence the PHA produced in Treviso pilot plant deserves further investigation. Up to now, tests for future applications evaluated the possibility of compounding PHA from OFMSW-WAS and other biodegradable polymers, as PBS (polybutylene succinate) or PBAT (polybutylene adipate terephthalate). Preliminary results showed that it is possible to obtain biodegradable films (for agricultural purposes) with acceptable mechanical and permeability properties through melt compounding and blown extrusion, starting from mixtures at different PHA content. Also packaging applications have been explored by Melendez-Rodriguez et al. (2020) on PHA from Treviso, carrying out a wide characterization on fiber-based continuous films obtained starting from electrospun PHA. The combination of electrospinning and a mild annealing postprocessing below the biopolymer's T_m was able to yield unique biomaterials with balanced mechanical, thermal and barrier properties (Melendez-Rodriguez et al., 2020). Furthermore, one of the main concerns relating to the use of waste as feedstock is the content of pollutants

that can migrate from the waste, through the technology chain, and into the end-products. PHAs from Treviso and Lisbon were analyzed in order to determine the presence of relevant contaminants, such as heavy metals (Astolfi et al., 2020) and polychlorinated biphenyls (PCB) (Riccardi et al., 2020). The total content of the analyzed metals and PCBs for all tested PHA types complied with the current regulations and guidelines. Therefore, the use of such waste as raw material for PHA production process appears to be safe for the environment and human health. As an added value, PHA characterized by low M_v or purity may be exploited as electron donor for biological reductive dechlorination, applied into permeable reactive barriers (PRBs) for groundwater remediation from chlorinated hydrocarbons (Baric et al., 2014).

CONCLUSION

This study showed the possibility to exploit different organic biowaste as raw material for biodegradable polymers, characterized by thermal and chemical properties comparable to commercial plastics. Indeed, the application of a stabilization method at the end of the accumulation step followed by an efficient extraction allowed to reach high purity of the end-product and to preserve the polymer properties, mostly the molecular weight. This latter was influenced by the type of feedstock, indeed on equal terms of monomeric composition with PHA from FW, a PHA with significantly higher M_v was produced from OFMSW-WAS. Monomeric composition was strongly influenced by the VFAs composition and the operative conditions, since C-samples have an opposite 3HV content (56–87% w/w) than TV and L-samples. Thermal properties are all in the same range for the whole samples set and comparable to those reported in literature. Moreover, melting enthalpy is dependent from 3HV content and the showed trend complies with that reported in literature. As a conclusion, the present study underlines that, from three different processes at pilot scale, different PHAs having adjustable properties can be produced with a long time stability. The real novelty of the work is to show how waste-derived biopolymers can achieve good characteristics and workability, and this information has a pivotal role in future development and optimization of these processes, including the kind of downstream and applications.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

LL: investigation, data curation, and writing – original draft. AM: supervision and visualization. GC: formal analysis. NF and BSF: resources. MR: resources and funding acquisition. MV: visualization. MM: funding acquisition and project administration. FV: conceptualization, data curation, writing – original draft, and supervision. All authors contributed to the article and approved the submitted version.

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REVIEW
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Challenges and Perspectives of Polyhydroxyalkanoate Production From Microalgae/Cyanobacteria and Bacteria as Microbial Factories: An Assessment of Hybrid Biological System

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Polyhydroxyalkanoates (PHAs) are the biopolymer of choice if we look for a substitute of petroleum-based non-biodegradable plastics. Microbial production of PHAs as carbon reserves has been studied for decades and PHAs are gaining attention for a wide range of applications in various fields. Still, their uneconomical production is the major concern largely attributed to high cost of organic substrates for PHA producing heterotrophic bacteria. Therefore, microalgae/cyanobacteria, being photoautotrophic, prove to have an edge over heterotrophic bacteria. They have minimal metabolic requirements, such as inorganic nutrients (CO_2, N, P, etc.) and light, and they can survive under adverse environmental conditions. PHA production under photoautotrophic conditions has been reported from cyanobacteria, the only candidate among prokaryotes, and few of the eukaryotic microalgae. However, an efficient cultivation system is still required for photoautotrophic PHA production to overcome the limitations associated with (1) stringent management of closed photobioreactors and (2) optimization of monoculture in open pond culture. Thus, a hybrid system is a necessity, involving the participation of microalgae/cyanobacteria and bacteria, i.e., both photoautotrophic and heterotrophic components having mutual interactive benefits for each other under different cultivation regime, e.g., mixotrophic, successive two modules, consortium based, etc. Along with this, further strategies like optimization of culture conditions (N, P, light exposure, CO_2 dynamics, etc.), bioengineering, efficient downstream processes, and the application of mathematical/network modeling of metabolic pathways to improve PHA production are the key areas discussed here. Conclusively, this review aims to critically analyze cyanobacteria as PHA producers and proposes economically sustainable production of PHA from microbial autotrophs and heterotrophs in "hybrid biological system."

Keywords: polyhydroxyalkanoates, photoautotrophic, heterotrophic, mixotrophy, hybrid biological system, two-module system, petri net

INTRODUCTION

Fossil fuel depletion and non-biodegradability of petro-chemical based plastics has created a scenario where we critically need an alternative. Petroleum based plastic has been extensively used worldwide because of their high durability and inexpensive production. But their persistence due to non-biodegradable nature has raised certain serious environmental concerns. Biodegradable plastics are the best "eco-friendly" alternatives of petrochemical based synthetic plastic for protection and sustainable development of the environment (Gill, 2014; Shen et al., 2020). Polyhydroxyalkanoates (PHAs) are the best candidates for this. These are the polymers of hydroxyacids produced by number of microorganisms including bacteria. PHAs have similar properties to petroleum derived synthetic plastics like polypropylene (PP) and it is 100% biodegradable in environment (Singh et al., 2015; Sharma et al., 2020). Bacteria belonging to a number of genus are reported to have PHA producing abilities. Out of these, *Cupriavidus*, *Pseudomonas*, *Bacillus,* and recombinant *Escherichia coli* are majorly used for PHA production through microbial fermentation (Singh et al., 2009; Akdoğan and Çelik, 2018; Brojanigo et al., 2020; Kalia et al., 2021). For PHA production, these organisms were able to utilize various substrates including pure (carbohydrates, fatty acids) and agro-industrial biowaste (Pea Shells, Sweet Potato, Whey, Beet molasses) as substrates (**Figure 1**; Singh et al., 2009, 2015; Kumar et al., 2013; Amaro et al., 2019; Bhatia et al., 2021; Kalia et al., 2021). But PHA production from these heterotrophic bacteria has been quite expensive which is due to the cost of carbon (C) substrates. Approximately 45% of the production cost is utilized in substrate procurement. Biowastes have been used as an effective resource and have helped substantially in cost reduction of PHA production process (Singh et al., 2015). But still there is a big economical gap in petroleum-based plastic and bioplastic. Autotrophic microorganisms provide a good alternative for economical PHA production having an edge over heterotrophic bacteria in certain aspects related to bioprocess development. Photoautotrophic organisms, e.g., cyanobacteria (collectively referred to "microalgae" along with eukaryotic microalgae in this review), are emerging as a strong contender for this purpose. Microalgae are an extremely diverse group and still have lots of opportunities unexplored (Borowitzka, 2013).

They present a strong candidature as microbial factory for various bioproducts due to their high growth rate, minimal nutrient requirements, low fresh water requirement, and high photosynthetic efficiency through which they can convert inorganic nutrients (CO_2) into organic reserves, utilizing light as energy source. The organic reserves produced during photosynthesis can then be further processed to PHA in these cells itself or can be used as feed for other microorganisms. However, autotrophic cultivation also possesses its limitations such as (1) stringent management of closed photobioreactors and (2) optimization of monoculture in open pond culture. Alternatively, bioprocess in which both autotrophic and heterotrophic microorganisms/cultivation can be used may utilize the resources optimally and helps in improving the economy of bioprocess. Thus, in this review we will discuss cyanobacteria as PHA-producing microbial factories, their potential, and challenges and analyze the perspectives of adopting a hybrid system involving both photoautotrophic and heterotrophic components so that their interactive relationship benefits the bioprocess system. Such hybrid system can be exploited under different cultivation regime, e.g., mixotrophic, successive two modules, consortium based, etc. PHA production from cyanobacteria has been reported to be affected by factors like culture conditions: N, P, light exposure, CO_2 dynamics, etc. In addition to that other factors like two-stage (growth and PHA accumulation) processes, metabolic inhibitors for other pathways and bioengineering have also been reported to have a positive impact on PHA production (Sharma et al., 2007; Bhati and Mallick, 2012, 2015, 2016; Kamravamanesh et al., 2017; Troschl et al., 2018). A new promisingly arising field is the application of mathematical/network modeling in this field which has increasingly been adopted to improve PHA production (Novak et al., 2015; Pillai et al., 2019). Here, we propose another tool "Petri Net" and its application in metabolic pathway or bioprocess modeling to improve PHA production.

MICROALGAE AS PHA MICROBIAL FACTORY

Considered among the fastest growing autotrophic organisms, microalgae are the global primary biomass producers having the ability to fix atmospheric CO_2 utilizing solar energy. With the ability to utilize minimal or readily available material, i.e., inorganic nutrients (CO_2), water, sunlight, wastewater, and having high photosynthetic efficiency, they are the strong candidate as "microbial factory" for the production of various bioproducts (Rahman et al., 2013, 2014; Singh and Mallick, 2017). This ability of microalgae to grow in adverse environment is demonstrated in different studies reporting their cultivation in urban wastewater, swine waste, dairy manure, cattle residues, and poultry litter (PL) (Olguín et al., 2003; Mulbry et al., 2008; Ansari and Fatima, 2019). A vast variety of high value natural products, e.g., pigments, carotenoids, proteins, enzymes, sugars, fatty acids, polysaccharides, phycobilins, sterols, vitamins, and many other unusual bioactive compounds, are produced by microalgae (Olaizola and Huntley, 2003; Ratledge, 2004; Singh et al., 2005; Mendes et al., 2009; Borowitzka, 2013; Kamravamanesh et al., 2018b). They don't need specialized fertile land and large freshwater supply and can be grown on ponds. Thus, microalgal biomass is ideal for PHA production and provides a low-cost economical bioprocess system (Rahman et al., 2014). Among them, only few eukaryotic algae are reported for PHA production, e.g., *Chlorella* (Roja et al., 2019). But cyanobacteria are taking the lead in this field (Carpine et al., 2015, 2017). Cyanobacteria are one of the largest bacterial group which are aquatic and photosynthetic known to be one of the oldest microorganisms and first photoautotrophic on earth. Thus, they played a vital role in the evolution of life creating an oxygen-rich environment and still are contributing to the carbon/oxygen cycle globally. Presence of phycocyanin pigment in them imparts them the characteristic blue–green color, and thus, they are

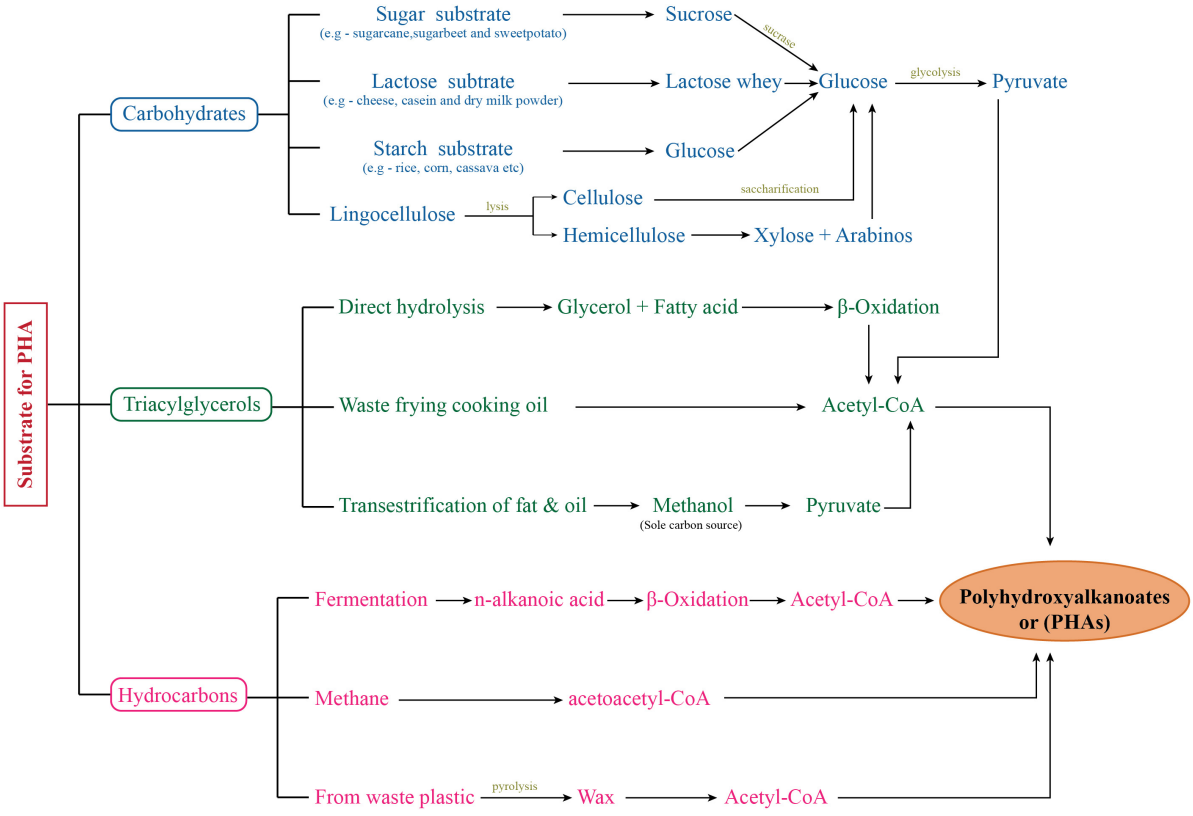

FIGURE 1 | Flow chart showing PHA production from diverse substrates.

known as blue–green algae. Fermentative PHA production is well known from heterotrophic bacteria. Similarly, most cyanobacteria also have the potential, although species specific even within same genera, to produce PHAs as intracellular carbon reserve. Like in heterotrophic bacteria, here also PHA is accumulated under nutrient (e.g., nitrogen and phosphorus) limiting conditions (Kaewbai-ngam et al., 2016). Such studies have reported the PHA production being influenced by various factors, e.g., nutrient availability, culture cultivation parameters (pH, light-dark cycles, temperature, gas/CO_2 flow), growth phase, and stationary phase duration (Sharma and Mallick, 2005a,b; Panda et al., 2006; Wagner et al., 2016; Troschl et al., 2017, 2018). In addition to this, microalgae can achieve wastewater remediation process along with PHA production through the assimilation of phosphorus and nitrogen as growth nutrients (Rahman et al., 2013). It is interesting to note that the microalgae grown in closed laboratory photobioreactor, open ponds, or in wastewater can achieve higher biomass economically. Thus, in addition to using this biomass as microbial factory for PHA production, this can also be harvested and processed for further utilization as substrate by other heterotrophic bacteria (Rahman et al., 2014). Similar to heterotrophic bacteria, along with PHAs other polymer reserves have also been reported in cyanobacteria, glycogen being the most significant of them (Kumar and Kim, 2018). It is interesting to note that in 1966 the first report of PHA production by cyanobacteria was documented for heterotrophic PHA production in a *Chlorogloea fritschii* with acetate as C-source; however, photoautotrophic PHA production was soon reported thereafter in 1971 in *Gloeocapsa* strain 6501 (Kamravamanesh et al., 2018a). Mixotrophy and chemoheterotrophy cultivation, favored over photoautotrophy, have also been documented in various studies (Wu et al., 2001; Sudesh et al., 2002; Sharma and Mallick, 2005b; Panda et al., 2006; Wagner et al., 2016).

PHA BIOSYNTHESIS

Polyhydroxyalkanoates production under heterotrophic microbial fermentative process has been explained quite extensively with number of researchers in this field. It is well known that the classical polyhydroxybutyrate (PHB) biosynthetic pathway has three basic steps catalyzed by three distinct enzymes: (1) condensation of two acetyl-CoA molecules forming one acetoacetyl-CoA catalyzed by *phaA* encoded β-ketothiolase, (2) reduction of acetoacetyl-CoA by *phaB* encoded NADPH-dependent acetoacetyl CoA dehydrogenase, and (3) polymerization of 3-hydroxyacid (3HA) units, i.e., (R)-3-hydroxybutyryl-CoA by *phaC* encoded PHA synthase. In addition to this basic pathway, 3-HA units can also be provided to PHA synthase (PhaC) by other secondary pathways involving methylmalonyl CoA pathway, *de novo* fatty acid synthetic

pathway, and fatty acid β-oxidation pathway. As evident, PHA synthase plays a central and critical role in PHA biosynthesis (Singh et al., 2009, 2015). Heterotrophic bacteria are well studied and explained to have three classes of PHA synthase having specificity to substrate (3-HA) C-chain length. These include Class I PhaC (scl-3HA), Class II PhaC (mcl-3HA), Class III PhaEC with two subunits (EC) (scl-3HA), and Class IV PhaRC with two subunits (RC) (scl-3HA). Based on the presence of these enzymes, a wide range of substrates including sugars (glucose, fructose, sucrose, maltose, and lactose), starch, glycerol, FAs and its derivatives, methanol, lignin, agricultural, industrial, and dairy by-products have been reported as potential substrates for PHA production (Kumar et al., 2019; Bhatia et al., 2021; Kalia et al., 2021). With the addition of different substrates combinations, precursor substrates, adopting different feeding regime PHA compositions and content have been observed to improve (Kumar et al., 2013, 2014; Singh et al., 2015; Ray and Kalia, 2017). **Figure 1** summarizes the PHA production by different bacteria on a wide range of substrates.

However, in cyanobacteria which can adapt for both photoautotrophic and heterotrophic cultivation, biosynthetic pathways for the utilization of exogenously provided C sources are more or less similar with the three basic steps discussed above. It is important to note that most of the information for PHA biosynthesis in cyanobacteria has been gained based on studies done on *Synechocystis* sp. PCC 6803. Here, synthase enzyme has two subunits PhaEC. Thus, PHA synthase in cyanobacteria belong to the type-III PHA synthases. Genes encoding the enzyme PhaA and PhaB are reported to be arranged in one operon and genes for PhaE and PhaC are arranged in one operon. Therefore, till now only SCL-PHAs are reported so far in the cyanobacterial species (Hai et al., 2001). Presence of a type-III PHA synthase has also been reported in *Synechococcus* sp. strains MA19 and PCC 6715, *Chlorogloeopsis fritschii* PCC 6912, *Anabaena cylindrica* SAG 1403-2, *Cyanothece* sp. strains PCC 7424, PCC 8303 and PCC 8801, and *Gloeocapsa* sp. strain PCC 7428. However, since cyanobacteria is a diverse group, the presence of other types of PHA synthase in them is still unclear and presents a great opportunity in near future (Troschl et al., 2017).

While in autotrophic cultivation of cyanobacteria CO_2, the C-source was fixed in Calvin–Benson–Bassham (CBB) cycle with the help of ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO). RuBisCO has higher efficiency for CO_2 than O_2 and is known to be responsible for assimilation of 90% of the carbon present in biomass on earth. Atmospheric CO_2 is transported across the wall with the help of inorganic carbon (Ci) transporters, thus helping to maintain local carbon concentration for RuBisCO. The output of the Calvin cycle, glyceraldehyde-3-phosphate after its conversion to 3-phosphoglycerate (PGA), can then enter into any of the three pathways for sugar metabolism, i.e., Entner–Doudoroff (ED) pathway, glycolysis, pentose phosphate pathway, and be finally converted to acetyl-CoA to be used in PHA synthetic pathway (Singh and Mallick, 2017; **Figure 2**).

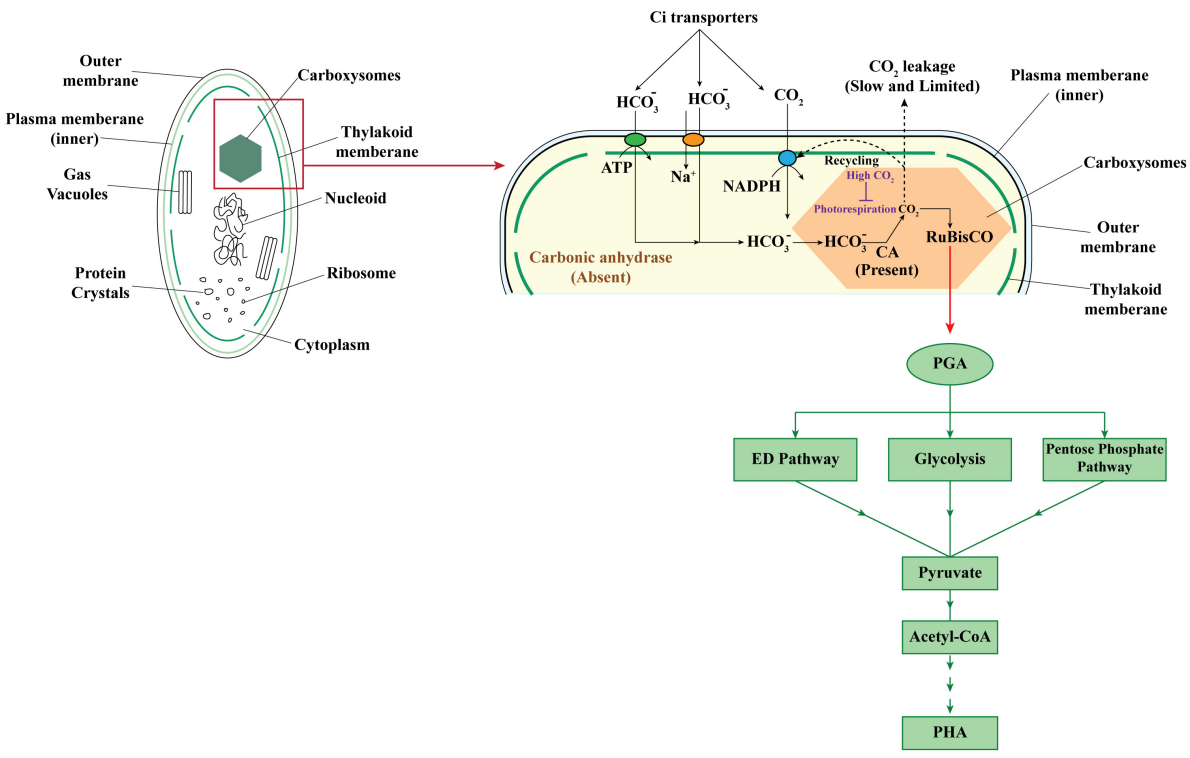

FIGURE 2 | Carbon concentrating mechanism in cyanobacteria for CO_2 fixation and further conversion to PHA.

PHA PRODUCTION IN CYANOBACTERIA

A number of bacteria under this group are reported so far for PHA production majorly including the genus *Nostoc*, *Arthrospira*, *Synechocystis,* and *Synechococcu*. Here the maximum yield varied from 1 to 78% depending on the cultivation conditions (**Table 1**). Invariably, PHA production was seen under nitogen (N) or phosphorus (P) limitation and during late exponential or stationary phase.

Nostoc

Nostoc is a diverse group of terrestrial as well as aquatic filamentous cyanobacteria occurring around freshwater sources. Since the first report of PHB production that was 8.6% of cdw by *Nostoc muscorum* under photoautotrophic conditions in 2005, number of species have been reported for PHA production under phosphate and nitrogen limitation (Sharma and Mallick, 2005a). Later, PHB content up to 19.27% with *N. ellipsosporum* and 22% with *N. muscorum* was reported under photoautotrophic conditions (Panda et al., 2005; Martins et al., 2017). Diverting the flux toward PHB production pathway by using metabolic inhibitors carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and dicyclohexylcarbodiimide (DCCD) resulted in PHB accumulation increment up to 21 and 17% from an initial PHB content of 8.5% of dry weight, respectively (Mallick et al., 2007). Sharma and Mallick (2005a,b) have reported the effect of culture conditions in improving the PHB production where it could be improved to 35% under mixotrophic condition with 0.4% glucose and acetate while a further improvement to 40–43% with gas exchange limitation under mixotrophy and chemoheterotrophy with 0.4% (w/v) acetate. Highest PHB production by *N. muscorum* was obtained up to 145.1 mg/L (16.6% of cdw) with 1% glucose and 1% acetate under phosphate starved conditions and CO_2 supply (Haase et al., 2012). In addition to nutrient limitation and mixotrophy/heterotrophy, reduction in dark period, i.e., optimizing the light-dark cycle also, has a positive impact on PHB production as observed on *N. muscorum* using response surface methodology (Sharma et al., 2007).

TABLE 1 | PHA production from cyanobacteria under different cultivation conditions (I): *Nostoc*, *Arthrospira*, *Synechocystis*, and *Synechococcus*.

Organism	Stimulatory culture condition/substrate	Yield % (dry cell weight)	Bioproduct	References
CO_2 as substrate: Photoautotrophic cultivation				
Nostoc	Additional CO_2	22	PHB	Haase et al., 2012
	Phosphate limitation	22.7	PHB	Panda et al., 2005
	-	19.27	PHA	Martins et al., 2017
	-	8.5	PHB	Sharma and Mallick, 2005a
Arthrospira	-	20.62	PHA	Martins et al., 2017
	Increased salinity	7.45	PHB	Shrivastav et al., 2010
	Phosphate limitation	3.5	PHB	Panda et al., 2005
	Nitrogen limitation	NA	PHB	Deschoenmaeker et al., 2016
Synechocystis	Nitrogen and phosphorous limitation	16.4	PHB	Kamravamanesh et al., 2017
	Nitrate limitation	12.5	PHB	Troschl et al., 2018
	Nitrogen and phosphorus limitation	11	PHB	Panda et al., 2006
	Nitrogen and phosphorus limitation	7.5	PHB	Wagner et al., 2016
	Photoautotrophic nitrogen limitation	4.1	PHB	Wu et al., 2001
	Nitrogen limitation	1	PHB	Carpine et al., 2015
Synechococcus	Phosphate limitation	55	PHB	Nishioka et al., 2001
Exogenous substrates: Heterotrophic/mixotrophic cultivation				
Nostoc	Acetate, glucose, valerate + nitrogen limitation	78	P(3HB-co-3HV)	Bhati and Mallick, 2015
	Mixotrophic with poultry litter	65	P(3HB-co-3HV)	Bhati and Mallick, 2016
	Acetate, and valerate + nitrogen and phosphorus limitation	60	P(3HB-co-3HV)	Bhati and Mallick, 2012
	Mixotrophic with glucose, acetate + gas exchange limitation	40	PHB	Sharma and Mallick, 2005b
	Acetate + glucose	40-43	PHB	Sharma and Mallick, 2005b
	Acetate, dark incubation after light-dark acclimatization	35	PHB	Sharma and Mallick, 2005a
	Acetate and propionate	31.4	P(3HB-*co*-3HV)	Mallick et al., 2007
	Acetate and glucose + phosphate limitation	16.6	PHB	Haase et al., 2012
	Acetate + glucose and dark incubation	45.6	PHB	Sharma et al., 2007
Arthrospira	Mixotrophic with acetate	3.0	PHB	De Philippis et al., 1992
Synechocystis	Fructose and acetate + phosphate and gas exchange limitation	38	PHB	Panda and Mallick, 2007
	Acetate + phosphate limitation	29	PHB	Panda et al., 2006
	Acetate + nitrogen limitation	7	PHB	Sudesh et al., 2001
	Digestate + mineral medium	6.6	PHA	Kovalcik et al., 2017
Synechococcus	Nitrogen limitation + dark incubation	27	PHB	Miyake et al., 1996

It is interesting to note that PHA copolymer poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid), P(3HB-co-3HV), production has also been reported from *Nostoc* but that is possible only under mixotrophic or heterotrophic condition with supplementation of propionate or valerate in culture medium. Reports have shown P(3HB-co-3HV) production on addition of these precursor substrates up to 28.2–31.4% (Mallick et al., 2007) and 58–60% of cdw under nitrogen and phosphate limitation, respectively, which further improved to a highest content of 78% of cdw under heterotrophic conditions (Bhati and Mallick, 2012, 2015). Poultry litter was used as nutrient supplement at the rate 10 g/L in culture along with glucose, acetate, valerate, and CO_2 and resulted in the production of P(3HB-co-3HV) copolymer yield of 774 mg L–1 (65–70% of dry cell wt.) (Bhati and Mallick, 2016). Notably, biomass achieved with *Nostoc* was not very high with yield around 1 g/L in most cases, and PHA accumulation in biomass was seen in late exponential phase.

Arthrospira

Arthrospira, earlier also known as *Spirulina*, are a type of filamentous bacteria found in alkaline (salt) lake but can be grown in freshwater also. *Arthrospira* at present is mainly cultivated as food supplement due to its protein and vitamin rich content. But it is a strong candidate to be used for economical PHA production due to the fact that it shows high growth rate with less water requirement. As reported, *Arthrospira* can achieve 5000–15000 tons dry weight production per year (Spolaore et al., 2006; Lu et al., 2011). The requirement of high alkalinity for its cultivation renders the culture free from other common contaminants. As a result, it can very well be used to maintain a stable and high biomass culture in open ponds. *Spirulina platensis* is one of the commonly explored species for PHB production (Panda et al., 2005). Ever since the first report of PHB production in 1982 which was achieved up to 6% cdw, there has been no significant improvement in PHA production under photoautotrophic conditions. Different strategies have been employed to improve the yield. PHB yield improvement was seen with nitrogen starvation (Deschoenmaeker et al., 2016), phosphate limitation (3.5% of cdw) (Panda et al., 2005), acetate supplement (2.5–3% of cdw) (De Philippis et al., 1992) increased salinity (14.7%) (Shrivastav et al., 2010). Highest yields were obtained with *Spirulina* sp. LEB 18 which produced 1.48 g/L/d PHB in 15 d equivalent to 20.62% of cdw (Martins et al., 2017). As evident, improvement in the yield of PHA production is the major issue related to *Arthrospira* and Mixotrophic or heterotrophic culture, and condition optimization has helped and can help further to improve the yields.

Synechocystis and *Synechococcus*

Synechocystis and *Synechococcus* are the genus of small-sized cyanobacteria found in both fresh and salt water. PHB production from *Synechocystis* was observed in 2001 where a yield of 4.1% was obtained under nitrogen-starved conditions (Wu et al., 2001). In this study, mixotrophic cultivation with glucose led to increase in biomass but not PHB yield (% of cdw). Here, the yield was improved to 9.5 and 15.2% of cdw under nitrogen limitation and 11.2% under phosphorus depletion in comparison to yield of 4.5% of cdw in balanced culture cultivation (Panda et al., 2006; Panda and Mallick, 2007; Wu et al., 2020). Samantaray et al. (2011) reported that heterotrophic cultivation with 0.4% acetate under dark incubation could improve the yield to 22% of cdw which was further improved to 38% of cdw when the stationary phase culture was subjected to phosphate and gas exchange limitation in the presence of acetate (0.4%) and fructose (0.4%) (Panda and Mallick, 2007). Till now *Synechocystis* PCC6803 is the most widely used strain for PHA production (Carpine et al., 2015). Closely related genus *Synechococcus* are also reported in literature for PHA production. *Synechococcus* sp. MA19, a thermophilic cyanobacterium which was a natural inhabitant of wet volcanic rock surface in Japan, produced PHB utilizing CO_2 up to 27% (w/w) (Miyake et al., 1996). The yields from this strain in photoautotrophic cultivation was further improved to 55% (dcw) amounting to 2.4 g/L under phosphate limitation. This is the highest PHA production reported so far from cyanobacteria under photoautotrophic conditions (Nishioka et al., 2001). *S. salina* was also reported to produce 7.5% of PHB which was further used after hydrothermal liquefaction for bio-oil production (Wagner et al., 2016). In another report it produced comparable yields of 4.8–9% of cdw reaching up to 2.0 g/L in 21 days of incubation in a large tubular photobioreactor with medium circulated, pH controlled through CO_2 flow, artificial illumination, and controlled temperature. Here, nitrogen limitation and illumination were observed to improve the PHA production while mixotrophy by the addition of acetate did not help due to contamination (Kovalcik et al., 2017). There has been not much reports for PHA production by *Synechococcus* sp., and hence, it leaves a lot of scope to try mixotrophic or heterotrophic cultivation to improve the production.

In addition to the above-mentioned genus that are majorly used for PHA production, other genus have also been reported to have PHA producing abilities. These genera include *Chlorogloea fritschii*, *Gloeocapsa* sp., *Oscillatoria limosa*, *Gloeothece* sp. PCC 6909, *Aulosira* sp., and *Calothrix* (Stal et al., 1990; Hai et al., 2001; Samantaray et al., 2011; Kaewbai-ngam et al., 2016; **Table 2**). *Chlorogloeopsis fritschii* PCC 6912, which is a thermophilic cyanobacteria, was reported to have 6% of PHB in its dried biomass (Hai et al., 2001), while *Brevibacillus invocatus* MTCC 9039 had PHB up to 3% of cdw accumulated in its stationary phase (Sankhla et al., 2010). Samantaray and Mallick (2012) reported the production of PHB simultaneously along with wastewater treatment in a biological recirculatory system by N_2-fixing cyanobacterium *Aulosira fertilissima.* This bacteria was also reported to produce 77% PHB under phosphate deficiency with 0.5% acetate and improved to 85% of cdw of PHB with 0.26% citrate, 0.28% acetate, and 5.58 mg/L K_2HPO_4 for an incubation period of 5 days (Samantaray et al., 2011). Similarly under nitrogen limitation, *Calothrix scytonemicola* TISTR 8095 could produce 25% of cdw (356.6 mg/L) PHB in 44 days (Kaewbai-ngam et al., 2016). *Anabaena cylindrica*, a filamentous cyanobacterium, could produce 2% of cdw PHB while P(3HB-co-3HV) was produced on supplementation of valerate and propionate (Lama et al., 1996).

TABLE 2 | PHA production from cyanobacteria under different cultivation conditions (II).

Organism	Substrate and culture condition	Yield % (dcw)	Bioproduct	References
CO_2 as substrate: Photoautotrophic cultivation				
Aulosira fertilissima	CO_2 + waste water	34.8 (41 g/m^2)	PHB	Samantaray et al., 2011
Calothrix scytonemicola	Nitrogen lim.	25.2	PHB	Kaewbai-ngam et al., 2016
Anabaena sp.	-	2.3	PHB	Gopi et al., 2014
Phormidium	-	2.3	PHB	Gopi et al., 2014
Exogenous substrates: Heterotrophic/mixotrophic cultivation				
Aulosira fertilissima	Waste water + citrate + acetate	87.22 (92 g/m^2)	PHB	Samantaray et al., 2011
	Acetate + phosphorous limitation	77	PHB	Samantaray and Mallick, 2012
	Fructose + valerate supplementation/phosphate deficiency	62-77	P(3HB-co-3HV)	Samantaray and Mallick, 2014
	Acetate + citrate	66	PHB	Samantaray and Mallick, 2012
	Citrate + phosphate limitation + dark	51	PHB	Samantaray and Mallick, 2012
Chlorogloeopsis fritschii	Mixotrophic with acetate	6.2	PHB	Hai et al., 2001
Scytonema geitleri	Acetate	7.12	PHB	Singh et al., 2019
Anabaena cylindrica	Acetate	2	PHB	Lama et al., 1996
	Acetate + propionate		PHB	Lama et al., 1996

Recombinant Cyanobacteria

Genetic engineering to modify microorganisms toward specific needs or property has been a well exploited practice in case of prokaryotic research and development. Since prokaryotes are relatively easy to manipulate due to simple genetic makeup in comparison to algae and plants, cyanobacteria also possess such attributes. Spontaneous transformability and short generation time have made *Synechocystis* sp. PCC6803 as the best studied model cyanobacterium not only for PHA production but other cyanobacteria related researches too. Moreover, *Synechocystis* sp. PCC6803 also has the honor of being the first photoautotrophic organism to have the complete genome sequenced in 1997 and first recombinant cyanobacteria used for PHA production (Wilde and Dienst, 2011; Troschl et al., 2017; Kamravamanesh et al., 2018a,b). As observed in studies discussed above, recombinant cyanobacteria also showed improvement with nutrient limitation and mixotrophic cultivation (**Table 3**). PHA biosynthesis can be improved by introducing multicopies of heterologous PHA synthase gene up to 11 wt% of the cdw (Sudesh et al., 2001). The ADP-glucose pyrophosphorylase gene (agp) was inserted with an erythromycin resistance cassette and under nitrogen depletion, photoautotrophic PHB production was up to 14.6% which improved under mixotrophic cultivation with acetate up to 18.6% (Wu et al., 2002). Inactivation of Thesll0783 gene by of kanamycin/bleomycin resistance cassettes and cultivation under nitrogen depletion indicated the importance of Thesll0783 gene in PHB production (Schlebusch and Forchhammer, 2010). The native SigE gene was overexpressed into *Synechocystis*, to control changes in sugar catabolism related to glycogen pathway which resulted in 1.4% of cdw phototrophically (Osanai et al., 2013). Similarly, overexpression of PHA biosynthetic operon from *Microcystis aeruginosa* NIES-843 in *Synechocystis*, resulted in 7% of cdw photoautotrophic PHB production under nitrogen depletion (Hondo et al., 2015). In another report, out of the four native genes chosen for overexpression (*phaAB*, *phaEC,* and *phaABEC*), maximum PHB content of 26% of cdw was achieved under nitrogen depletion with *phaAB* overexpression as compared to 9.5% of cdw in wild-type which further improved to 35% under mixotrophic conditions with supplementation of 0.4% acetate under nitrogen depletion (Khetkorn et al., 2016). Although not PHA, Wang et al. (2013) have reported 3-hydroxybutyrate by phaEC inactivated *Synechocystis* having heterologous expression of thioesterase gene (*tesB*) from *E. coli* and *phaAB* gene from *Cupriavidus necator* H16 under photoautotrophic utilizing CO_2. Overexpression of PHA synthase from *Chromobacterium* sp. USM2 ($phaC_{Cs}$), acetoacetyl-CoA synthase from *Streptomyces* sp. CL190 ($nphT7_{Ss}$), and *C. necator* acetoacetyl-CoA reductase ($phaB_{Cn}$) genes under the control of the light-inducible *psbAII* promoter under direct photosynthesis produced 14% cdw yield, among the best, under photoautotrophic cultivation and were observed to further improve to 41% on addition of acetate 0.4% (Lau et al., 2014). Recently, a strategy of increasing acetyl-CoA level by inducing deletions of phosphotransacetylase (Pta) and acetyl-CoA hydrolase (Ach) and the expression of a heterologous phosphoketolase (XfpK) from *Bifidobacterium breve* with CO_2 as feed produced 12% of cdw (232 mg/L) (Carpine et al., 2017). Optimization of acetyl CoA reductase binding site with CO_2 produced 1.84 g/L PHB with productivity of 263 mg/L/d (Wang et al., 2018).

Genetic manipulations have also been successfully employed in *Synechococcus*. *Synechococcus* sp. PCC7942 having PHB synthesizing genes from *Alcaligenes eutrophus* produced 3.01% of cdw PHB under photoautotrophic and nitrogen-starved conditions which was further improved 25.6% of cdw on supplementation of acetate under nitrogen-starvation (Takahashi et al., 1998). Recombinant cyanobacteria *Synechococcus* sp. PCC7002 *recA* null mutation with the *E. coli* recA gene achieved 52% of cdw PHA from carbon dioxide in Antibiotics-free cultivation (Akiyama et al., 2011). *Synechococcus PCC7942* having heterologous expression of *C. necator* PHA operon showed improved PHA accumulation, i.e., from 3 to 25% of cdw (Drosg et al., 2015) and introduction of GABA shunt with CO_2 feed led to PHA yield of 4.5% cdw (Zhang et al.,

TABLE 3 | PHA production from recombinant cyanobacteria under different cultivation conditions.

Organism	Recombinant factor/Culture media	Yield % (dry cell weight)	Biopolymer	References
CO_2 as substrate: Photoautotrophic cultivation				
Synechocystis	Random UV mutagenesis	37	PHB	Kamravamanesh et al., 2018a
	Optimized ribosome binding site for *phaB1*	35	PHB	Wang et al., 2018
	Overexpression of native *phaA* and *phaB* Nitrogen deprivation	26	PHB	Khetkorn et al., 2016
	Overexpression of the sigma factor *sigE* Nitrogen limitation	25	PHB	Osanai et al., 2013
	agp gene—deletion-mutant	14.9	PHB	Wu et al., 2002
	Overexpression $phaC_{Cs}$, $nphT7_{Ss}$, and $phaB_{Cn}$ under the control of the *psbAII* promoter Increased CO_2	14–16%	PHB	Lau et al., 2014
	xfpk overexpression in double *pta* and *ach* mutant	12	PHB	Carpine et al., 2017
	Overexpression of PHA biosynthetic operon from *Microcystis aeruginosa*	7.0	PHB	Hondo et al., 2015
	Knockout mutant of the sll0783 gene Nitrogen limitation	NA	PHB	Schlebusch and Forchhammer, 2010
Synechococcus	*Pha genes in complemented* cyanobacterial *recA* null mutant with the *E. coli recA* Nitrogen limitation	52	PHB	Akiyama et al., 2011
	PHA biosynthesis genes from C. necator	1.85	PHB	Takahashi et al., 1998
Exogenous substrates: Heterotrophic/mixotrophic cultivation				
Synechocystis	Overexpression $phaC_{Cs}$, $nphT7_{Ss}$ and $phaB_{Cn}$ under the control of the *psbAII* promoter Acetate + gas exchange limitation	41	PHB	Lau et al., 2014
	Overexpression of native *phaA* and *phaB* Acetate and nitrogen Limitation	35	PHB	Khetkorn et al., 2016
	agp gene—deletion-mutant Acetate	18.6	PHB	Wu et al., 2002
	PHA biosynthetic genes of *C. necator* Acetate and nitrogen limitation	11	PHB	Sudesh et al., 2002
	PHA biosynthetic operon from *C. necator* Acetate and nitrogen limitation	11	PHB	Sudesh et al., 2001
Synechococcus	PHA biosynthesis genes from *C. necator* Acetate and nitrogen limitation	26	PHB	Takahashi et al., 1998

2016). In an interesting report, polymer with 4-hydroxybutyrate (4HB) monomer also, i.e., copolymer P(3HB-co-4HB) was produced with the introduction of PHA biosynthetic genes from *Chlorogloeopsis fritschii* PCC 9212 and *ccmR* gene deletion in *Synechococcus* sp. PCC 7002. This study provided PHA content of ∼4.5% of cdw and was an indication of possibility of P(3HB-co-4HB) production in cyanobacteria using light and CO_2 (Zhang et al., 2015).

HYBRID BIOLOGICAL SYSTEM

Although the PHA production in heterotrophic cultivation have attained a significant level as far as the yield per unit feed are concerned. However, still the feed procurement cost is one of the major concerns making it an expensive practice to follow. As discussed till now, it is quite clear that due to the high photosynthetic efficiency, minimal nutrient requirement, high growth per hectare and CO_2 sequestering ability, cyanobacteria has gained the attention of researchers in biotechnology across the globe. In addition to that other properties such as no specific agricultural land requirement as compared to plants, efficient waste water utilization are also notably interesting. Possibilities of genetic engineering have added to its popularity providing a novel low cost expression system. Thus, PHA production from cyanobacteria eliminates the factors causing high production cost, i.e., exogenous C substrates, nutrient maintenance, etc. But, as with any other system photoautotrophic PHA production also has its limitations which need to be addressed while developing a sustainable system. It is quite evident that photoautotrophic cultivation in laboratory photobioreactors are helpful in maintaining monoculture but need continuous illumination which requires high maintenance, thus causing high production cost. In comparison, open pond cultures are economical but maintaining a contamination free monoculture is a challenge. Moreover, the uncertainty due to fluctuation in temperature, pH, light intensity, and CO_2 fixation caused by diurnal and seasonal changes leads to less efficient system (Kamravamanesh et al., 2018b).

Thus, the need of the hour is to develop a hybrid biological system that can address the challenges and utilizes the opportunities present in both photoautotrophic and

heterotrophic cultivation. Various reports have shown that PHA during photoautotrophic cultivation is lower and can be achieved up to 55% only in *Synechococcus* sp. (Nishioka et al., 2001). Thus, here we suggest the following cultivation regimes and strategies to improve PHA production.

Mixotrophy

Invariably in all the cases reported so far mixotrophic cultivation or heterotrophic cultivation have resulted in significant improvement in PHA yields (Sudesh et al., 2002; Sharma and Mallick, 2005b; Panda et al., 2006; Wagner et al., 2016). There has been 2–9-fold improvement in PHA yield (% of cdw). This suggests a possibility to use photoautotrophic and heterotrophic component (cultivation) in same system. The proposed system having a cyanobacteria as PHA producer may be subjected to photoautotrophic cultivation during day time/light phase followed by the introduction of heterotrophic cultivation during night time/dark phase (Hai et al., 2001; Sharma and Mallick, 2005b; Sharma et al., 2007; Bhati and Mallick, 2016; Kovalcik et al., 2017). During light phase, cyanobacteria will utilize CO_2 as carbon source, and in dark phase, exogenous C sources (glucose, acetate, copolymer precursors, etc.) may be added in media. An alternative improvement takes high biomass growth and lower PHA yield in cyanobacteria under photoautotrophic cultivation under consideration. In this system (**Figure 3A**), cyanobacteria may be subjected to photoautotrophic conditions during day time/light phase without any N/P limitation to attain high biomass till the late exponential or stationary phase and then subjected to heterotrophic conditions in night time/dark phase under N/P limitations. This may constitute a feast (dark) and famine (light) phase, promoting PHA production in microorganisms.

Photoautotrophic-Heterotrophic Consortium

The main aim of developing a consortium is to have a more efficient system through the division of labor that gives enhanced accessibility of resources, enhanced productivity, efficient nutrient cycling, community stability and non-competitive partitioning, and distribution of carbon or energy source between members of community based on metabolic functionality. Out of the different interaction aspects, mutualistic relationship is the most widely exploited for numerous biotechnology studies such as bioprocess, biofuel, and other value-added products formation (Bernstein and Carlson, 2012). Photosynthetic consortium having algae and bacteria has been reported for PHA production. Here, PHA production was obtained up to 20–30% of cdw without oxygen supplementation. In this study the consortium was subjected to feast and famine regime where the PHA production was achieved in feast phase during dark cultivation. During famine phase in illuminated cultivation, accumulated PHA can be oxidized and consumed releasing the reducing equivalents and CO_2. The reducing equivalents produced during this famine phase are used in PHA production process in feast phase, while the CO_2 released is used by algae for photosynthesis. During photosynthesis, algae released O_2 which reduced the aeration requirement and was also used for PHA oxidation. Thus, this study provided an evidence of efficient resource utilization by consortium (Fradinho et al., 2013a,b). Another consortium of microalga *Chlorella vulgaris* and the bacterium *Pseudomonas aeruginosa* was developed by Guerra-Renteria et al. (2019) for nitrogen and phosphorus uptake from wastewater. Thus the consortium can be used for organic waste degradation also where oxygen release from algae is utilized by bacteria to degrade organic matter simultaneously CO_2 release from the bacteria is utilized by microalgae as raw material to complete photosynthesis. This symbiotic interaction helps to reduce wastewater treatment efforts by natural way. A recent study has reported lichen-associated *Pseudomonas* having the ability to detoxify and utilize naphthalene and anthracene for energy reserve storage and produce PHA with 3-HHx unit up to 30.62 and 19.63% of 3-HHx, respectively (Nahar et al., 2019). The proposed system has consortium of microalgae/cyanobacteria and bacteria as two components so as to utilize resources under both light and dark phase (**Figure 3B**).

Two Module System

Since the PHA yield in fast growing photoautotrophic cultivation, the PHA yields are low. Thus, either the high biomass or the organic products released by them can alternatively be used as substrate itself. Hydrothermal liquefaction (HTL) is the efficient strategy to obtain organic compounds from cyanobacterial

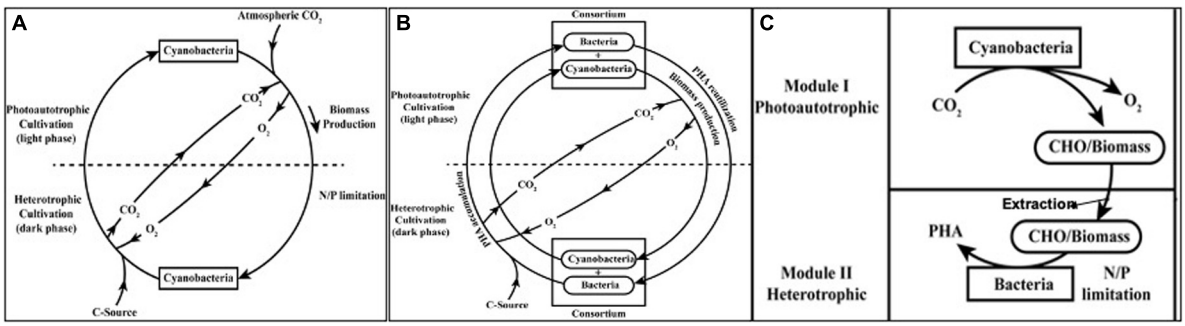

FIGURE 3 | Strategies and culture regime of "hybrid biological system" for PHA production improvement. **(A)** Mixotrophy; **(B)** Photoautotrophic-heterotrophic consortium; and **(C)** Two module system.

biomass, e.g., propylene and bio oil (biofuel) production was shown from cyanobacterial biomass processed through HTL. This suggested PHB as a feedstock for biorefinery as propylene level was found to be proportional to the accumulated amount of PHB (Wagner et al., 2016). One of the study revealed that microalgae grown in wastewater can be harvested using different methods. The harvested biomass can further be used as a substrate for growth of genetically engineered *E. coli* and PHB production (Rahman et al., 2014). Using defatted biomass also gives an attractive option as reported by Goo et al. He showed 82% biopolymer yield using defatted mass of microalgae *Dunaliella tertiolecta* with different salt concentration (Goo et al., 2013). In a recent report, PHA production was reported in two modules with synthetic mixed culture of *Synechococcus elongatus cscB* and *Pseudomonas putida* cscAB. Here in the first module, *Synechococcus elongatus* cscB was cultivated under photoautotrophic condition where it fixes CO_2 to convert it into sucrose and release it into culture medium. In the second module, *Pseudomonas putida* csc AB was used under heterotrophic cultivation to utilize sucrose produced in first module for PHA production under nitrogen limitation (Lowe et al., 2017). The proposed system, depicted in **Figure 3C**, may have photoautotrophic cyanobacterial growth in one module, and then in second module after the processing of its biomass, it can be utilized by heterotrophic bacteria as substrate for PHA production.

Metabolic Network Modeling

A number of factors and parameters have been reported in literature those are affecting the growth and PHA production in microalgae. Some of them are temperature, light intensity, pH, salinity, CO_2 exchange level, nitrogen level, phosphorous level etc. These factors are more detrimental when operating in a large scale or open pond culture. Even if, we use the above proposed strategies involving the use of consortium or two module system, optimization of these factors and additionally the metabolic interaction of member microorganisms, is a critical step and requires lot of efforts and resource utilization affecting the final performance and efficiency of any bioprocess. In the effort of developing a bioprocess for PHA production we need to have an insight into the enzymatic and metabolic pathways of the PHB biosynthesis. We need to select or manipulate genetically an optimal strain with maximum productivity. In addition to these, other requirements like the selection of inexpensive substrate, culture condition optimization of media, process parameter optimization for large scale production and designing a bioreactor ought to be managed carefully (Troschl et al., 2017). In present research world, mathematical or *in silico* tools are proving to be a boon in such studies, as these not only can handle integration and combination of large number of parameters or interactive connections to be analyzed but also reduce the time taken for experimental exercises to completely analyze that much number of combinations. Microbial consortia engineering through studying the highly complex interactions between populations and also with environment is increasingly been accepted to have potential to construct sustainable bioprocesses with enhanced metabolic productivity. Mathematical models including differential equations and stochastic methods have been used in microbial ecology modeling (Bernstein and Carlson, 2012). A number of mathematical models used in different studies for PHA production include kinetic, dynamic, cybernetic models (Dias et al., 2008; Jiang et al., 2011; Novak et al., 2015). Flux balance analysis (FBA) or in other words linear programming (LP) based on objective function and constraints is used in multiple studies, e.g., to predict the relative abundance of *Desulfovibrio vulgaris* (sulfate reducing bacteria) and *Methanococcus maripaludis* (methanogen) based on the analysis of mutual metabolic exchange between them (Stolyar et al., 2007), dynamic modeling for simulation of two different co-cultures for synergistic co-fermentation of xylose + glucose mixture with ethanol (Hanly and Henson, 2011, 2013), synthetic or semisynthetic co-cultures of *E. coli* analysis for xylose, and glucose utilization in *S. cerevisiae*. Another stoichiometric model is the elementary flux mode analysis (EFMA) that includes metabolic pathway network modeling and analysis (Volkova et al., 2020). EFMA has been used to study mass and energy flows through microbial community of a phototrophic, biofilm community (Taffs et al., 2009).

Somehow, majority of mathematical tools are not easy to use for non-mathematical background people. Thus, network-based modeling is a convenient option in such case. Metabolic network modeling-guided strains' design or development is gaining interest and has been used for n-butanol, 1,3-propanediol, glycerol, limolene, and isoprene (Hendry et al., 2020). Genome-scale metabolic network reconstructions and constraint-based analyses have been used in number of studies for metabolic modeling (Lee et al., 2020). Similar strategy was used to predict and design a strain that has a force C-flux toward malonyl-CoA for enhanced production of polyketides and biofuels (Xu et al., 2011). Recently, a novel strain design algorithm OptRAM (Optimization of Regulatory and Metabolic Networks) was developed with integrative regulatory and metabolic network modeling. This tool has helped the researchers to identify and design strain for Succinate 2,3-butanediol and ethanol production in yeast (Shen et al., 2019). Similarly, mathematical and metabolic engineering were used for PHA production and recovery process improvement (Novak et al., 2015; Pillai et al., 2019). In another study, analysis and prediction of metabolic flexibility of *Candidatus Accumulibacter phosphatis*, a phosphate accumulating organism, was done based on redox factor preferences of different oxidoreductase (da Silva et al., 2020).

For network-based modeling, here we propose another tool Petri Net for such analysis. Petri Nets (PN) are directed bipartite graph in with vertices as two disjointed sets, i.e., places and transitions. Arcs connect vertices, i.e., transitions with places and places with transitions. The distribution of tokens, i.e., quantities of particular components over the places corresponds to the state of the modeled system. In biological representation "places" (circles) correspond to biological or chemical components, "Transitions" (rectangles) to processes (e.g., reactions) (Kansal et al., 2015). A transition is active only under certain dynamics and firing rules causing the flow of tokens, i.e., information and tokens are consumed from the input places through the transitions. PN provide a competent method for both

qualitative (structural topology) and quantitative analysis (token distribution). Snoopy, MARCIE, WoPeD, and Pathway Logic Assistant are some of the tools used for petri net modeling in computational biology (Bartocci and Lio, 2016). Petri Net has the advantage of graphical representation of complex networks which is compatible to computational simulations and is user friendly to a non-mathematical population (Koch, 2015). It has been used to model various biological pathways/processes, e.g., metabolism, signal transduction, gene regulation, protein complex assembly, metabolic disorders for biomedical data prediction, and enzyme kinetic modeling (Koch et al., 2017). Recently, PN are used for modeling of biological pathways (PHA; C3 pathway), networks for processes involved in diagnostics, treatment, and multi drug resistance in tuberculosis (Gupta et al., 2019; Singh and Jha, 2019; Singh et al., 2020).

CONCLUSION

In spite of having an advantage associated with photoautotrophic metabolism that eliminates the cost attributed to C substrate, cyanobacteria still have lower yield of photoautotrophic PHA production. However, still a sustainable biological system can be constructed if we can efficiently use the abilities and attributes related to photoautotrophic and heterotrophic PHA production. The proposed hybrid biological system involving mixotrophy, photoautotrophic–heterotrophic consortium and two module system along with the optimization of process parameters, and utilization of metabolic network modeling can achieve the required sustainability. Such biological system can provide an added advantage, as it not only has economical production of PHA but due to CO_2 sequestering capabilities can help in reducing global warming effect and can reduce pollution by providing alternative of synthetic plastic.

AUTHOR CONTRIBUTIONS

RA, ST, and MS have contributed toward conceptualization and framing the review content for PHA production from microbes. GS has contributed for the conceptualization and integration of mathematical/metabolic model aspect. All authors contributed to the article and approved the submitted version.

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Fed-Batch *mcl*-Polyhydroxyalkanoates Production in *Pseudomonas putida* KT2440 and Δ*phaZ* Mutant on Biodiesel-Derived Crude Glycerol

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Crude glycerol has emerged as a suitable feedstock for the biotechnological production of various industrial chemicals given its high surplus catalyzed by the biodiesel industry. *Pseudomonas* bacteria metabolize the polyol into several biopolymers, including alginate and medium-chain-length poly(3-hydroxyalkanoates) (*mcl*-PHAs). Although *P. putida* is a suited platform to derive these polyoxoesters from crude glycerol, the attained concentrations in batch and fed-batch cultures are still low. In this study, we employed *P. putida* KT2440 and the hyper-PHA producer Δ*phaZ* mutant in two different fed-batch modes to synthesize *mcl*-PHAs from raw glycerol. Initially, the cells grew in a batch phase (μ_{max} 0.21 h^{-1}) for 22 h followed by a carbon-limiting exponential feeding, where the specific growth rate was set at 0.1 (h^{-1}), resulting in a cell dry weight (CDW) of nearly 50 (g L^{-1}) at 40 h cultivation. During the PHA production stage, we supplied the substrate at a constant rate of 50 (g h^{-1}), where the KT2440 and the Δ*phaZ* produced 9.7 and 12.7 gPHA L^{-1}, respectively, after 60 h cultivation. We next evaluated the PHA production ability of the *P. putida* strains using a DO-stat approach under nitrogen depletion. Citric acid was the main by-product secreted by the cells, accumulating in the culture broth up to 48 (g L^{-1}) under nitrogen limitation. The mutant Δ*phaZ* amassed 38.9% of the CDW as *mcl*-PHA and exhibited a specific PHA volumetric productivity of 0.34 (g L^{-1} h^{-1}), 48% higher than the parental KT2440 under the same growth conditions. The biosynthesized *mcl*-PHAs had average molecular weights ranging from 460 to 505 KDa and a polydispersity index (PDI) of 2.4–2.6. Here, we demonstrated that the DO-stat feeding approach in high cell density cultures enables the high yield production of *mcl*-PHA in *P. putida* strains using the industrial crude glycerol, where the fed-batch process selection is essential to exploit the superior biopolymer production hallmarks of engineered bacterial strains.

Keywords: *mcl*-poly(3-hydroxyalkanoates), fed-batch, crude glycerol, *pseudomonas putida*, PHA depolymerase

INTRODUCTION

The worldwide manufacture of petrochemical plastics reaches over 359 million tons annually as these materials are essential in our current lifestyle (Poblete-Castro et al., 2020a; Tournier et al., 2020). Unfortunately, synthetic plastics are not prone to biodegradation and massively accumulate in the oceans and soil ecosystems (Zettler et al., 2013; Lau et al., 2020). Factors like temperature and radiation decompose the polymers in small particles (<5 mm), entering now into the food chain (Law and Thompson, 2014). This phenomenon is detrimental to preserving natural environments and human health (Smith et al., 2018), demanding actions to move toward a circular bioproduction economy. To this end, commercial substitutes of petrochemical plastics are the biodegradable poly(3-hydroxyalkanoates) (PHAs). These biopolymers possess physical and mechanical properties similar to oil-based plastics displaying thermal malleability and elasticity and having suitable breaking points for thermoforming (Laycock et al., 2013). The industrial sectors that exploit these biopolymers include food, textile, agriculture, biomedicine, and electronics (Raza et al., 2018).

PHAs are naturally occurring inclusion bodies during nutrient imbalance formed in microbes' cytosolic space and reported as energy reservoirs (Madison and Huisman, 1999) and essential elements to cope with different stress agents (Obruca et al., 2017). A manifold of companies currently manufactures PHAs at a large scale (Borrero-de Acuña et al., 2017). However, industrial production of PHAs is costly, given high operational expenses because of the employed carbon substrate and downstream processing (Chen et al., 2020). Hence, to overcome these setbacks, waste materials arise as low-cost carbon substrates to sustain bacterial growth and renewable polyester production (Cesário et al., 2014; Nielsen et al., 2017; Borrero-de Acuña et al., 2019). Combining engineered microbial cell factories with high cell density fermentations is a robust approach to achieve elevated PHA productivities (Choi et al., 2020).

In the last decade, the biodiesel industry has generated large quantities of crude glycerol, an inevitable by-product resulting from the esterification process of fatty acids (Garlapati et al., 2016). Given the high glycerol surplus, the product market price is continually dropping (Zhang et al., 2020), making it an attractive substrate to derive biochemicals (Kaur et al., 2020). Microbial catabolism of crude glycerol presents some challenges since the biodiesel by-product contains methanol, traces of diesel, and heavy metals (Mothes et al., 2007; Samul et al., 2014). Remarkably, *Pseudomonas* strains can endure these toxic materials (Poblete-Castro et al., 2017) and fuel glycerol metabolic products into PHA biosynthetic pathways enabling the synthesis of *medium-chain* length (*mcl*-PHAs) (Kenny et al., 2012; Pappalardo et al., 2014; Fu et al., 2015; Liu et al., 2018) and copolymers of short-*co*-medium-chain length polyesters (Orellana-Saez et al., 2019; Pacheco et al., 2019). Batch and fed-batch production of *mcl*-PHA is feasible using crude glycerol in *Pseudomonas putida* where the strain KT2440 formed 34% of the cell dry weight (CDW) as polyester with a final product titer of 1.45 (g L^{-1}) in 75 h (Poblete-Castro et al., 2014a). A newly isolated soil strain from Thailand, *Pseudomonas* sp. ASC2, proved to synthesize 3.02 (g L^{-1}) of *mcl*-PHAs in flask experiments. In a high cell density fermentation, *P. putida* GO16 attained 19 (g L^{-1}) biomass using the waste polyol, showing a specific PHA volumetric productivity of 0.13 (gPHA L^{-1} h^{-1}) (Kenny et al., 2012).

While the polymerization of PHAs in *P. putida* is well characterized and relies on the PHA synthase proteins PhaC1 and PhaC2, the not yet fully understood depolymerization process is a result in part of the enzymatic action of PHA depolymerase (PhaZ, PP_5004) (Arias et al., 2013). Different metabolic stimuli govern gene regulatory crosstalk, with the polymerization vs. depolymerization of PHA kinetics still unclear (Karmann et al., 2017; Velázquez-Sánchez et al., 2020). The accumulating evidence suggests that the polymerization regulatory control depends on the type of carbon substrate (De Eugenio et al., 2010). Inactivation of the *phaZ* gene in *P. putida* KT2442 boosted *mcl*-PHA production of cells grown on fatty acids (Cai et al., 2009). Conversely, non-related PHA carbon sources, glucose or gluconate, did not yield higher PHA titers in this mutant. Glycerol-grown cells of a *phaZ*-lacking strain of KT2440 produced 36% more biopolymer than the wild type using crude glycerol (Poblete-Castro et al., 2014a). Using fatty acids as substrates, a *phaZ* minus strain of KT2440 formed more than 70 (g L^{-1}) of unsaturated *mcl*-PHA in fed-batch cultures (Vo et al., 2015). These experiments laid the groundwork for challenging the production capacities of the *phaZ*-deficient mutant in high cell density cultures. Here, we assessed different feeding strategies for high titer PHA production in *P. putida* strains on industrial crude glycerol. The DO-stat fed-batch fermentation is best suited to bioconvert raw glycerol into the elastomer polyesters where the Δ*phaZ* mutant and the parental KT2440 reached *mcl*-PHA specific volumetric productivities of 0.34 and 0.23 (g L^{-1} h^{-1}), respectively.

RESULTS AND DISCUSSION

Constant-Feeding PHA Synthesis Under Nitrogen Limitation

P. putida KT2440 and a *phaZ*-deficient mutant strain demonstrated in a previous study to synthesize efficiently *mcl*-PHAs from crude glycerol in batch cultures (Poblete-Castro et al., 2014a). We now challenged these natural polyester producers in the process of choice for the industrial production of PHAs, the fed-batch culture. Applying different feeding strategies using industrial crude glycerol without any modification (Cremer Oleo, GmbH, Germany), we developed a three-stage fermentation where the two initial phases aimed to form biomass and the third stage *mcl*-PHA under nitrogen depletion. The batch cultures started with a biomass concentration of 0.13 (g L^{-1}) in a 4 L working volume bioreactor. The culture broth initially contained 20 (g L^{-1}) crude glycerol and 1 (g L^{-1}) of glucose to prevent a characteristic extended lag phase (Escapa et al., 2012; Beckers et al., 2016), usually taking more than 10 h of *P. putida* cells growing on glycerol as the sole C source (Poblete-Castro et al., 2020b). As the *P. putida* cells propagate, foam developed

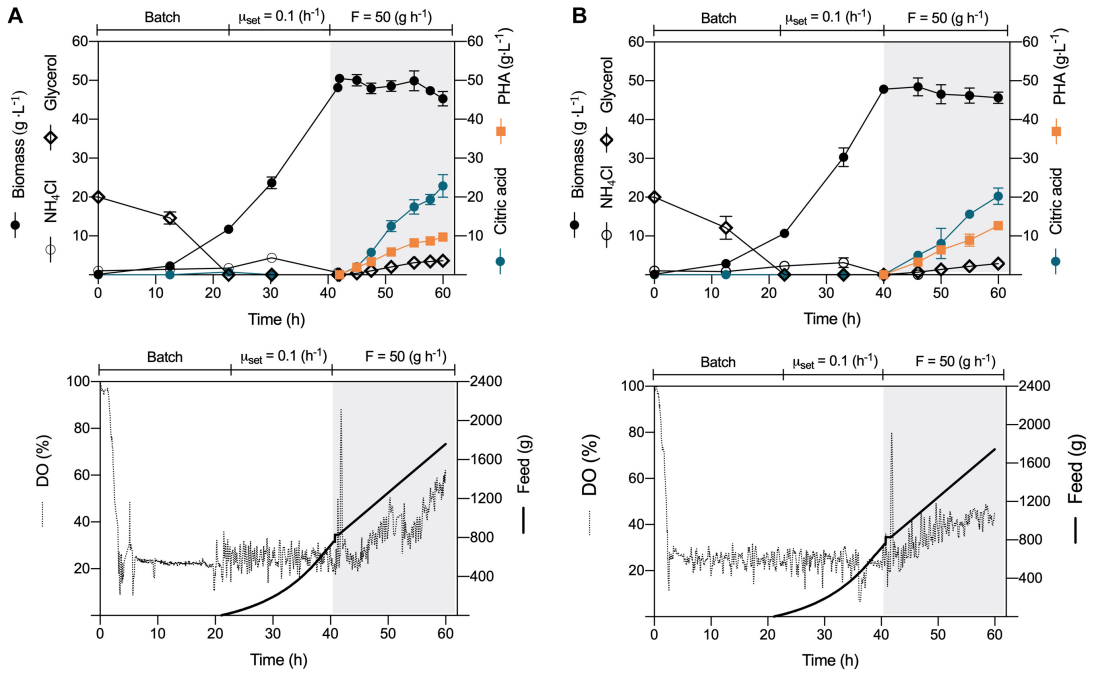


FIGURE 1 | Constant-feeding fed-batch *mcl*-PHA production. The fermentation process comprised three phases: (i) batch, (ii) exponential feeding, and (iii) constant feeding of crude glycerol (50 g h^{-1}) under nitrogen depletion. Time profile of **(A)** *P. putida* KT2440 and **(B)** *phaZ*-knockout mutant. The data represent the mean values and standard deviation from two independent experiments.

in the bioreactor (at 3 h), which dispersed once we provided antifoam (200 μL L^{-1}). After 22-h cultivation, *P. putida* KT2440 and Δ*phaZ* mutant had similar maximum specific growth rates of 0.21 h^{-1} (**Figure 1A**), reaching a CDW of 11.5 g L^{-1} (**Figures 1A,B**). Then, we started feeding glycerol exponentially, setting the specific growth rate at nearly 50% of μ_{max} (0.1 h^{-1}) (**Figure 1**). During this phase, no glycerol accumulated in the culture broth or the cells secreted by-products due to the coupled catabolism and anabolism under carbon limiting conditions (Poblete-Castro et al., 2012; González-Cabaleiro et al., 2015).

At 40 h cultivation, we no longer provided glycerol and ammonium; we instead provided a substrate pulse to attain 10 (g L^{-1}) of glycerol within the bioreactor. This procedure triggered nitrogen limitation, and once glycerol was under the detection limit (after 1 h), we fed the substrate, but this time at a constant mass flow rate of 50 (g h^{-1}). During the biopolymer production phase, we upheld the airflow rate, providing only filter air instead. The DO evolution showed a marked increase as the cells accumulated higher amounts of PHAs through the process, showing a reduced O_2 demand as the cells no longer duplicate (**Figures 1A,B**). The *P. putida* strains secreted citric acid as the main co-product as glycerol accumulated in the medium, showing a final yield on the polyol of 0.18 ± 0.01 (g g^{-1}) and 0.16 ± 0.02, for the wild-type and the mutant strain, respectively (**Table 1** and **Figure 1**). Indeed, nitrogen depletion of glycerol-grown *P. putida* cells inhibits the TCA cycle enzymes like isocitrate dehydrogenase (ICD), slowing the carbon flux through the oxidative route (Beckers et al., 2016). The biomass suffered a slight reduction in KT2440 and the *phaZ*-knockout mutant from ∼50 (g L^{-1}) to 45.4 and 46.7 (g L^{-1}), respectively, a common trend of cells enduring overflow metabolism (Xu et al., 1999; Poblete-Castro et al., 2014b). At the end of the fermentation process (60 h), the wild-type KT2440 synthesized 9.7 (gPHA L^{-1}) with a biopolymer content of 21.4% of the CDW (**Figure 1A** and **Table 1**), while the Δ*phaZ* deletion mutant achieved 12.7 (gPHA L^{-1}), amassing 27.2% of the cell biomass as polyester (**Figure 1B** and **Table 1**). Finally, the monomeric composition of the generated biopolymers by constant-feeding strategy was dominated by 3-hydroxydecanoate with a 75.4 and 76.1% relative molar fraction in the wild-type and Δ*phaZ* mutant, respectively (**Table 1**). The rest of the monomers had the following decreasing proportion within the polymeric chain in both tested strains: 3-hydroxyoctanoate, 3-hydroxydodecanoate, 3-hydroxy-5-cis-dodecanoate, 3-hydroxyhexanoate, and 3-hydroxytetradecanoate.

PHA Production Using a Dissolved-Oxygen-Stat Feeding (DO-Stat) Under Nitrogen Limitation

Despite the sound PHA volumetric concentration obtained in this study operating the constant-feeding scheme, the *P. putida* strains did not amass the PHA content previously reported in batch cultures (Poblete-Castro et al., 2014a). Thus, to fully harness the biopolymer production capacities of the Δ*phaZ* mutant and the wild type, a dissolved oxygen-stat (DO-stat) feeding approach was applied. This well-controlled strategy has proven to yield higher PHA amount and content in engineered

TABLE 1 | Medium-chain length (*mcl*-PHA) production in *Pseudomonas* strains under various fermentation modes utilizing crude glycerol.

Strain	Production mode	Biomass	PHA	PHA content	$Y_{CIT/Gly}$ Citrate yield	$Y_{PHA/Gly}$ PHA yield	Specific PHA volumetric productivity	Monomeric composition (%)					References
		(g L^{-1})	(g L^{-1})	(%wt)	(g g^{-1})	(g g^{-1})	(g L^{-1} h^{-1})	C6	C8	C10	C12	C12:1	C14	
P. putida KT2440	Constant feeding	45.3	9.7	21.4	0.18	0.08	0.16	1.2	15.3	75.4	5.7	2.4	0.3	This study
Δ*phaZ*	Constant feeding	46.7	12.7	27.2	0.16	0.10	0.21	1.3	16.1	76.1	5.9	0.6	N.D.	This study
P. putida KT2440	DO-stat	49.5	13.8	27.9	0.36	0.11	0.23	0.8	15.8	73.3	5.6	4.5	0.4	This study
Δ*phaZ*	DO-stat	52.4	20.4	38.9	0.32	0.13	0.34	1.2	16.7	76.2	5.4	0.5	N.D.	This study
P. putida GO16	Fed batch	19.0	6.3	33.2	N.D.	N.S	0.13	3	18	35	13	15	7	Kenny et al., 2012
Pseudomonas sp. ASC2	Batch	10.7	3.0	28.2	N.D	N.S.	0.04	N.D.	4.8	N.D.	N.D.	95.2	N.D.	Muangwong et al., 2016

N.D., Not detected; N.S., Not shown.

P. putida strains on glucose and aromatics (Poblete-Castro et al., 2014b; Borrero-de Acuña et al., 2020). For biomass formation, we repeated the same growth conditions as those employed during the constant-feeding approach, where a mixture of pure oxygen and air was provided to avoid oxygen limitation. Nitrogen was always sufficient at this stage, reaching both strains biomass productions of nearly 50 (g L^{-1}) (**Figure 2**). At this point, we provided a pulse of glycerol (10 g L^{-1}) to enable the cells to consume the remaining ammonium, and to get a better DO response, we provided only filter air as carried out in the constant-feeding cultivations. Likewise, citrate began to accumulate in the culture broth due to the metabolic shift provoked by nitrogen limitation (**Figure 2**). As a response to glycerol exhaustion, the DO saturation increased; thus, every time the dissolved oxygen exceeded a value of 70%, an automated addition of the substrate (20 g L^{-1} glycerol) occurred in an interval of 15 min (**Figure 2**). This feeding operation did not reduce the formed biomass, and the intracellular polyester synthesis boosted, maintaining a rapid pace of accumulation until the termination of the process (**Figure 2**). After 20 h of feeding driven by the DO response, the wild-type strain attained a biomass titer of 49.5 (gCDW L^{-1}), of which 13.8 (g L^{-1}) consisted of *mcl*-PHA (**Figure 2A** and **Table 1**). Similarly, the depolymerase-deficient mutant achieved a biomass yield of 52.4 (gCDW L^{-1}) but comprised an enhanced biopolymer yield of 20.4 (g L^{-1})—overall PHA content 38.9%wt (**Figure 2B**). The specific PHA volumetric productivity (0.34 g L^{-1} h^{-1}) reached by the Δ*phaZ* mutant is the highest reported today to derive *mcl*-PHA using industrial crude glycerol (**Table 1**). *P. putida* KT2440 and its *phaZ* knockout mutant strain secreted 48 (g L^{-1}) of citric acid in the DO-stat stage (**Figure 2**). However, citrate yields on glycerol obtained in the fed-batch cultivations were lower (**Table 1**) than the values previously reported (0.5 g g^{-1}) in batch cultures (Poblete-Castro et al., 2014a). The co-production of citrate not only is detrimental in terms of carbon loss for biopolymer synthesis but also impacts negatively to the PHA production process as production costs boost, and the addition of the base solution to maintain the optimal pH exerts a dilution effect lowering the PHA volumetric productivities (Warnecke and Gill, 2005).

It is clear that further metabolic engineering efforts must avoid the entry of the carbon flux into the TCA cycle during the biopolymer production phase to diminish organic acid formation in *P. putida* KT2440. There are several routes to achieve this, beginning with the overexpression of acetyl-CoA carboxylase (ACC), which converts acetyl-CoA into malonyl-CoA, where the latter is the main precursor for PHA synthesis of substrates metabolized through the central carbon metabolism (Rehm et al., 1998; Poblete-Castro et al., 2013). Another path is to block the entry of acetyl-CoA into the Krebs cycle by inactivating *in vivo* the citrate synthase enzyme using RNA interference or antisense RNA (Desai and Papoutsakis, 1999; Ko et al., 2020), as the genetic systems can be activated during the nitrogen-limiting phase, which may have no negative impacts on biomass formation in the initial nutrient-sufficient phase. Finally, inspection of the monomer composition of the biosynthesized PHA highly resembled the previous feeding regime's values. The

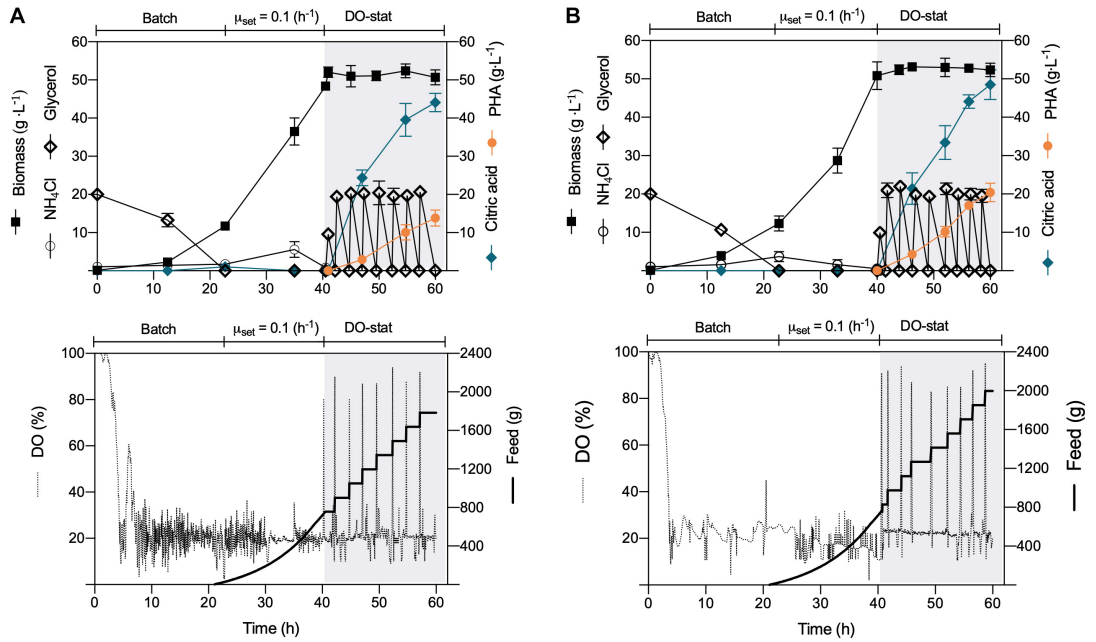


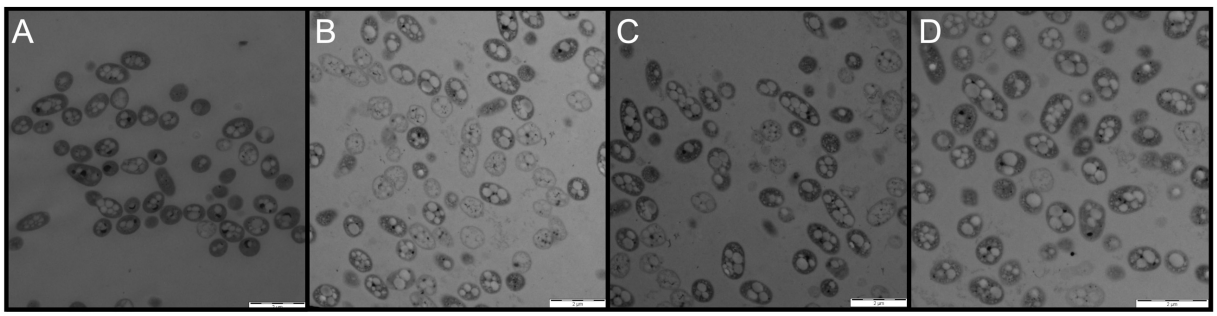
FIGURE 2 | DO-stat fed-batch *mcl*-PHA production. The culture comprised three phases: (i) batch, (ii) exponential feeding, and (iii) DO-stat response for substrate feeding under nitrogen limitation. Time profile of **(A)** *P. putida* KT2440 and **(B)** *phaZ*-knockout mutant. The data represent the mean values and standard deviation from two independent experiments.

most abundant hydroxy acid encountered in both strains was the C10 (**Table 1**). This monomer's relative molar fraction in the parental strain was 73.3%, whereas the Δ*phaZ* knockout mutant presented 76.2% (**Table 1**).

Visualization of the Biosynthesized *mcl*-PHA and Physical Properties

Crude glycerol contains harmful compounds, including methanol, salts, and heavy metals (Mothes et al., 2007; Samul et al., 2014). These elements impair cell growth and evoke stress responses at the proteome and transcriptome levels (Manara et al., 2012; Fu et al., 2015; Bojanovič et al., 2017). This toxic feedstock might also influence cell morphology and the polymerization process of the polyesters. **Figure 3** depicts micrographs acquired by transmission electron microscopy taken at the maximum PHA formation point (60 h) in the constant feeding (3A, KT2440 and 3B, Δ*phaZ mutant*) and DO-stat fermentations (3C, KT2440 and 3D, Δ*phaZ mutant*). Neither the parental strain nor the *phaZ*-disrupted mutant displays significant cellular morphological variance or altered PHA inclusion bodies (**Figure 3**). Conversely to a previous study on crude glycerol, the cells showed no aggregation (Poblete-Castro et al., 2014a), indicating robust growth and proper mixing. Impurities present in the substrate source as black dots were distinctive in both growing *P. putida* strains. Notably, intracellular PHA structures had no apparent alteration by deleting the polymer disassembling enzyme PhaZ (**Figures 3B,D**). As previously noted, *P. putida* strains grown on crude glycerol presented larger numbers of intracellular PHA granules than those cultivated on pure glycerol (Poblete-Castro et al., 2014a), which are more unevenly shaped (**Figures 3A–D**). The PHA morphology depends on various factors, including the level of expression of granular associate proteins like phasins and PHA polymerase and depolymerase (Jendrossek, 2009), which in turn rely on the culture conditions and the growth substrate (De Eugenio et al., 2010). Under stress, PHA-producing bacteria alter the PHA content and its morphology as a response mechanism improving the survival rate when cells thrive under high salt concentrations and low and elevated temperatures or encountering toxic compounds (Obruca et al., 2020). As raw glycerol contains methanol and heavy metal, this could explain the intracellular granule's observed alterations.

We also unveiled the thermal properties of the biosynthesized *mcl*-PHA via differential scanning calorimetry (DSC) of purified biopolymer sampled at 60-h cultivation from the bioreactors. The obtained glass transition points (*Tg*) were very similar among the produced *mcl*-PHAs (−10.5 to −11.9°C) showing a single *Tg* characteristic of a copolymer of medium-chain length polyesters (Cheema et al., 2012; Pacheco et al., 2019; **Figure 3E**). As distinctive elastomers, the melting endotherms were in the range of 56.7–59.3°C (Chan Sin et al., 2010; Gumel et al., 2012). Most importantly, the molecular weight of the purified PHA was higher than 400 KDa (**Figure 3E**), a prerequisite for processing these kinds of macromolecules industrially (Mothes et al., 2007), especially as softener materials (Muangwong et al., 2016; Liu et al., 2018). Together, the PHA thermal and physical properties did not vary significantly between the polyesters synthesized by wild-type and the *phaZ* minus mutant (**Figure 3E**). A *Pseudomonas resinovorans* strain



E

Strain	Fermentation mode	*Tg* (°C)	*Tc* (°C)	*Tm* (°C)	Mw (KDa)	Mn (KDa)	PDI
KT2440	Constant-feeding	-10.5±0.2	Not observed	56.7±0.4	462	193	2.4
Δ*phaZ*	Constant-feeding	-11.5±0.1	Not observed	58.5±0.5	493	198	2.5
KT2440	DO-stat	-11.3±0.2	Not observed	57.1±0.4	473	201	2.4
Δ*phaZ*	DO-stat	-11.9±0.2	Not observed	59.3±0.4	488	190	2.6

FIGURE 3 | Transmission electron micrographs of *P. putida* cells in fed-batch fermentation taken at 60 h fermentation. **(A)** *P. putida* KT2440 constant feeding, **(B)** *phaZ*-knockout mutant constant feeding, **(C)** *P. putida* KT2440 DO-stat, and **(D)** *phaZ*-knockout mutant DO-stat. **(E)** Thermal properties of the biosynthesized *mcl*-PHA: Transition glass temperature (*Tg*), melting temperature (*Tm*), and crystallization temperature (*Tc*). Molecular weight (*Mw*), average-number molecular weight (*Mn*), and polydispersity index (PDI). The values represent mean and standard deviation from two replicates.

lacking the *phaZ* gene presented the same molecular weights and thermal properties of the accumulated PHA as the parental strain (Solaiman et al., 2003). Further, given the high polydispersity index values (PDI > 2), the synthesized *mcl*-PHA on crude glycerol would also perform appropriately in extruders as the elastomers exhibit narrow average-number molar mass distribution (Mn, 190 KDa), where these values are relatively close to commercial polyesters (Debuissy et al., 2017). While extracting the PHA from the cells, we could appreciate the elastomer film that remained after chloroform evaporation in a Petri dish for purification. Film materials are paramount in the packaging and agroindustry sectors. A filmable *mcl*-PHA was synthesized by *Pseudomonas mediterranea* using raw glycerol (Pappalardo et al., 2014). The obtained *mcl*-PHA presented nearly the same proportion of C10 in the monomeric chain than the filmable biopolymer, and above all, both elastomers display *Tm* values higher than 50°C and Mw of 400 KDa.

CONCLUSION

In this study, we demonstrated that the applied feeding strategy during *mcl*-PHA synthesis in *P. putida* strains using the crude glycerol as substrate influences the amount of formed polyester but not their physical properties. We proved that a DO-stat fed-batch process is a more suitable feeding strategy than the constant-feeding approach to metabolize the toxic crude glycerol resulting in nearly 50% more biopolymer at the end of the fermentation in the *phaZ*-deficient mutant and the wild-type KT2440. The attained specific PHA volumetric productivity by Δ*phaZ* knockout mutant (0.34 g L^{-1} h^{-1}) is a step further in the quest to derive *mcl*-PHA from the polyol of the biodiesel industry in a more cost-effective fashion. There is still room for improvement concerning PHA production since the *P. putida* cells secreted high citric acid levels. Further metabolic engineering endeavors must reduce the carbon wastage and redirect carbon flux toward polyester biosynthetic pathways to mining the cell factory's PHA production performance.

METHODS

P. putida Strains

The wild-type *P. putida* KT2440 (DSM 6125) was obtained from the DSMZ collection, Germany, and the Δ*phaZ* mutant was constructed in a previous study (Poblete-Castro et al., 2014a). These strains were used for the different fed-batch fermentations.

Culture Conditions

Strains were stored in 25% glycerol at −80°C as glycerol stocks. Cells were routinely streaked onto Luria–Bertani (LB) agar plates and grown overnight to isolate single colonies. For any shake flask cultivation, *P. putida* strains were aerobically grown at 180 rpm and 30°C. Single colonies were picked from the plate and transferred into a 50 mL shake flask containing 10 mL of LB liquid medium. Defined minimal medium (M9) containing 6 (g L^{-1}) crude glycerol (Cremer Oleo, GmbH, Hamburg, Germany) was employed for subsequent pre-culture cultivation. The industrial glycerol contains 80% glycerol, 0.5% methanol, 10% ash, 3% organic matter, and 6.5% water. The M9 medium composition

consisted (per liter) of 12.8 g $Na_2HPO_4{\cdot}7H_2O$, 3 g KH_2PO_4, 4.7 g $(NH_4)_2SO_4$, and 0.5 g NaCl. After autoclave sterilization, the medium was supplemented with filtered trace elements [6.0 $FeSO_4{\cdot}7H_2O$, 2.7 $CaCO_3$, 2.0 $ZnSO_4{\cdot}H_2O$, 1.16 $MnSO_4{\cdot}H_2O$, 0.37 $CoSO_4{\cdot}7H_2O$, 0.33 $CuSO_4{\cdot}5H_2O$, and 0.08 H_3BO_3 (mg L^{-1})] and 0.12 g of $MgSO_4{\cdot}7H_2O$. Ten milliliters of M9 medium in a 50 mL shake flask was inoculated with the overnight LB-grown culture at an initial OD at 600 $_{nm}$ of 0.2 and incubated overnight. A second pre-culture was initiated by transferring a predetermined volume of the previous one into 300 mL of M9 medium containing 10 g L^{-1} crude glycerol in 1 L baffled Erlenmeyer flasks and cultivated overnight.

Fed-Batch Cultivations

The fed batches were seeded with the second pre-culture to attain an initial cell density of (OD_{600} 0.26). The fed-batch reactor contained M9 medium supplemented with 20 g L^{-1} crude glycerol, 0.12 g L^{-1} $MgSO_4{\cdot}7H_2O$, and 8 ml of the trace element solution. Overall, 4 L of working volume was set up in a 15 L vessel (B10 stirred tank bioreactor, Biologische Verfahrenstechnik, Basel, Switzerland) to conduct the fermentation processes. The airflow rate was maintained at 10 L min^{-1} (air-to-pure oxygen ratio was set to 10:1) during the biomass production phase. In the PHA production phase, the airflow rate was kept, but pure oxygen was no longer needed, and the reactor was sparged only with compressed air, providing better DO response when the carbon substrate was supplied. The temperature was set at 30°C and 12.5 (w/v) of NH_4OH was added as required to stabilize pH to 6.8 ± 0.1 during the course of the biomass formation phase and as nitrogen supply to avoid N limitation. Then, in the PHA production phase, the base was replaced by NaOH 10% (w/v). When required, Tego Antifoam (Evonik, Germany) was supplemented to prevent foaming (200 μL L^{-1}). The agitation speed was automatically adjusted to 800 rpm in order to keep the dissolved oxygen level above 20% air saturation.

The feeding solution contained per liter: 780 g crude glycerol and 12 g $MgSO_4{\cdot}7H_2O$. An exponential feeding strategy was applied during the biomass production phase, following an exponential function (Eq. 1).

$$F(t) = \mu_{set}(V_0.X_0)e^{(\mu\ set.t)}(S_0.Y_{xs})^{-1} \quad (1)$$

Hereby, F is the feed rate (L h^{-1}), μ_{set} is the desired specific growth rate (set to 0.1 h^{-1}), S_0 is the substrate concentration of the feed medium (780 g L^{-1} crude glycerol), t is the time after feed start (h), Y_{xs} is the biomass yield on crude glycerol taken from Poblete-Castro et al. (2014a), V_0 is the initial volume of the culture (L), and X_0 is the initial biomass level (g cells L^{-1}).

Biomass Quantification and Analytical Procedures

The optical density ($OD_{600\ nm}$) was registered over time in a spectrophotometer (Ultraspec 2000; Hitachi, Japan) to determine cell growth. Ten milliliters of cells was harvested at 9,000×g for 10 min at 4°C and washed once with distilled water prior to CDW gravimetric quantification in pre-weighed tubes. The cell pellet was dried to constant weight at 100°C. A photometric test (LCK 303 kit, Hach Lange, Danaher, United States) served to measure offline the ammonium levels in the supernatant. Supernatant samples were withdrawn and accordingly diluted to analyze the crude glycerol and organic acid (citrate, isocitrate, succinate, fumarate, malate, pyruvate, and oxaloacetate) concentrations by HPLC Agilent 1260 (Agilent, Krefeld, Germany). The HPLC system was equipped with an 8-mm Rezex ROA-organic acid H column (Phenomenex, United States), which was operated at 65°C. The mobile phase consisted of 0.013 N H_2SO_4 at a 0.5 ml min^{-1} flow coupled with a RID detector system (Agilent serie1260).

PHA Characterization and Quantification

The polyesters were firstly methanolized in order to determine the PHA monomeric compositions and the intracellular PHA content. For this, 10 ml of culture was transferred to a falcon tube and cells were harvested at 9,000 × g for 10 min at 4°C (Eppendorf 5810 R, Hamburg, Germany). Pellets were washed once with distilled water. The supernatants were discarded, and the pelleted cells were stored at −20°C until needed. The methanolysis procedure was performed as previously specified (Borrero-de Acuña et al., 2014). Gas chromatography (GC) coupled with mass spectrometry (MS) was used to analyze the methyl esters of monomers. One milliliter of the organic phase was injected into a Varian GCMS system 450GC/240MS ion trap mass spectrometer (Varian Inc., Agilent Technologies) at a split ratio of 1:10. The software employed to process the resulting data was the MS Workstation 6.9.3 (Varian Inc., Agilent Technologies). The different compounds, i.e., the methyl esters of 3-hydroxyexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate, 3-hydroxydodecanoate, 3-hydroxy-5-cis-dodecanoate, and 3-hydroxytetradecanoate, were split by using a FactorFour VF-5ms capillary column (30 m × 0.25 mm i.d. × 0.25 mm film thickness), including calibration with commercial PHB (Sigma−Aldrich, MI, United States) and purified *mcl*−PHA from a previous work (Oliva-Arancibia et al., 2017). The carrier gas helium was set to a flow rate of 0.9 ml min^{-1}. The temperatures for the injector and transfer line were established at 275 and 300°C, respectively. The oven temperature was stepwise programmed as follows: 40°C for 2 min, rising progressively from 40 to 150°C at a rate of 5°C min^{-1} and ultimately increasing to 280°C at a constant rate of 10°C min^{-1}. To capture positive ions, an electron ionization at 70 eV was settled, while the mass spectra were registered by scanning ions of m/z 50 to m/z 650. The PHA concentration was determined by the method described by Lageveen et al. (1988). The percentage of biopolymer in relation with the CDW in two biological replicates was averaged out to ascertain PHA content (wt%).

Transmission Electron Microscopy

Prior to fixation, the bacteria were cooled down to 4°C. Next, 2% of glutaraldehyde and 5% of formaldehyde (5%) were added. Cells were subsequently washed with cacodylate buffer (0.01 mol L^{-1} cacodylate, 0.01 mol L^{-1} $CaCl_2$, 0.01 mol L^{-1} $MgCl_2$ $6H_2O$, and 0.09 mol L^{-1} sucrose, pH 6/9) and stained for

1 h at room temperature with 1% aqueous osmium solution. Dehydration was achieved by adding acetone at increasing concentrations (10, 30, 50, 70, 90, and 100%) and incubating the samples for 30 min at each time. Solely the dehydration with 70% acetone containing 2% uranyl acetate was allowed overnight. The Spurr formula for hard resin was applied to infiltrate samples with an epoxy resin. A diamond knife was used to slice the samples into ultra-thin sections, which were further counterstained with a mixture of uranyl acetate and lead citrate. A TEM910 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) was operated at an acceleration voltage of 80 kV to acquire images. Digital imaging of ultra-thin sections was acquired with a Slow-Scan CCD-Camera (ProScan, 1,024 × 1,024, Scheuring, Germany) with ITEM-Software (Olympus Soft Imaging Solutions, Munster, Germany).

Differential Scanning Calorimetry Analysis

The glass transition, crystallization, and melting temperatures (*Tg*, *Tc*, and *Tm*, respectively) of each sample were determined by a Mettler–Toledo DSC 821e. The following five cycles were performed: (i) a first heating from −40 to 200°C at 10°C min^{-1}, (ii) an isotherm for 3 min, (iii) a cooling from 200 to −40°C at 10°C min^{-1}, (iv) an isotherm for 3 min, and (v) a second heating from −40 to 200°C at 10°C min^{-1}.

Size Exclusion Chromatography Measurements

The weight average molecular weights (Mw) and the respective polydispersity indices of the samples were determined by size exclusion chromatography (SEC) along with a static light scattering Dawn EOS in line with an Optilab DSP interferometric refractometer (both were obtained from Wyatt Technology) using $CHCl_3$ as the mobile phase and a calibration curve constructed using polystyrene standard samples. The SEC measurement was performed on a Dionex P590A liquid chromatography pump equipped with a guard column and two PLgel 5-mm Mixed C (300 × 7.5 mm) columns in series with a Viscotek differential refractometer. The eluent was $CHCl_3$ at a flow rate of 1.0 mL min^{-1} at 25°C. Polystyrene standards with a molecular weight range of 1,020–1,944,000 were used to generate a universal calibration curve.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

IP-C conceived and performed the fed-batch fermentation and analytics. CS performed the thermal analysis. MR carried out the TEM studies. JB-dA and IP-C interpreted the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Substrate-Flexible Two-Stage Fed-Batch Cultivations for the Production of the PHA Copolymer P(HB-*co*-HHx) With *Cupriavidus necator* Re2058/pCB113

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Recent studies of the impact and dimension of plastic pollution have drawn the attention to finding more sustainable alternatives to fossil-based plastics. Microbially produced polyhydroxyalkanoates (PHAs) biopolymers are strong candidates to replace conventional plastic materials, due to their true biodegradability and versatile properties. However, widespread use of these polymers is still hindered by their high cost of production. In the present study, we target high yields of the PHA copolymer poly(hydroxybutyrate-*co*-hydroxyhexanoate) [P(HB-*co*-HHx)] using a substrate-flexible two-stage fed-batch approach for the cultivation of the recombinant *Cupriavidus necator* strain Re2058/pCB113. A more substrate-flexible process allows to cope with constant price fluctuations and discontinuous supply of feedstocks on the market. Utilizing fructose for biomass accumulation and rapeseed oil for polymer production resulted in a final biomass concentration of 124 g L^{-1} with a polymer content of 86 wt% holding 17 mol% of HHx. Productivities were further optimized by operating the biomass accumulation stage in a "drain and fill" modus where 10% of the culture broth was recycled for semi-continuous biomass accumulation, after transferring 90% to a second bioreactor for PHA production. This strategy succeeded in shortening process times rising productivity yields to ∼1.45 g L^{-1} h^{-1}.

Keywords: *Cupriavidus necator*, *Ralstonia eutropha*, PHA, poly(hydroxybutyrate-*co*-hydroxyhexanoate), two-stage fed-batch, substrate-flexible, rapeseed oil, high-cell-density cultivation

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INTRODUCTION

Out of the 407 million tons of plastics that are yearly produced worldwide over 35% are used as packaging materials, designed for immediate disposal after a single use (United Nations Environment Programm, 2018). Nevertheless, none of the commonly used plastics are biodegradable. As a result, they accumulate in landfills or the natural environment causing serious contamination problems (Jambeck et al., 2015; Geyer et al., 2017). In this scenario, a shift to sustainable plastic production that relies on renewable resources and does not threaten the environment is urgent. Key to the development of biologically derived biodegradable polymers is a class known as polyhydroxyalkanoates (PHAs). These linear polyesters are produced

as water-insoluble storage polymers by a wide range of bacteria during nutrient limiting or stress conditions and the presence of excess carbon (Lenz and Marchessault, 2005; Dias et al., 2006; Obruca et al., 2020). Depending on the length of the side chain, PHA can be classified into *short-*, *medium-,* and *long-chain-length-* (*scl-*, *mcl-,* and *lcl-*)-polymers (Steinbüchel et al., 1992). PHAs exhibit thermoplastic and in the case of *mcl*-PHAs also elastomeric properties, similar to those of petroleum-based plastics. This makes PHAs suitable for a wide range of applications (Philip et al., 2007; Noda et al., 2010). After being disposed in environments with high microbial activity such as the soil, marine water or even in sewage sludge PHAs biodegrade easily within months (Ong et al., 2017; Meereboer et al., 2020).

Despite the considerable advantages of PHAs in terms of low environmental impact and highly tunable mechanical properties, production capacities for these polymer, although being one of the fastest growing amongst biopolymers, only account for around 1% of the global bioplastics extent (Vandi et al., 2018; European bioplastics, 2019). Barriers hindering PHA commercialization are mostly related to relatively higher costs, which makes it challenging to compete with low-priced petroleum-based plastics that are produced on a very large scale (Możejko-Ciesielska and Kiewisz, 2016; Sabapathy et al., 2020).

Cupriavidus necator (formerly *Ralstonia eutropha*) is the most studied organism for PHA production, mainly due to its ability to store the polymer up to 90% of its cell dry weight (CDW) under an ample spectrum of carbon sources and its diverse genetic modifiability (Pohlmann et al., 2006; Reinecke and Steinbüchel, 2008; Choi et al., 2020). The recombinant *C. necator* strain used in this study (Re2058/pCB113) was engineered to produce the poly(hydroxybutyrate-*co*-hydroxyhexanoate) [P(HB-*co*-HHx)] when fed with fatty acids containing feedstocks (Budde et al., 2011). Enhanced mechanical and thermal properties are depictured by this copolymer in comparison to poly(hydroxybutyrate) [P(HB)], which is typically produced by the wild-type strain or by this recombinant strain in the absence of an oleaginous substrate. The choice of a suitable carbon source is a main aspect in the optimization of PHA production, since it represents the highest cost driving factor, besides the downstream process (Koller et al., 2017). In this aspect, several inexpensive plant oils, predominantly palm oil and derivatives from the palm oil industry but also waste oleaginous feedstocks like waste cooking oils and animal by-products have been broadly used due to low prices and higher conversion rates to PHA in comparison to sugars (López-Cuellar et al., 2010; Riedel and Brigham, 2020). Nevertheless, it was pointed out that more flexible processes need to be developed in order to cope with constant price fluctuations and discontinuous supply of these feedstocks (Rodriguez-Perez et al., 2018). Developing a substrate-flexible process, where different substrates can be used for biomass accumulation and PHA production will reduce the dependency of a single feedstock. In addition, regarding bioprocess optimization to further decrease costs, only recently advanced cultivation strategies, like repeated batch and repeated fed-batch with bioreactors operated in a "drain and fill" modus were approached in order to achieve higher productivity yields and avoid the downtime between batches which results in high operation times (Koller, 2018).

In this study we report substrate-flexible two-stage fed-batch cultivations for the production of P(HB-*co*-HHx) using fructose during the biomass accumulation stage and rapeseed oil for polymer production. In order to achieve high space time yields (STY) a "drain and fill" modus is proposed for semi-continuous biomass production during the initial stage. Taking advantage of the high-cell-density achieved during the first stage, the second stage is run without previous sterilization of the in-series bioreactors.

MATERIALS AND METHODS

Bacterial Strain, Preculture Conditions and Growth Media

Experiments were performed with the recombinant *C. necator* strain Re2058/pCB113, which produces the PHA copolymer P(HB-*co*-HHx), when grown on fatty acid containing feedstocks and polyhydroxybutyrate (PHB) when grown on fructose (Budde et al., 2011).

The seed train followed for the bioreactor cultivations consisted of two steps. First, a 125-mL Ultra Yield Flask™ (Thomson Instrument Company, United States) containing 10 mL dextrose-free trypsic soy broth (TSB) medium and supplemented with 10 μg mL^{-1} gentamicin sulfate and 200 μg mL^{-1} kanamycin sulfate was inoculated with a single colony from an actively growing TSB agar plate, sealed with an AirOtop™ membrane (Thomson Instrument Company, United States) and incubated for 17 h until reaching OD_{583} of 4–5.

Secondly, 1 mL of the pre-seed culture was used to inoculate a 500-mL DURAN® baffled flask containing 100 mL phosphate buffered minimal medium that was sealed with an AirOtop membrane and incubated for around 26 h until OD_{583} of 4–5. The minimal media contained for each liter: 33.5 mL 1 M NaH_2PO_4, 64.5 mL 0.5 M Na_2HPO_4, 5.2 mL 0.5 M K_2SO_4, and 1 mL 1 M NaOH that were autoclaved together and then supplemented with the left sterile components: 20 mL 50% (w v^{-1}) fructose, 20 mL 11.2% (w v^{-1}) urea (or 20 mL 20% (w v^{-1}) ammonium chloride for optimization of the biomass production stage), 10 mL 39 g L^{-1} $MgSO_4$, 10 mL 6.2 g L^{-1} $CaCl_2$, 1 mL 10 mg mL^{-1} gentamicin sulfate and 1 mL trace element solution. The trace element solution consisted of 15 g L^{-1} $FeSO_4{\cdot}7H_2O$, 2.4 g L^{-1} $MnSO_4{\cdot}H_2O$, 2.4 g L^{-1} $ZnSO_4{\cdot}7H_2O$, and 0.48 g L^{-1} $CuSO_4{\cdot}5H_2O$ dissolved in 0.1 M hydrochloric acid. Incubation of the precultures was always performed at 30°C and 200 rpm in an orbital shaker (25 mm amplitude, INFORS HT Multitron Standard, Infors AG, Switzerland).

For bioreactor cultivations the unsterile components were sterilized *in situ* followed by addition of the sterile components to an initial volume of 0.5 L. Inoculation was performed with 20 mL of the pre-seed culture to an initial $OD_{583} \approx 0.2$. After 24 h of cultivation $MgSO_4$, $CaCl_2$, K_2SO_4, and trace elements

were added to initial concentrations in order to avoid limitation of these nutrients.

Bioreactor Cultivation Conditions

For fed-batch cultivations 1-L Multifors parallel benchtop bioreactors (Infors AG, Switzerland) were used. The cultivation temperature was kept constant at 30°C and the pH maintained at 6.8 ± 0.1 through controlled addition of 1 M H_3PO_4 and 2 M NaOH (or 25% (v v^{-1}) ammonia for pH-controlled feeding). Stirring was performed using two six-blade Rushton impellers. The initial stirring speed was set to 200 rpm, whereas the initial flow rate was set to 0.05 vvm. Via an automatized cascade, aeration was increased up to 0.5 vvm and later stirring was increased up to 1,500 rpm in order to prevent dissolved oxygen (DO) values from dropping below 40%. Foam was mechanically broken as described previously (Riedel et al., 2012). Additionally, silicon oil was added as antifoam when needed (maximum total amount added of 1 mL). The fed-batch cultivations were always performed in biological duplicates (two independent bioreactor cultivations) and consisted of two stages: a biomass accumulation stage and a PHA production stage.

Evaluation of Nitrogen Feeding in the Biomass Accumulation Stage

The biomass accumulation stage was conducted as indicated below with varying concentrations of nitrogen source in the feeding solution: without ammonium chloride, with 1% (w v^{-1}) ammonium chloride and with 2% (w v^{-1}) ammonium chloride. The cultivations were run for 48–50 h and the accurate concentration for ensuring availability of nitrogen throughout the complete biomass stage was tested. In the cultivations run afterward, ammonium chloride was replaced by corresponding concentrations or urea.

Two-Stage Fed-Batch Cultivation

Biomass accumulation stage

Initial batch phase until depletion of the 1% (w v^{-1}) fructose present in the bioreactor followed by the automated start (triggered by sudden DO increase) of exponential feeding with 50% (w v^{-1}) fructose and 0.56% (w v^{-1}) urea at the specific growth rate μ_{set} according to

$$\mu_{set} = 0.75 \cdot \mu_{max} \quad (1)$$

$$F(t) = F_0 \cdot e^{\mu_{set} \cdot t} \quad (2)$$

The initial feed rate (L h^{-1}) was calculated according to

$$F_0 = \frac{\mu_{set}}{Y_{X/S} \cdot S_i} (X_0 \times V_0) \quad (3)$$

where $Y_{X/S}$ the biomass/substrate yield (calculated from the batch phase), S_i the concentration of the carbon source in the feeding solution, and X_0 and V_0 the biomass concentration (calculated from a correlation between previous OD_{583} and CDW measured values) and bioreactor liquid volume at the end of the batch phase, respectively. During this stage the pH was controlled through addition of 25% ammonia in order to avoid nitrogen limitation. Feeding was performed until the measured OD_{583} exceeded 100 which marked the beginning of the second stage.

PHA production

PHA accumulation was triggered by nitrogen limitation. Therefore, the pH-control was switched from ammonia to 2 M NaOH. During this stage, a total amount of 135 g L^{-1} rapeseed oil were fed to the culture in a constant manner during the first 12 h. The culture was grown for further 32–36 h until the complete oil present in the bioreactor was consumed and the cells had achieved the highest PHA content.

Repeated Fed-Batch Cultivation With Semi-Continuous Biomass Accumulation

Three complete cycles of biomass accumulation and PHA production were run operating the biomass accumulation bioreactor in a "drain and fill" modus. The biomass accumulation stage was followed as indicated above until $OD_{583} > 100$ and then 90% of the broth was transferred to a second bioreactor for PHA accumulation. To this end external periplasmic pumps were used. The main bioreactor was then refilled with sterile fresh media to a starting volume of 0.5 L and the biomass accumulation stage was repeated. Taking advantage of the high-cell-density achieved during the first stage, the second stage was run without previous sterilization of the bioreactors.

Analytical Methods

For sampling, aliquots of 8 mL from bioreactor cultures were sampled in pre-weighted 15-mL tubes. The samples were centrifuged for 15 min and 4°C at 6,500 × *g*. The pellets were washed with 7 mL cold water (for samples during biomass accumulation) or with a mixture of 5 mL cold water and 2 mL cold hexane to remove residual oil (for samples during PHA production, when rapeseed oil was used as carbon source) and then dried at 80°C for CDW determination. The content and composition of PHA from dried cells was determined using a methanolysis protocol and gas chromatography as described previously (Bartels et al., 2020). The residual cell dry weight (RCDW) was defined as CDW minus the PHA content in g L^{-1}. At every sampling point the OD_{583} of the culture broth was measured in duplicates, manually with a spectrophotometer (Ultraspec 3000, GE Healthcare, CT, United States; OD_{583}-Photometer) and additionally with the automated pipetting system (Cedex Bio HT Analyzer®, Roche Diagnostics International AG, Switzerland; OD_{583}-Cedex). Furthermore, 1 mL of the supernatant was filtered through an 0.2 µL PES syringe filter and used for fructose determination via HPLC-RID. Chromatography was run with 20 µL injection volume at 80°C for 62 min on an Agilent Hi-Plex Ca column. The eluent was DI H_2O with an 0.6 mL min^{-1} flux. Unfiltered supernatant was measured with the Cedex Bio HT Analyzer to assess consumption of NH_3, Mg^{2+}, PO_4^{3-}, and Ca^{2+}.

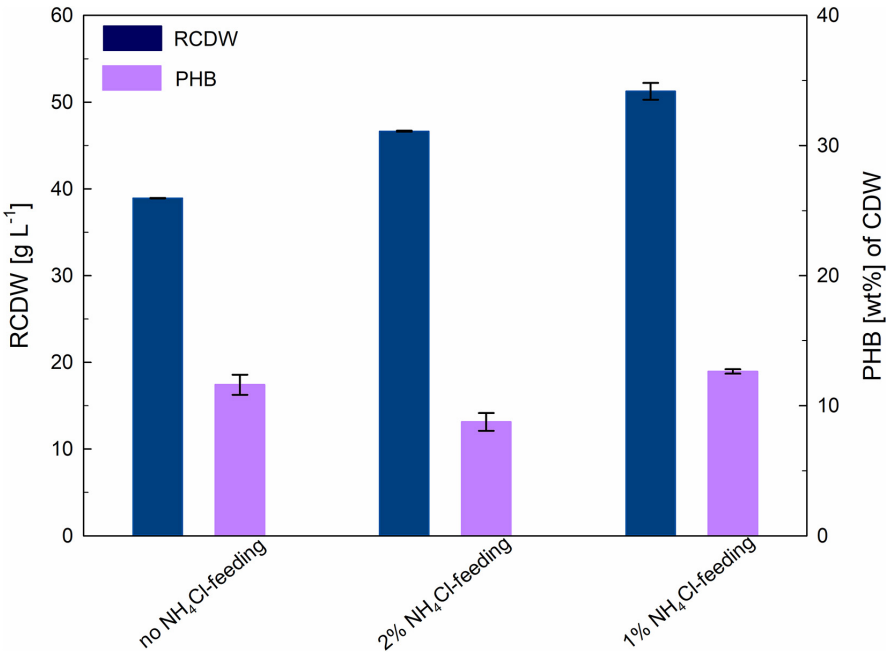

FIGURE 1 | Optimization of biomass production stage: the achieved residual cell dry weight (RCDW; g L^{-1}) and PHB content (wt%) after 48–50 h of cultivation is indicated for every condition. Error bars indicate standard deviation from two independent bioreactor cultivations.

RESULTS

Evaluation of Nitrogen Feeding in the Biomass Accumulation Stage

For an initial evaluation of the optimal conditions for ensuring nitrogen availability throughout the complete biomass accumulation stage, three different strategies were evaluated; providing nitrogen only through the pH-controlled feeding with 25% (v v^{-1}) ammonia, adding also 1% (w v^{-1}) ammonium chloride to the fructose feeding solution and adding 2% (w v^{-1}) ammonium chloride to the fructose feeding solution. It is important to mention that after nitrogen limitation the bacteria immediately cease growth and engage polymer accumulation, this limiting final biomass yields. **Figure 1** shows an overview of the final RCDW and PHB values yielded by each cultivation after 48–50 h.

When no nitrogen source was added to the fructose feeding solution (see **Supplementary Figure 1**), NH_3 concentrations measured showed a notorious decrease after the beginning of the fed-batch phase (from 120 to 3 mM). The inability of the pH-controlled nitrogen feeding to keep up with the nitrogen consumption of the culture triggered a premature accumulation of PHB, which lowered the final biomass yield of the cultivation. After 48 h a RCDW of 38 g L^{-1} with a PHB content of 12 wt% had been reached.

In order to circumvent nitrogen limitation during the biomass accumulation stage, addition of two different concentrations of ammonium chloride to the fructose feeding solution was tested (see **Supplementary Figure 2**). When 1% (w v^{-1}) ammonium chloride was added, the feeding succeeded in compensating the consumption of the cells; although NH_3 concentration slightly dropped during the fed-batch phase it never went below 80 mM. This cultivation showed the highest biomass yield with 51 g L^{-1} RCDW and a PHB content of 12 wt% after 50 h of cultivation. Regarding the feeding with 2% (w v^{-1}) ammonium chloride, it could be observed that during the fed-batch phase NH_3 quickly accumulated in the bioreactor doubling the initial concentrations. Final titers after 50 h of cultivation showed a RCDW of 45 g L^{-1} with a PHB content of 11 wt% of CDW.

Exponential feeding with 50% (w v^{-1}) fructose and 1% (w v^{-1}) ammonium chloride in addition to the pH-controlled feeding with 25% (v v^{-1}) ammonia was chosen as the best strategy to ensure nitrogen availability throughout the complete biomass production stage. In the following cultivations the fructose feeding was supplemented with 0.56% (w v^{-1}) urea (corresponding to 1% (w v^{-1}) ammonium chloride (187 mM NH_4^+).

Two-Stage Fed-Batch Cultivation

An overview of the substrate-flexible two-stage fed-batch strategy for P(HB-*co*-HHx) production with fructose feeding for the biomass accumulation stage and rapeseed oil feeding for polymer production is presented in **Figure 2**.

The cultivation was performed in biological duplicates (two independent bioreactor cultivations) and the results are depictured below (**Figure 3**). The batch phase lasted ∼21 h and showed a maximal growth rate of 0.22 h^{-1} and an average yield of 0.44 (g biomass/g fructose). After depletion of the 1% (w v^{-1}) fructose present in the bioreactor exponential feeding with 50% (w v^{-1}) fructose and 0.56% (w v^{-1}) urea at a μ_{set} of 0.15 h^{-1} was

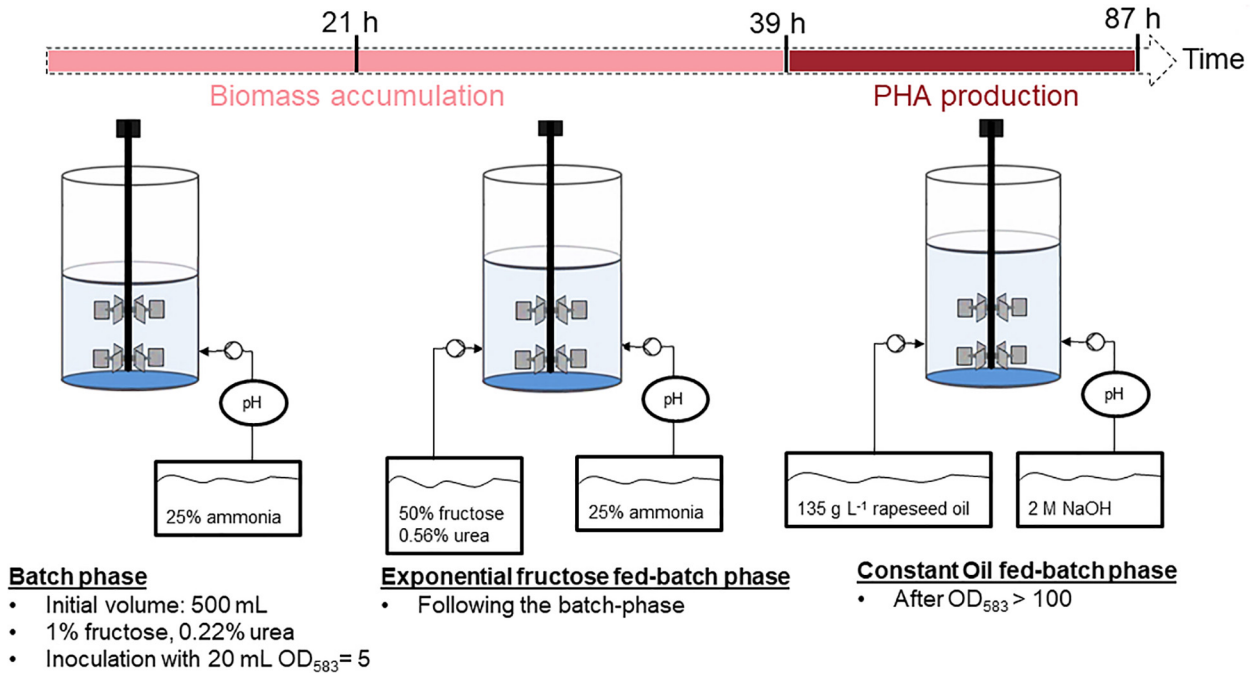

FIGURE 2 | Overview of the substrate-flexible two-stage fed-batch strategy.

performed for 18 h. During this period around 100 mL of feeding solution was provided to the culture. Fructose remained limiting during the complete fed-batch phase with HPLC measurements showing undetectable concentrations. Furthermore, ammonia measurements showed permanent availability of nitrogen with a minimal concentration of 30 mM NH_3. It is important to mention that the availability of the nitrogen source, urea, was detected only indirectly. Urea is taken up by the bacteria and hydrolyzed by the cytoplasmic urease enzyme complex, leading to one CO_2 and two ammonium molecules (Beckers et al., 2004). In this study, only measurements of NH_3 were performed. After 39 h of cultivation 36 g L^{-1} CDW with a PHB content of 9.3 wt% of CDW had been attained. After measuring optical densities greater than OD_{583} = 100 the second stage of the cultivation was started triggering nitrogen limitation by changing the pH-control to NaOH and feeding with rapeseed oil to enable the incorporation of the *mcl*-HHx-monomers into the PHA polymer. During the first 12 h of this stage a total amount of 135 g L^{-1} rapeseed oil were fed at a constant feeding rate. Nitrogen had been depleted within 6 h and after a total period of 87 h a final CDW of 124 g L^{-1} and a P(HB-*co*-HHx) content of 86.1 wt% of CDW with an HHx level of 16.9 mol% had been achieved. The P(HB-*co*-HHx) STY was calculated to be 1.22 g L^{-1} h^{-1}.

Repeated Fed-Batch Cultivation With Semi-Continuous Biomass Accumulation

While the two-stage fed-batch cultivation aimed to evaluate the feasibility of achieving high P(HB-*co*-HHx) titers using fructose for the biomass accumulation stage and rapeseed oil for polymer production and served as a reference for this process, the repeated fed-batch cultivations performed later in the study and presented here engaged with the goal of developing a more time-effective process with optimized STY.

To this end, a strategy consisting of a repeated fed-batch based on a "drain and fill" operation modus for semi-continuous biomass accumulation was developed for the first stage of the process. 90% of the high-cell-density culture delivered from the first stage was then transferred into a second bioreactor, which needed no previous sterilization and served for polymer accumulation in the second stage of the process. Three complete cycles of biomass accumulation and polymer production were conducted over a total period of 1 week. An overview of the chosen strategy, including the times needed for each cycle is provided in **Figure 4**. A detailed sketch of the cultivations is provided in **Supplementary Figure 3**.

Biomass Accumulation

Figure 5 delivers key information gained from the repeated fed-batch strategy that was applied for the three cycles of semi-continuous biomass accumulation. The first cycle of biomass accumulation (0–36 h) involved an initial batch phase of 22 h that was characterized by a long lag phase of around 11 h after which the culture showed a μ_{max} of 0.23 h^{-1}. After fructose depletion, exponential feeding at a μ_{set} of 0.15 h^{-1} was applied for 14 h. During this first fed-batch phase fructose remained at undetectable concentrations and NH_3 measurement showed permanent availability of nitrogen with a maximal value of 45 mM. At the end of the first biomass accumulation cycle 31.1 g L^{-1} CDW with a PHB content of 14.3 wt% had been reached. After this timepoint 90% of the high-cell-density culture

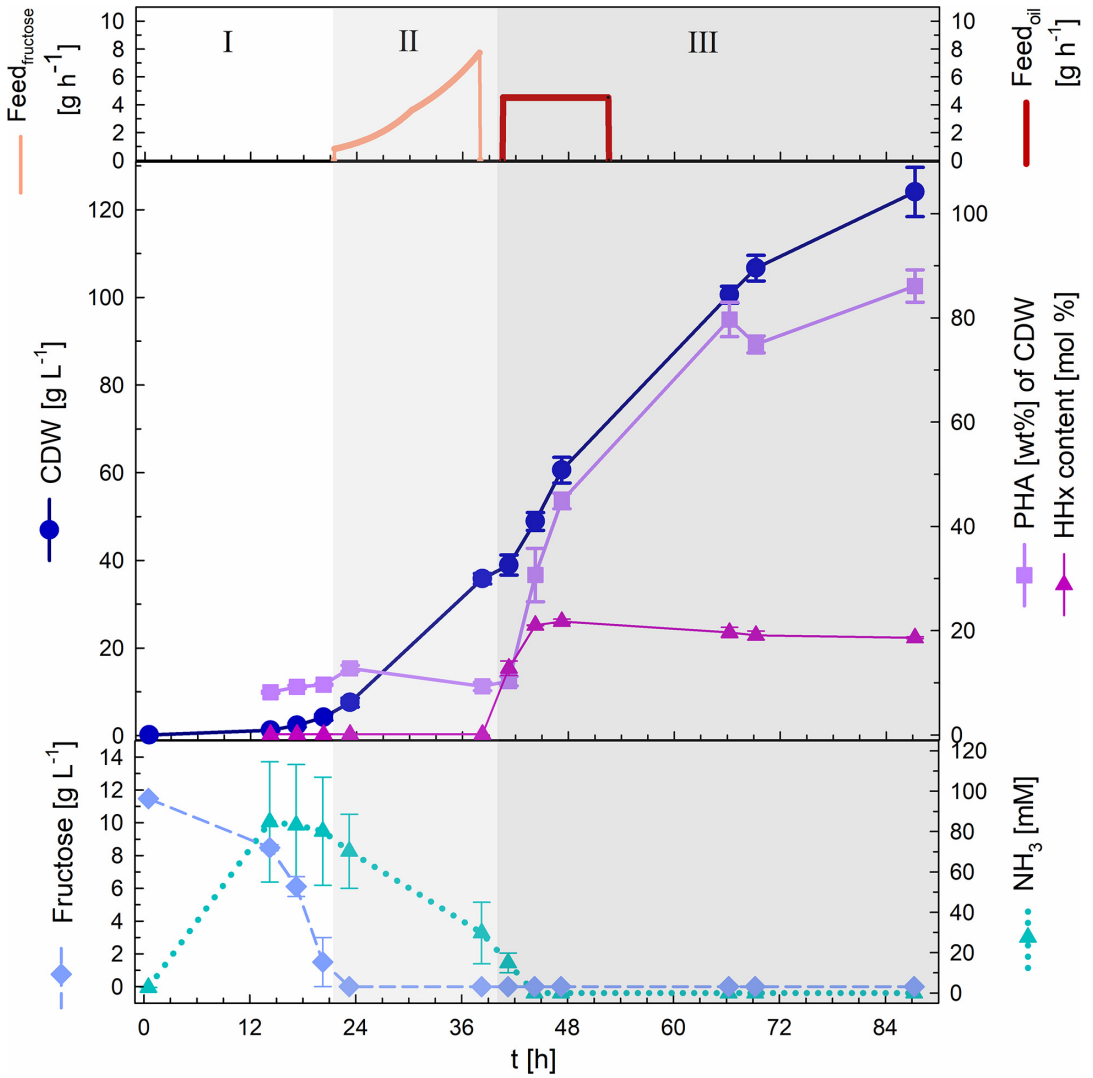

FIGURE 3 | Substrate-flexible two-stage fed-batch cultivation. Upper graph: fructose feed (g h^{-1}) and oil feed (g h^{-1}). Middle graph: cell dry weight (CDW; g L^{-1}), PHA content (wt%) and HHx content (mol%). Lower graph: fructose (g L^{-1}) and NH_3 (mM) concentrations. Biomass accumulation stage: batch phase (I) and exponential fructose fed-batch phase (II). PHA production stage: constant rapeseed oil fed-batch phase (III). Error bars indicate standard deviation from two independent bioreactor cultivations.

broth was withdrawn and the left 60 mL were recycled for the next cycle of biomass accumulation that was started after refilling the bioreactor with fresh media to an initial volume of 0.5 L.

The second cycle of biomass accumulation (36.5–60.5 h) showed a comparably much shorter batch phase of only 4 h (with no detectable lag phase) within which the complete 10 g L^{-1} fructose provided in the fresh media had been consumed. Feeding was then performed for 20 h at again, a μ_{set} of 0.15 h^{-1}. At the end of the second fed-batch phase fructose and NH_3 had accumulated in the bioreactors reaching 8 g L^{-1} and 137 mM respectively. Biomass measurements showed final values of 28 g L^{-1} with a PHB content of 19.4 wt%.

For the third and last cycle of biomass accumulation (61–84 h), 60 mL of culture broth from the second cycle were again recycled. The third batch phase lasted 7 h and was followed by 19 h of exponential fed-batch phase. At the end of the fed-batch phase fructose and NH_3 had accumulated in the bioreactors (21 g L^{-1} fructose and 90 mM NH_3). Final values of the third biomass accumulation cycle showed 24 g L^{-1} CDW and a PHB content of 19.1 wt%.

Recycling 10% of the biomass at the end of each cycle reduced batch times needed for initial biomass accumulation from 22 h (1st cycle) to 4–7 h (2nd and 3rd cycle).

PHA Production

During the second stage of the cultivation, which was conducted in a second bioreactor, polymer accumulation was triggered by nitrogen limitation. Nitrogen source was no longer provided and feeding with rapeseed oil permitted the incorporation of *mcl*-monomers into the PHA polymer. **Figure 6** illustrates such stage,

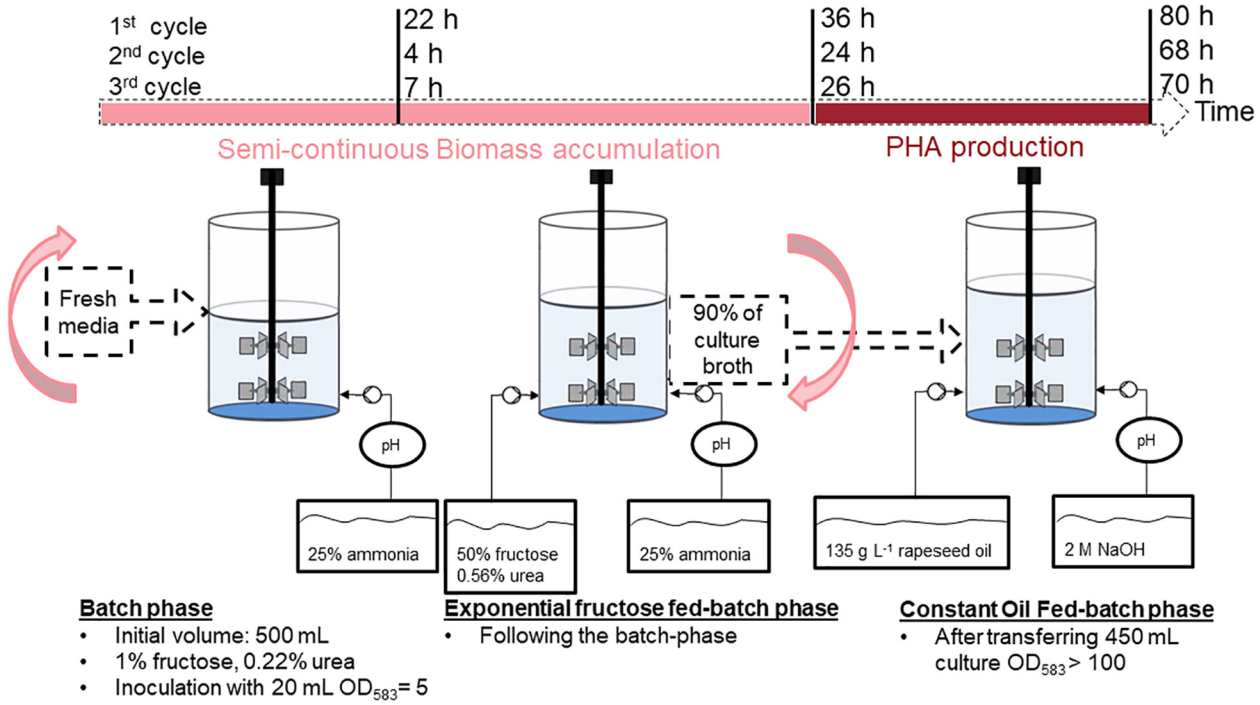

FIGURE 4 | Overview of the substrate-flexible two-stage repeated fed-batch cultivation strategy with semi-continuous biomass accumulation.

in this case, the data presented was gained from the first cycle of polymer production.

The culture broth withdrawn from the first biomass accumulation stage that presented relatively low NH_3 concentrations (30 mM) showed nitrogen depletion after 4 h after which time P(HB-*co*-HHx) enrichment was set off reaching maximal concentrations of 82.9 wt% with an HHx level of 18.1 mol%. At the end of the first cycle 119 g L^{-1} CDW had been attained.

In order to guarantee high P(HB-*co*-HHx) production during the second stage of the process, it was mandatory to withdraw from the first stage a culture broth that contained no excess nitrogen concentrations (see **Table 1**). It was observed that NH_3 concentrations that did not exceed 45 mM (see two-stage fed-batch and repeated fed-batch 1st cycle) were consumed within the first few hours of the second stage after which still enough carbon source was fed to trigger the accumulation of high PHA concentrations. When overfeeding during the first stage of the process resulted in ammonia accumulation in the bioreactor (see repeated fed-batch 2nd and 3rd cycle) polymer production during the second stage was strongly hindered.

Comparison of Growth and PHA Production During All Cultivations

First, the biomass accumulation stage was optimized to RCDW > 50 g L^{-1} by ensuring nitrogen availability throughout the complete stage, thus retarding polymer production (**Figure 1**). It was determined, that supplementing the fructose feeding with 187 mM ammonium in addition to the pH-controlled feeding with 25% (v v^{-1}) ammonia was the best strategy to avoid premature polymer enrichment. Moving forward, after reaching high biomass concentrations, this was followed by a polymer production stage with rapeseed oil feeding. A CDW of 124 g L^{-1} and a P(HB-*co*-HHx) content of 86.1 wt% with an HHx level of 16.9 mol% after 87 h with a STY of 1.22 g L^{-1} h^{-1} was achieved (**Table 1** and **Figure 3**). Next, a repeated two-stage fed-batch cultivation with semi-continuous biomass accumulation was developed. By variation of the biomass accumulation stage into cyclic mode ("drain and fill"), the need of new precultures and the non-productive time of cleaning and setting up the bioreactor for the initiation of a fresh cultivation could be avoided. The culture lag phase, of around 11 h, observed at the beginning of the batch phase in the first approach was significantly reduced by recycling 10% of the high-cell-density biomass of each cycle (**Figure 5**). This served as inoculum when the bioreactor was refilled with fresh media whereas 90% of the culture broth was transferred into a second bioreactor for polymer accumulation. By this, a time reduction of the batch phase from 22 h to 4–7 h was observed in the cyclic approach (**Figure 5**). Taking only the reduction of the batch phase into consideration the STY of the process could be increased by 20% to a potential STY of ∼1.45 g L^{-1} h^{-1}.

DISCUSSION

The purpose of our study was to develop a substrate-flexible two-stage fed-batch process where fructose was only utilized for cell growth and P(HB-*co*-HHx) accumulation was only triggered

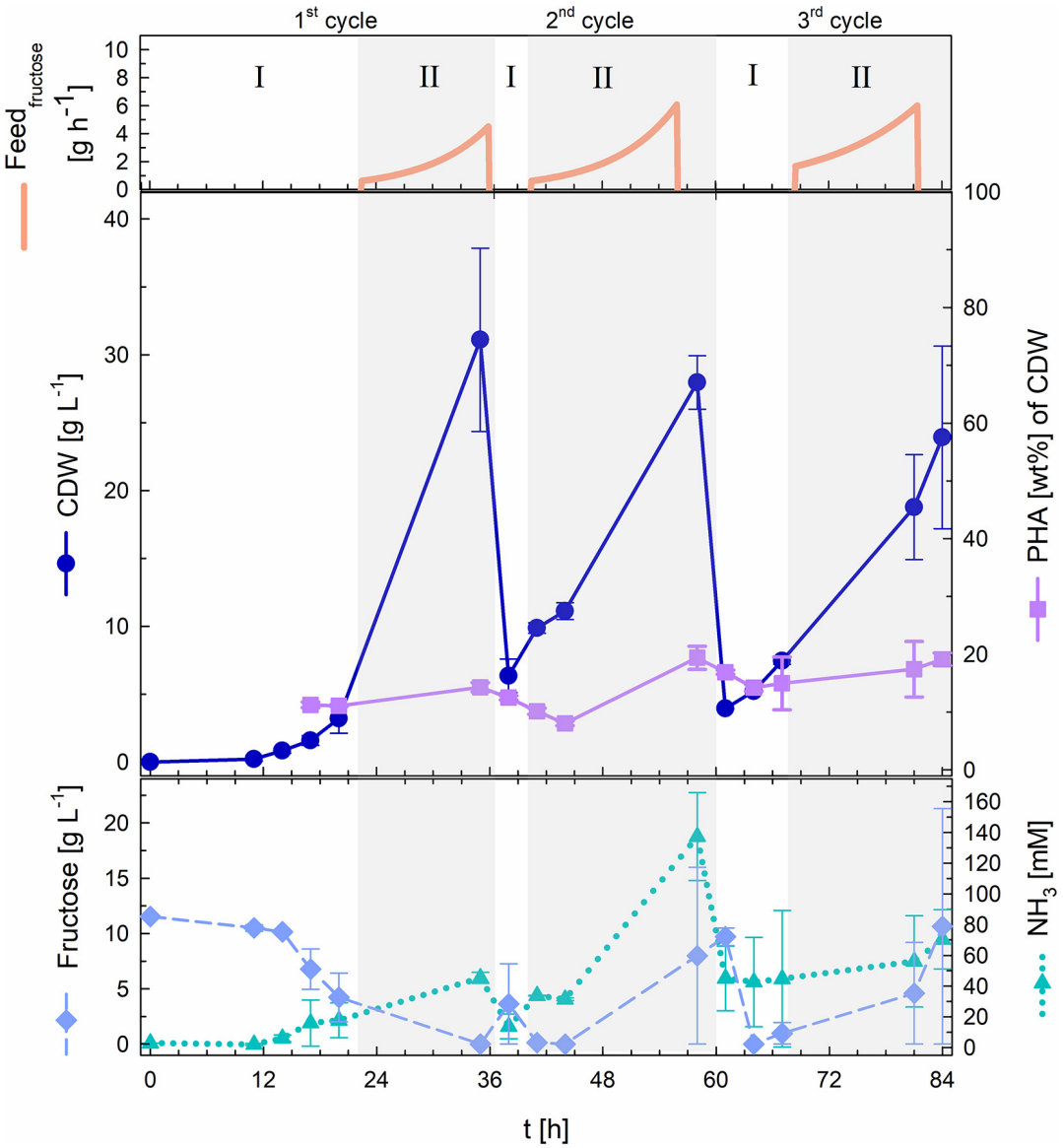

FIGURE 5 | Repeated fed-batch cultivation with semi-continuous biomass accumulation. Upper graph: fructose feed (g h^{-1}). Middle graph: cell dry weight (CDW; g L^{-1}) and PHA content (wt%). Lower graph: fructose (g L^{-1}) and NH_3 (mM) concentrations. Three cycles of biomass accumulation: batch phase (I) and exponential fructose fed-batch phase (II). Error bars indicate standard deviation from two independent bioreactor cultivations.

in combination with rapeseed oil feeding. The P(HB-*co*-HHx) yields from rapeseed oil where between 0.70–0.76 g g^{-1} with HHx contents of around 17 mol%. This strongly correlates to results obtained in high-cell-density cultivations with the same strain, where only plant oil was used as the carbon source (Riedel et al., 2012; Madison et al., 2014). Previously published studies with *C. necator* Re2058/pCB113 showed a decrement of the HHx content when using mixtures of sugars and plant oils during the whole cultivation (Murugan et al., 2016, 2017; Purama et al., 2018). This effect also occurred during the PHA production phase of the 2nd and 3rd cycle, where fructose was still present due to overfeeding of the biomass accumulation stage (**Figure 5** and **Table 1**). Early PHA production with *C. necator* Re2058/pCB113 was reported previously and is attributed to the PHA production genes being located on an overexpression plasmid (Budde et al., 2011). However, interestingly, in this study the HHx content did not decrease during the 1st cycle and the two-stage fed-batch (without fructose overfeeding), although the strain had already accumulated around 20 wt% PHB during the biomass accumulation from fructose (**Figures 3**, **5 Table 1**). Therefore, the method presented herein is able to increase substrate flexibility without affecting final yields and polymer composition. However, the effect on the thermal, physical, mechanical features and the molecular weight has to be investigated in further studies. The weight average molecular weight of P(HB-*co*-HHx) produced with *C. necator* Re2058/pCB113 has been reported to be in the

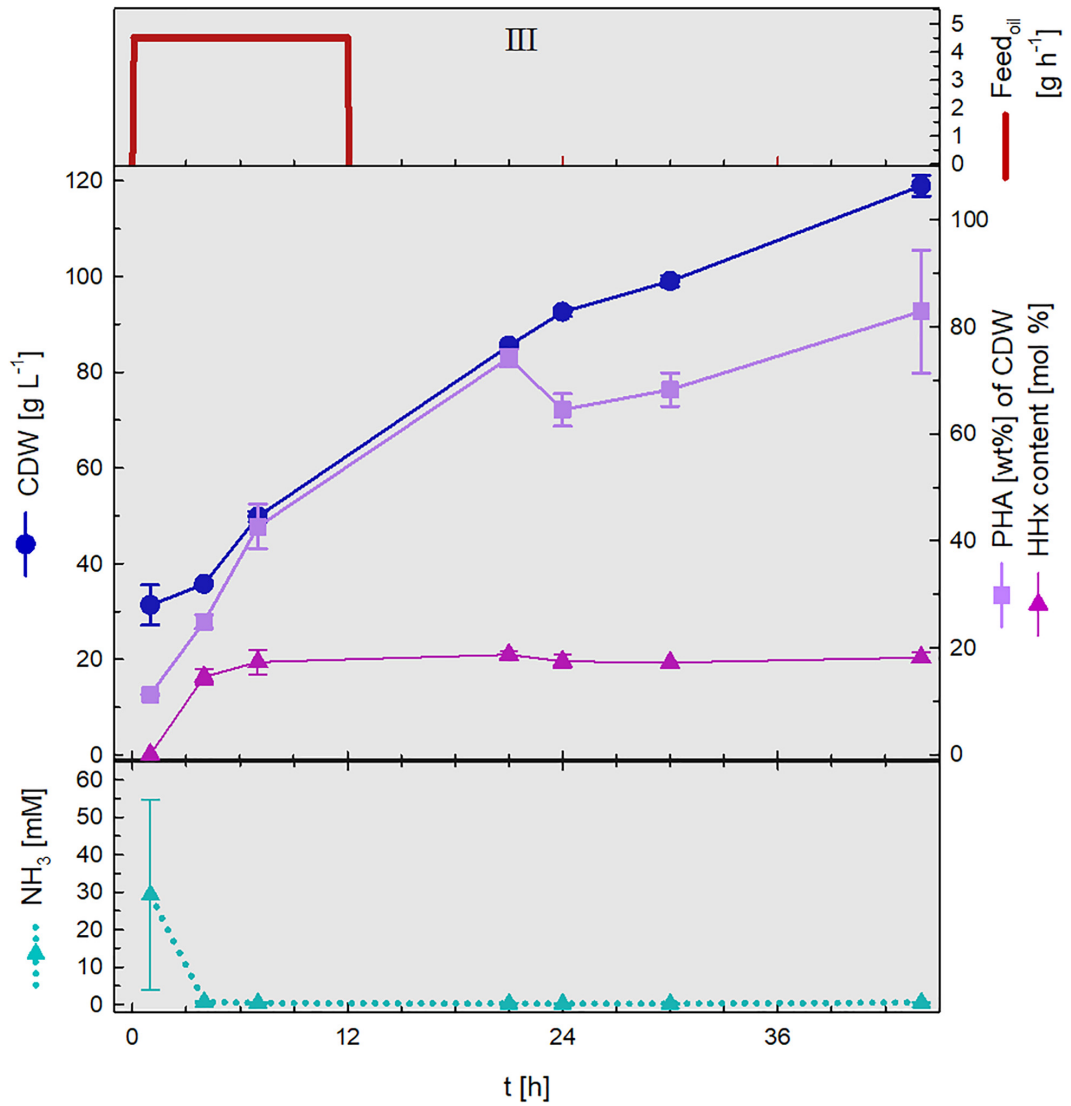

FIGURE 6 | PHA production stage. Upper graph: oil feed (g h^{-1}). Middle graph: cell dry weight (CDW, g L^{-1}), PHA content (wt%) and HHx content (mol%). Lower graph: NH_3 (mM) concentrations. Constant rapeseed oil fed-batch phase (III) of the 1st cycle. Error bars indicate standard deviation from two independent bioreactor cultivations.

TABLE 1 | Comparison of growth and PHA production during all cultivations.

	Biomass accumulation stage				PHA production stage			Overall
	CDW (g L^{-1})	PHB (wt%)	NH_3 (mM)	Duration (h)	CDW (g L^{-1})	P(HB-*co*-HHx) (wt%)	HHx (mol%)	STY (g L^{-1} h^{-1})
Two-stage fed-batch	35.8 ± 1.2	9.3 ± 0.8	29.7 ± 15.3	39	124.0 ± 5.6	86.1 ± 3.1	16.9 ± 0.2	1.22
Repeated fed-batch 1st cycle	31.1 ± 6.7	14.3 ± 0.8	45.4 ± 3.6	36	118.9 ± 2.1	82.9 ± 11.5	18.1 ± 0.9	1.23
Repeated fed-batch 2nd cycle	27.9 ± 1.9	19.4 ± 2.0	137.3 ± 28.6	24	35.8 ± 10.1	33.9 ± 29.2	20.6 ± 2.4	0.18*
Repeated fed-batch 3rd cycle	23.9 ± 6.7	19.1 ± 1.1	70.4 ± 19.3	26	53.6 ± 18.7	56.3 ± 18.4	13.5 ± 2.3	0.43*

CDW and PHA concentrations, including HHx content for the production stage, where P(HB-co-HHx) was synthesized, are displayed. In addition, final PHA STYs are shown for each run. NH_3 accumulation at the end of the biomass accumulation stage is presented in order to understand the low polymer yields(*) attained in the 2nd and the 3rd cycle of the repeated fed-batch. In addition, the duration of the biomass accumulation stage shows the advantage of the repeated fed-batch approach. Measurements represent means from duplicate cultivations ± are indicating minimum and maximum values.

range of 3.0–6.0 × 10^5 Da when grown on plant oils as sole carbon source (Riedel et al., 2012; Zainab-L et al., 2018). Using mixtures of palm oil and fructose or seed oil and molasses as carbon sources for *C. necator* Re2058/pCB113 cultivations (total CDW < 10 g L^{-1}), lead to average molecular weights in the range of 5.5–8.3 × 10^5 with controllable HHx-contents between 4–28 mol% HHx (Murugan et al., 2017; Purama et al., 2018).

Efforts in optimizing the initial growth stage with fructose feeding succeeded in rising the RCDW up to 50 g L^{-1} before the main polymer production phase (**Figure 1**). To avoid premature nitrogen limitation, urea was chosen as an additional nitrogen source beside pH-controlled ammonia feeding. We took notice, that during the 2nd and 3rd cycle of biomass accumulation overfeeding led to accumulation of NH_3 in the bioreactors (**Table 1** and **Figure 5**). Recent studies have suggested that high NH_3 levels may trigger a stress response, involving the formation of (p)ppGpp alarmone, that could trigger preliminary PHB accumulation without nutrient starvation (Gutschmann et al., 2019). The high concentrations of NH_3 affected the overall yield of this cycles that were characterized by comparably lower CDWs and polymer accumulation. In the future, it could be considered to apply urea feeding only for the 1st cycle of cell growth which as this cycle showed to be considerably longer and thus is more prone to undergo nutrient limitations.

The very high STY of 1.22 g PHA L^{-1} h^{-1} accomplished in this study is comparable to other published high-cell-density cultivations from plant oils (Kahar et al., 2004; Obruca et al., 2010; Riedel et al., 2012; Arikawa and Matsumoto, 2016; Gutschmann et al., 2019). Productivities were further optimized by adopting an advanced cultivation strategy ("drain and fill") for semi-continuous biomass production in the first stage of the process. Doing these, initial long lag phases (**Figures 3**, **5**) could be avoided reducing the overall process time in ∼20%, showing the potential to increase the STY to ∼1.45 g P(HB-*co*-HHx) L^{-1} h^{-1}. However, it is important to note that this fed-batch productivity is reported on a timescale from inoculation to harvest. To be able to compare values from the fed-batch approach and the repeated fed-batch approach with semi-continuous biomass accumulation, productivity should be amortized over time from one harvest to the next, considering the downtime for cleaning, setup, sterilization, and preparation of the inoculum for the subsequent cultivation (Blunt et al., 2018). In our study the P(HB-*co*-HHx) production was triggered under unsterile conditions in separate bioreactors. This could avoid sterilization costs, a major price factor in biotechnological processes (Wang et al., 2014).

Only recently repeated batch and fed-batch strategies have been reported for optimized PHA production (Singhaboot and Kaewkannetra, 2015; Gahlawat et al., 2017). To the best of our knowledge, to date, this is the first report on a repeated fed-batch strategy where the "drain and fill" protocol is used for semi-continuous biomass accumulation whereas high polymer concentrations, of over 100 g L^{-1} P(HB-*co*-HHx) are attained in-series bioreactors. According to Ienczak et al. (2013), even a total PHA production of ∼200 g L^{-1} would be possible based on the achieved high RCDW (∼50 g L^{-1}) in this study.

CONCLUSION

As a conclusion, the data presented herein describes the production of the PHA copolymer P(HB-*co*-HHx) utilizing fructose and rapeseed oil as feedstocks in different stages of the process. Optimized polymer productivities of ∼1.45 g L^{-1} h^{-1} with a total PHA production up to 100 g L^{-1} were reached through a repeated fed-batch process with semi-continuous biomass accumulation. The new method described in this study not only reduced the process times related to long lag-phases at the beginning of each batch (time reduction of ∼20%) but also circumvented the need of laborious pre-seed cultures. Taking advantage of the high-cell-densities achieved before triggering polymer accumulation (RCDW > 30 g L^{-1}) performing the second stage in unsterile bioreactors allowed the sparing of sterilization costs and time. Results suggest that applying the method presented here could contribute to reduce production costs and, in this way, accelerate the commercialization of a sustainable PHA-bioplastic.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SR contributed to the conception and design of the study. LS and SW carried out the experiments and analysis of the data. LS and SR prepared the first draft of the manuscript. SR and PN were responsible for the project administration and funding acquisition. All authors contributed to the manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2021.623890/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Polyhydroxybutyrate (PHB) Production Using an Arabinose-Inducible Expression System in Comparison With Cold Shock Inducible Expression System in *Escherichia coli*

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Cupriavidus necator strain A-04 has shown 16S rRNA gene identity to the well-known industrial strain *C. necator* H16. Nevertheless, the cell characteristics and polyhydroxyalkanoate (PHA) production ability of *C. necator* strain A-04 were different from those of *C. necator* H16. This study aimed to express PHA biosynthesis genes of *C. necator* strain A-04 in *Escherichia coli* via an arabinose-inducible expression system. In this study, the PHA biosynthesis operon of *C. necator* strain A-04, consisting of three genes encoding acetyl-CoA acetyltransferase ($phaA_{A\text{-}04}$, 1182 bp, 40.6 kDa), acetoacetyl-CoA reductase ($phaB_{A\text{-}04}$, 741 bp, 26.4 kDa) and PHB synthase Class I ($phaC_{A\text{-}04}$, 1770 bp), was identified. Sequence analysis of the $phaA_{A\text{-}04}$, $phaB_{A\text{-}04}$, and $phaC_{A\text{-}04}$ genes revealed that $phaC_{A\text{-}04}$ was 99% similar to $phaC_{H16}$ from *C. necator* H16. The difference in amino acid residue situated at position 122 of $phaC_{A\text{-}04}$ was proline, whereas that of *C. necator* H16 was leucine. The intact $phaCAB_{A\text{-}04}$ operon was cloned into the arabinose-inducible araBAD promoter and transformed into *E. coli* strains Top 10, JM109 and XL-1 blue. The results showed that optimal conditions obtained from shaken flask experiments yielded 6.1 ± 1.1 g/L cell dry mass (CDM), a PHB content of 93.3 ± 0.9% (w/w) and a productivity of 0.24 g/(L·h), whereas the wild-type *C. necator* strain A-04 accumulated 78% (w/w) PHB with a productivity of 0.09 g/(L·h). Finally, for the scaled-up studies, fed-batch cultivations by pH-stat control in a 5-L fermenter of *E. coli* strains XL1-Blue harboring pBAD/Thio-TOPO-$phaCAB_{A\text{-}04}$ and pColdTF-$phaCAB_{A\text{-}04}$ in MR or LB medium, leading to a PHB production of 31.4 ± 0.9 g/L at 54 h with a PHB content of 83.0 ± 3.8% (w/w), a CDM of 37.8 ± 1.2 g/L, a $Y_{P/S}$ value of 0.39 g PHB/g glucose and a productivity of 0.6 g PHB/(L·h) using pColdTF-$phaCAB_{A\text{-}04}$ in MR medium. In addition, PHB production was 29.0 ± 1.1 g/L with 60.2 ± 2.3% PHB content in the CDM of 53.1 ± 1.0 g/L, a $Y_{P/S}$

value of 0.21 g PHB/g glucose and a productivity of 0.4 g PHB/(L·h) using pBAD/Thio-TOPO-*phaCAB*$_{A-04}$ in LB medium. Thus, a relatively high PHB concentration and productivity were achieved, which demonstrated the possibility of industrial production of PHB.

Keywords: polyhydroxybutyrate, araBAD promoter, *Cupriavidus necator*, recombinant *E. coli*, pH stat feeding strategy

INTRODUCTION

Microplastic pollution continues to induce negative impacts that harm both animals and ecosystems. Nevertheless, the plastic manufacturing sector has moved to a circular economy that calls for upstream and downstream industry collaborations in chemical and mechanical recycling to create new resins offered with recycled and biobased content (Hahladakis et al., 2020). However, awareness among consumers regarding the efficient postuse of petrochemical plastics, the benefits of green material and government regulations that encourage people to adopt eco-friendly alternatives still promote the demand for bioplastics and biodegradable plastics. Recently, global bioplastic production capacity has been estimated to increase from 2.11 million tons in 2019 to approximately 2.43 million tons in 2024. Bioplastics can widely be used in a wide variety of markets and applications, from packaging, catering products, consumer electronics, automotive, agriculture, textiles and a number of other segments. Bioplastics consist of diverse materials and are primarily focused on renewable materials, including starch, cellulose, or bioethanol. Among them, polyhydroxyalkanoates (PHAs) and innovative biopolymers, such as biobased polypropylene, have received the highest attention and showed strong growth rates. Biodegradable plastics, which include PHAs, polylactic acid (PLA), starch blends and others, account for 55.5% (over 1 million tons) of the global bioplastic production capacities. The production of biodegradable plastics is expected to increase to 1,33 million in 2024, especially due to PHA's significant growth rates (Bioplastics, 2020).

Nowadays, PHAs are one of the most preferable solutions to alleviate the conventional plastic pollution, especially for the preparation of PHAs microbeads that is required for the biodegradation of microbeads in marine environment. PHAs are a class of well-known microbial polyesters that possess unique advantages of thermoplastic, biocompatible and biodegradable properties. Based on their true biodegradable properties in various environments, PHAs are considered environmentally sustainable biodegradable plastics. However, PHAs have not yet become competitive and ecofriendly alternative materials to petrochemical plastics compared with semi-biosynthetic polymers, PLA and polybutylene succinate (PBS). The major problem is their high cost of production resulting from raw materials, solvent used in polymer extraction and purification, long cultivation time in the wild-type strain and small amount of obtained PHAs, even at its high PHA content from non-growth-associated PHA-producing strains. Recently, metabolic engineering approaches in which the PHA biosynthesis genes *phaCAB* and other related genes are cloned into *Escherichia coli* have been used to increase the efficiency of PHA production.

In our previous studies, we reported new isolated *Cupriavidus necator* strain A-04 that has shown the ability to produce PHAs [formerly reported as *Alcaligenes* sp. A-04 and *Ralstonia eutropha* A-04 (Chanchaichaovivat, 1992; Chanprateep et al., 2008)]. First, it has shown the ability to produce PHB from fructose and molasses. In 5 L fermentor, batch cultivation, the PHB produced was 7.21 g/L with PHB content of 82.12% (w/w) at 72 h when ammonium sulfate and fructose were 1.5 and 30.0 g/L, respectively. The amount of PHB produced from cane molasses was less than that obtained from fructose. The amount of PHB produced from molasses was 2.3 g/L with PHB content of 53.66% (w/w) at 72 h when 20% (w/v) of molasses was used as a carbon source and 0.1 g/L of ammonium sulfate was a nitrogen source (Phonprapai, 1994). It has also shown the ability to produce copolymer of poly(3-hydroxybutyrate-co-3-hydroxuvalerate) [P(3HB-*co*-3HV)], poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P(3HB-*co*-4HB)], and terpolymer of poly(3-hydroxybutrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) [P(3HB-*co*-3HV-*co*-4HB) with a wide range of monomer compositions from various organic acids (Surathikajon, 1994; Chanprateep and Kulpreecha, 2006; Chanprateep, 2010). Then, it was taxonomic characterization and it possessed 99.78% 16S RNA sequence similarity with *C. necator* H16, but it differed in PHA production ability based on PHB yield ($Y_{P/S}$, g-PHB/g-carbon) from butyric, valeric, γ-hydroxybutyric acid, and fructose (Chanprateep et al., 2008). It could incorporate a high mole fraction of monomeric 4-hydroxybutyrate monomeric into the poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] copolymer under a C/N ratio of 20 and the biocompatibility of P(3HB-*co*-4HB) produced was also reported (Chanprateep et al., 2010). Recently, it has also shown the ability to grow on pineapple wastes hydrolysate that contains mixtures of sugars and tolerate levulinic acid and 5-hydroxymethyl furfural, and a detoxification step was not required prior to the conversion of cellulose hydrolysate to PHB (Sukruansuwan and Napathorn, 2018). Therefore, we aimed to study the PHA biosynthesis operon of *C. necator* strain A-04 and to realize the capability of the PHA biosynthesis operon of *C. necator* strain A-04 when it was heterologously expressed in recombinant *E. coli*. There have been a number of exciting developments in the field of metabolic regulation in recent years, but only a few reports are based on the use of arabinose-inducible expression systems for PHA production (Horng et al., 2010; Wu et al., 2016). The objective of this work was to analyze *phaCAB* genes from the isolated

C. necator strain A-04 and clone these genes for expression in an arabinose-inducible araBAD promoter (pBAD/Thio-TOPO vector) that have not been reported for PHB production. Here, we investigated the optimal conditions; L-arabinose concentration, four different media, glucose concentration for $phaCAB_{A-04}$ expression under pBAD/Thio-TOPO vector in shake flask cultivation. In fed-batch cultivation, $phaCAB_{A-04}$ expression under araBAD promoter was compared with the cspA promoter (a cold- and isopropyl-β-D-thiogalactoside (IPTG)-inducible vector, pColdTF) (Boontip, 2020) using a pH stat fed-batch cultivation strategy. Finally, high cell density of cell dry mass (CDM) 51.2 ± 1.0 g/L with PHB 29.0 ± 1.1 g/L was obtained in LB medium.

MATERIALS AND METHODS

Strains and Plasmids

All bacterial strains and plasmids used in this study are listed in **Table 1**. *C. necator* strain A-04, which is a soil-isolated bacterium possessing 99.84% 16S rRNA identity to *C. necator* H16, was used as a wild-type PHB-producing strain (Chanprateep et al., 2008). *E. coli* JM109 [F′*traD36 proA+B+lacI*^{q}*(lacZ)*Δ*M15/*Δ(*lac-proAB*) *glnV44 e14- gyrA96 recA1 relA1 endA1 thi hsdR17*] (Promega Corporation, Madison, WI, United States) was used for general genetic manipulation and for the production of PHB. *E. coli* Top10 [F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80 l*ac*ZΔ M15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(ara-leu)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G (Invitrogen, Carlsbad, CA, United States) was used for PHB production. *E. coli* XL1 Blue [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*Z*Δ*M15* Tn*10* (Tet^r)] (Promega Corporation, Madison, WI, United States) was used for PHB production in a 5 L fermenter. The pCR4-TOPO vector (Invitrogen, Carlsbad, CA, United States) was used for cloning the *phaCAB* and *phaC* genes. The pBAD/Thio-TOPO vector (Invitrogen, Carlsbad, CA, United States) was used as a final vector for the *phaCAB* expression plasmid. *C. necator* strain A-04 was cultivated in pre-culture medium consisting of 10 g/L yeast extract, 10 g/L polypeptone, 5 g/L beef extract, and 5 g/L $(NH_4)_2SO_4$ at 30°C at 200 rpm for 24 h. *E. coli* JM109 and *E. coli* Top10 were cultivated at 37°C in Luria-Bertani (LB) medium containing 10 g/L casein peptone, 10 g/L sodium chloride and 5 g/L yeast extract. The antibiotic ampicillin (100 μg/mL) or carbenicillin (50 μg/mL) was added to the medium to maintain the stability of the plasmids.

TABLE 1 | Bacterial strains and plasmids used in this study.

Strains/ plasmids	Relevant description	Reference/source
Strain		
Cupriavidus necator strain A-04	Wild Type	Chanprateep et al., 2008
Escherichia coli JM109	F′*traD36 proA+B+ lacI*^{q}*(lacZ)*Δ*M15/*Δ*(lac-proAB) glnV44 e14- gyrA96 recA1 relA1 endA1 thi hsdR17*	Promega Corporation, Madison, WI, United States
Escherichia coli TOP10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80 l*ac*ZΔ M15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(ara-leu)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen, Carlsbad, CA, United States
Escherichia coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*Z?M15* Tn*10* (Tet^r)	Promega Corporation, Madison, WI, United States
Plasmids		
pGEM-T	Cloning vector, Amp^r	Promega Corporation, Madison, WI, United States
pBAD/Thio-TOPO	Amp^r, *araC* gene, *araBAD* promoter	Invitrogen, Carlsbad, CA, United States
pBAD/Thio-TOPO-$phaCAB_{A-04}$	pBAD/Thio-TOPO derivative, carrying C-terminal 6HIS- and N-terminal thioredoxin fused *phaCAB* from *C. necator* strain A-04	This study
pBAD/Thio-TOPO-$phaC_{A-04}$	pBAD/Thio-TOPO derivative; *araBAD* promoter, *phaC* from *C. necator* strain A-04	This study
pColdTF-$phaCAB_{A-04}$	pColdTF derivative, carrying N-terminal 6His-fused *phaCAB* from *C. necator* strain A-04	Boontip, 2020

Amplification of PHA Biosynthesis Genes From *C. necator* Strain A-04

Genomic DNA of *C. necator* strain A-04 was extracted by standard procedures (Sambrook and Russell, 2001). A partial fragment of the *phaC* gene from the genomic DNA of *C. necator* A-04 was amplified using two degenerate primers (F): 5′-TAYATHYTNGAYYTNCARCCNT-3′ and (R): 5′-CGYAGTHYCACAYYAGGTCG-3′, which were based on highly conserved regions of the *phaC* genes of *Comamonas acidovorans* (accession no. AB009273), *Alcaligenes* sp. strain SH-69 (accession no. U78047), *Alcaligenes eutrophus* (accession no. J05003), *Aeromonas caviae* (accession no. D88825) *Rhodococcus rubber* (accession no. X66407), *Chromatium vinosum* (accession no. 01112), and *C. necator* H16 (accession no. AM260479.1) (Sudesh et al., 1998). Next, the partial sequences (469 bp) of *phaC* were detected with degenerated PCR primers, and the subsequent sequences were obtained using the GenomeWalker^{TM} Universal Kit (Clontech Laboratories Inc., United States). In addition, two target-specific primers (forward primer (F0): 5′-TACATCCTGGACCTGCAGCC-3′ and reverse primer (R4): 5′-CGTAGTTCCACACCAGGTCG-3′) were designed based on the determined sequences. A GenomeWalker^{TM} Universal Kit was completed according to the manufacturer's protocols. The amplified full-length genes of the entire *pha* locus containing the $phaC_{A-04}$, $phaA_{A-04}$, and $phaB_{A-04}$ genes of *C. necator* strain A-04 were cloned into a pGEM-T easy vector (Promega, Madison, WI, United States) followed by transformation of the vectors into *E. coli* JM109 prior to DNA sequencing. DNA sequencing was performed at the Macrogen service center (Macrogen Inc., Seoul, Korea). The nucleotide sequences of $phaC_{A-04}$, $phaA_{A-04}$, and $phaB_{A-04}$ reported here were deposited in the GenBank online database under accession numbers FJ897463.1, FJ897461.1, and

FJ897462.1, respectively. In addition, the nucleotide sequences of the NAD-dependent 4-hydroxybutyrate dehydrogenase gene of *C. necator* strain A-04 were deposited in the GenBank online database under accession number MH243762.1.

Construction of the Plasmids and Cloning of the *phaCAB* and *phaC* Genes

Specific primers for the amplification of *phaCAB* were designed according to the sequences obtained. The oligonucleotide primers for the amplification of the *phaCAB* gene fragment were (F-phaCAB): 5′-ATGGCGACCGGCAAAG-3′ (forward primer) and (R-phaCAB): 5′- TCAGCCCATATGCAGGCC-3′ (reverse primer). The oligonucleotide primers for the amplification of the *phaC* gene fragment were (F-phaCAB): 5′-ATGGCGACCGGCAAAG-3′ (forward primer) and (R-phaC): 5′-TCATGCCTTGGCTTTGACG-3′ (reverse primer). PCR using the respective primers for the amplification of *phaCAB* and *phaC* genes from the genomic DNA of *C. necator* strain A-04 was carried out to obtain the ligated DNA fragments using Ex Taq^{TM} DNA Polymerase (TaKaRa Bio Inc., Otsu, Japan) for ligation. The fragment was ligated to pBAD/Thio-TOPO® (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's protocol. A new plasmid named pBAD/Thio-TOPO-$phaCAB_{A\text{-}04}$ with the entire $phaCAB_{A\text{-}04}$ operon and pBAD/Thio-TOPO-$phaC_{A\text{-}04}$ with $phaC_{A\text{-}04}$ were transformed using a heat shock method (Sambrook and Russell, 2001) into *E. coli* JM109 and *E. coli* TOP10. The colonies were screened on LB agar containing carbenicillin (50 μg/mL), and the inserts were confirmed through PCR and restriction enzyme digestion.

Expression of pBAD/Thio-TOPO-$phaCAB_{A\text{-}04}$ in *E. coli* JM109 and *E. coli* TOP10 and Comparison of Their PHB Production From Glucose

Shaken flask experiments were performed in 500-mL Erlenmeyer flasks containing 100 mL of medium. *E. coli* JM109 and *E. coli* TOP10 transformants containing pBAD/Thio-TOPO-$phaCAB_{A\text{-}04}$ or pBAD/Thio-TOPO-$phaC_{A\text{-}04}$ plasmid were grown in LB medium containing carbenicillin 50 μg/mL at 37°C and 200 rpm for 16–18 h. Subsequently, cells were harvested by centrifugation, washed to remove the medium and resuspended in 5 mL of 0.85% sodium chloride solution. The cells were separately inoculated into four different media: LB (10 g/L casein peptone, 10 g/L sodium chloride and 5 g/L yeast extract), superbroth (SB) (32 g/L tryptone, 5 g/L sodium chloride and 20 g/L yeast extract), 2YT (16 g/L tryptone, 5 g/L sodium chloride and 10 g/L yeast extract), and terrific broth (TB) (12 g/L tryptone, 4 mL glycerol and 24 g/L yeast extract) containing L-arabinose 0.2% (w/v), glucose 20 g/L and carbenicillin 50 μg/mL to evaluate suitable media for PHB production. Next, five concentrations of L-arabinose, 0.002, 0.02, 0.2, 0.5, and 2% (w/v), were separately added when an optical density at a wavelength of 600 nm (OD_{600}) of 0.5–0.6 was obtained to induce the arabinose-inducible araBAD promoter for $phaCAB_{A04}$ or $phaC_{A04}$ expression. Finally, the inoculum size was investigated by means of a colony forming unit (CFU) varying from 4.5×10^8, 3.2×10^9, and 1.5×10^{10} CFU in LB medium containing L-arabinose 1.0% (w/v), glucose 20 g/L and carbenicillin 50 μg/mL. Culture samples were taken at 12 h intervals for 96 h.

PHA Granule Observation by Transmission Electron Microscopy

Polyhydroxyalkanoate production was confirmed by comparative staining using the modified Sudan Black B staining method (López-Cortés et al., 2008). Cell morphology and PHA granules were also observed by transmission electron microscopy (TEM) at Scientific and Technological Research Equipment Centre Chulalongkorn University, Bangkok, Thailand. For TEM, diluted culture from production medium was fixed in 2% (v/v) glutaraldehyde containing 2% (v/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and was postfixed in 1% (w/v) osmium tetroxide. The cells were dehydrated in an ascending series of ethanol concentrations from 35 to 100% and embedded in Spurr resin (EMS, PA, United States). Thin sections were prepared with an LKB 2088 Ultratome V (Surrey, United Kingdom), stained with 2% (w/v) uranyl acetate and 2% (w/v) lead citrate and examined with TEM (JEM 2100, JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 80 kV (Muangwong et al., 2016).

Conditions for PHB Production in a 5-L Fermenter

In this study, competent *E. coli* XL1 blue cells were transformed with pBAD/Thio-TOPO-$phaCAB_{A\text{-}04}$ and pColdTF-$phaCAB_{A\text{-}04}$. Seed cultures were prepared in LB medium containing 50 μg/mL carbenicillin or 100 μg/L ampicillin and 20 g/L glucose at 30°C and 200 rpm for 10 h at 200 rpm. For fed-batch culture, LB medium supplemented with 20 g/L glucose and MR medium supplemented with 20 g/L glucose and 10 mg/L thiamine were compared. MR medium, pH 7.0, consisted of the following: 13.5 g/L KH_2PO_4, 4.0 g/L $(NH_4)_2HPO_4$, 1.4 g/L $MgSO_4{\cdot}7H_2O$, 1.7 g/L citric acid and 10 mL/L trace metal solution. The trace metal solution contained the following (in 5 M HCl): 10.0 $FeSO_4{\cdot}7H_2O$, 2.0 $CaCl_2$, 2.2 $ZnSO_4{\cdot}7H_2O$, 0.5 $MnSO_4{\cdot}4H_2O$, 1.0 $CuSO_4{\cdot}5H_2O$, 0.1 $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.02 $Na_2B_4O_7{\cdot}10H_2O$ (Kahar et al., 2005). The seed culture at 5% (v/v) was inoculated into a 5-L bioreactor (MDL500, B.E. Marubishi Co., Ltd., Tokyo, Japan) containing an initial volume of 2 L of LB medium supplemented with 50 μg/mL carbenicillin or 100 μg/L ampicillin and 20 g/L glucose in comparison with MR medium supplemented with 10 mg/L thiamine, 50 μg/mL carbenicillin or 100 μg/L ampicillin and 20 g/L glucose. Fed-batch cultures were carried out at 30°C, and the pH was maintained at 7.0 by automatic addition of 28% (v/v) ammonia hydroxide solution. The dissolved oxygen was monitored and controlled at above 10% air saturation by varying agitation and aeration. The feeding solution used for the fed-batch cultivation contained the following: 700 g/L glucose, 15 g/L $MgSO_4{\cdot}7H_2O$ and 0.25 g/L thiamine (Kahar et al., 2005). A pH stat feeding strategy using a pH electrode (Broadley-James Corporation, Irvine, CA, United States) based on increasing

pH due to glucose depletion was used in this study. When the pH was greater than 7.0, an appropriate volume of feeding solution was fed to supply glucose to the culture medium. The fermenter was coupled to a process control system (Bio Process Controller, MDIAC-S5, B.E. Marubishi Co., Ltd., Tokyo, Japan). The agitation speed and the air flow rate were 500 rpm and 1 mL/min, respectively. After an OD_{600} of 0.5 was obtained, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.5 mM. Culture samples were collected at 6 h intervals for 72 h. For pColdTF-*phaCAB*$_{A-04}$, after an OD_{600} of 0.5 was obtained, the cultivation temperature was reduced from 37 to 15°C for 30 min. Next, IPTG was added to the culture at a final concentration of 0.5 mM. After IPTG addition, the cultivation temperature was shifted from 15 to 37°C and maintained at 37°C for 72 h (Boontip, 2020). Culture samples were collected at 6 h intervals for 48 h.

Analytical Methods

Cell growth was monitored by the CDM, which was determined by filtering 5 mL of the culture broth through pre-weighed cellulose nitrate membrane filters (pore size 0.22 μm; Sartorius, Göttingen, Germany). The filters were dried at 80°C for 2 days and stored in desiccators. The net biomass was defined as the residual cell mass (RCM), which was calculated by subtracting the amount of PHB from the CDM. The PHB in dried cells was methyl-esterified using a mixture of chloroform and 3% (v/v) methanol-sulfuric acid (1:1 v/v) (Braunegg et al., 1978). The resulting monomeric methyl esters were quantified by a gas chromatograph (model CP3800, Varian Inc., Walnut Creek, CA, United States) using a Carbowax-PEG capillary column (0.25-μm df, 0.25-mm ID, 30 m length, Agilent Technologies, Inc., Santa Clara, CA, United States). The internal standard was benzoic acid, and the external standard was PHB (Sigma-Aldrich Corp., St. Louis, MO, United States). The total reducing sugar concentration was determined using a 3,5-dinitrosalicylic acid (DNS) assay (Miller, 1959). The NH_4^+ concentration in the culture medium was determined using a colorimetric assay (Kemper, 1974).

PHA Extraction and Purification

Harvested cells were dried and extracted with 1,3-dioxolane, and PHB was recovered from 1,3-dioxolane by precipitation in water. The precipitation step was repeated three times (Yabueng and Napathorn, 2018).

Chemical Structure Analysis

^{1}H and ^{13}C NMR spectra of PHA samples were recorded on a Varian Inova 500 MHz instrument. The chemical shifts are reported in parts per million (ppm) relative to chloroform as an internal reference. Spectra were recorded using 5% (w/w) polymer solutions in CDCl3 with the following parameters: 25°C, pulse of 90 degrees, width of 8003.2 Hz, 5.0 s relaxation delay, and 0.2 Hz line broadening. Two-dimensional ^{1}H-correlation spectroscopy (2D-^{1}H-COSY) was performed in combination with ^{1}H NMR on a Bruker AM 400 MHz FT-NMR (Bruker BioSpin Corporation, Woodland, TX, United States).

Thermal Analysis by Differential Scanning Calorimetry (DSC)

A 10-mg sample of PHB was encapsulated in an aluminum sample vessel and placed in the sample holding chamber of the differential scanning calorimetry (DSC) apparatus (DSC7, PerkinElmer, Inc., Waltham, MA, United States). STARe software (version SW 10.00; Mettler-Toledo International Inc., Columbus, OH, United States) was used to operate the DSC apparatus at the Petroleum and Petrochemical College, Chulalongkorn University. The previous thermal history of the sample was removed before the thermal analysis by heating the sample from ambient temperature to 180°C at 10°C/min. Next, the sample was maintained at 180°C for 5 min before cooling at 10°C/min to −50°C. The sample was then thermally cycled at 10°C/min to 180°C. The melting peak temperature, denoted by T_m, was given by the intersection of the tangent with the furthest point of an endothermic peak and the extrapolated sample baseline. The glass transition temperature, denoted by T_g, could be estimated by extrapolating the midpoint of the heat capacity difference between glassy and viscous states after heating of the quenched sample.

Data Analysis

All the data presented in this manuscript are representative of the results of three independent experiments and are expressed as the mean values ± standard deviations (SDs). Analysis of variance (one-way ANOVA) followed by Duncan's test for testing differences among means was conducted using SPSS version 22 (IBM Corp., Armonk, NY, United States). Differences were considered significant at $P < 0.05$.

RESULTS

Cloning of *phaCAB*$_{A-04}$

Based on our study, the element composition of biomass represented by $CH_aO_bN_c$ for *C. necator* H16 was $CH_{1.77}O_{0.50}N_{0.24}$ with ash content of 5.31% (w/w) and C/N ratio based on biomass element composition was 4.09 whereas those for *C. necator* strain A-04 was $CH_{1.66}O_{0.45}N_{0.16}$ with ash content of 2.865% (w/w) and C/N ratio of 6.37. For identification of the PHA biosynthesis genes of *C. necator* strain A-04, a partial fragment of the *phaC*$_{A-04}$ gene (469 bp) was amplified from the genomic DNA using degenerate primers based on the highly conserved regions of *phaC* genes of *C. acidovorans* (accession no. AB009273), *Alcaligenes* sp. strain SH-69 (accession no. U78047), *A. eutrophus* (accession no. J05003), *A. caviae* (accession no. D88825), *R. rubber* (accession no. X66407), *C. vinosum* (accession no. 01112), and *C. necator* H16 (accession no. AM260479.1), as shown in **Figure 1**. In addition, after optimizing PCR conditions, it was found that the partial fragment of the *phaC*$_{A-04}$ gene (469 bp) and *phaC*$_{H16}$ gene (as a positive control, 460 bp) could be amplified from the genomic DNA using different $MgCl_2$ concentration where *C. necator* H16 required higher $MgCl_2$ concentration than that of *C. necator* strain A-04. Although, there seems to be

FIGURE 1 | Alignment of the deduced amino acid sequence of the PHA synthase from *Comamonas acidovorans* (accession no. AB009273), *Alcaligenes* sp. strain SH-69 (accession no. U78047), *A. eutrophus* (accession no. J05003), *Aeromonas caviae* (accession no. D88825), *Rhodococcus rubber* (accession no. X66407), *Chromatium vinosum* (accession no. 01112), and *C. necator* H16 (accession no. AM260479.1) (Sudesh et al., 1998). Amino acids that are identical between all the PHA synthases are identified by black boxes, whereas conserved and similar amino acids are highlighted in light gray. The red box means the selected conserved sequence regions that were used for designing degenerate primers.

a low opportunity to obtain new results contributing to the existing knowledge, it is very challenging to us to express PHA biosynthesis genes of *C. necator* strain A-04 in recombinant *E. coli* based on an academic point of view.

Next, the entire sequence of $phaCAB_{A\text{-}04}$ was obtained using a gene walk kit by digesting genomic DNA with SmaI. The resulting 1.7 kb PCR product was obtained when the outer forward primer (F3: 5′-TCACGCTGCTGACCACGCTGCTGGACTTTG-3′) was used with outer adaptor primer 1 (AP1: 5′-GTAATA CGACTCACTATAGGGC-3′), whereas a PCR product as large as 4.0 kb was obtained when the nested forward primer (F4: 5′-TTGAGCTGGCCAATACCTTCTCGTTCTTGC-3′) was used with nested adaptor primer 2 (AP2: 5′-ACTATAGGGC ACGCGTGGT-3′). Analysis of the entire nucleotide sequence of the PCR product revealed that $phaA_{A\text{-}04}$ and $phaB_{A\text{-}04}$ were located downstream of the $phaC_{A\text{-}04}$ gene, and the molecular organization of the $phaCAB_{A\text{-}04}$ operon from *C. necator* strain A-04 is shown in **Figure 2**. The PCR product of the expected size (4.0 kb) was recovered, purified, and cloned into the pBAD/Thio-TOPO vector. They were transformed into *E. coli* TOP10, JM109 and XL-1 Blue as the expression host strains. Twenty of five-hundred transformants were screened for PHA production by Nile blue A staining of the colonies and investigated the light intensity under blue light (470 nm) (BluPAD dual LED blue/white light transilluminator, Bio-helix, Taiwan) using Image-J program. Five clones that showed either similar or higher levels of fluorescence intensity that increased with the increase in PHA content of PHA than that of *C. necator* A-04 were selected and further analyzed to ensure the correctness of sequences. There have been relatively few reports on the use of

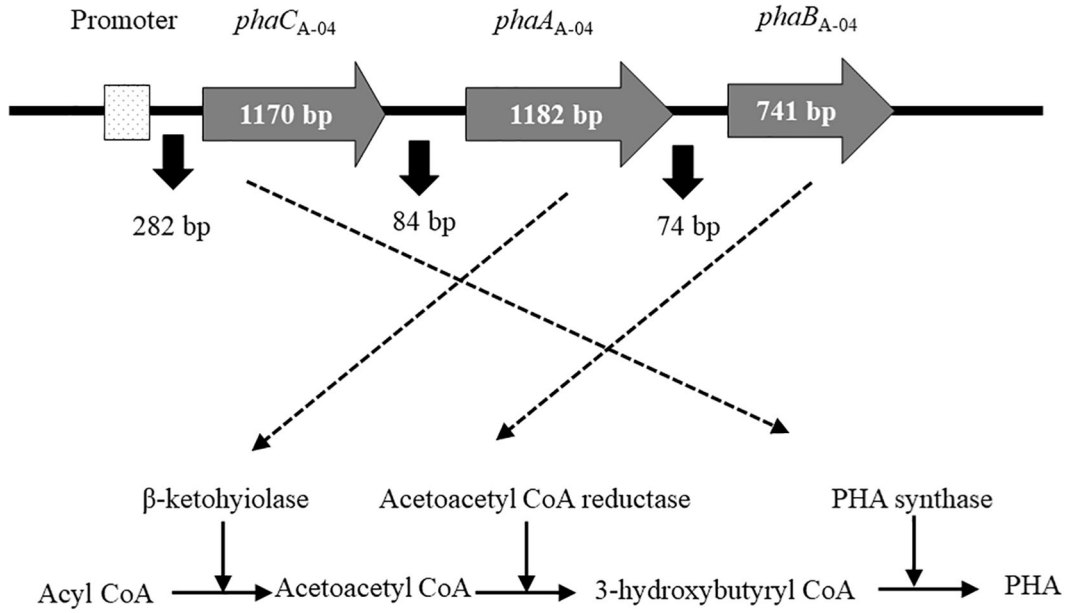

FIGURE 2 | Diagram of the molecular organization of *phaCAB*$_{A-04}$ biosynthesis genes from *C. necator* strain A-04. PHA synthase (*phaC*$_{A-04}$), acetyl-CoA acetyltransferase (*phaA*$_{A-04}$), acetoacetyl-CoA reductase (*phaB*$_{A-04}$) genes and promoter (P$_{A-04}$).

arabinose promoter for PHB production in recombinant *E. coli* (Horng et al., 2010; Wu et al., 2016). In this study, an arabinose-inducible araBAD promoter (pBAD/Thio-TOPO vector) that has not been reported for PHB production was used to produce PHB in *E. coli* TOP10, JM100 and XL-1 Blue. For a comparative study, the expression plasmid pColdTF (cspA promoter, cold shock and IPTG inducible) harboring *phaCAB*$_{A-04}$ genes was transformed into *E. coli* XL-1 Blue.

Selection of Growth Medium

The choice of growth media has a significant influence on cell growth and PHB production. Thus, selection of suitable growth medium and conditions for PHB production in *E. coli* TOP10 was carried out by comparison of kinetic parameters. The results are shown in **Figure 3**. Terrific broth (TB) medium provided better growth conditions than the other three types of media. The specific growth rate of *E. coli* TOP10 harboring pBAD/Thio-TOPO-*phaCAB*$_{A-04}$ in TB medium was 0.035 (1/h), followed by SB medium (0.027 (1/h)), 2YT (0.024 (1/h)), and LB medium (0.007 (1/h)). However, the LB medium appeared to promote PHB production in *E. coli* TOP10, resulting in a specific production rate of 0.048 g-PHB/g-CDW/h, whereas the specific production rate in TB medium was 0.012 g-PHB/g-CDW/h. TB medium contains the highest amount of yeast extract with additional glycerol, which makes it suitable for the long-term growth of cells but does not promote PHB production. The LB medium contained the lowest amount of yeast extract and tryptone with a slightly higher amount of sodium chloride and was found to promote PHB production better than TB, 2YT, and SB media. **Figures 3C–F** show time courses of PHB granules accumulated in *E. coli* TOP10 grown in LB medium at 6, 12, 18, and 24 h visualized by Sudan Black B staining. Finally, LB medium was chosen for further optimization (Nambu et al., 2020).

Optimal Conditions for *phaCAB*$_{A-04}$ Genes Expression

To optimize PHB production in recombinant *E. coli*, many variables need to be systematically investigated. In this study, arabinose concentration, length of induction, inoculum size and glucose concentration were investigated in detail.

First, the arabinose concentration was varied from 0.002, 0.02, 0.2, 0.5, and 2% (w/v) for pBAD/Thio-TOPO-*phaCAB*$_{A-04}$-expressing *E. coli* TOP10. **Figure 4A** represents the effect of arabinose concentration on the relationship between the specific growth rate and specific production rate. The specific PHB production rate increased proportionally with increasing L-arabinose concentration from 0.002 to 1% (w/v), and then the specific PHB production rate decreased at an L-arabinose concentration of 2.0 (w/v). It was clearly observed that 1.0% (w/v) L-arabinose gave the highest amount of PHB at 5.6 $\pm$ 0.4 g/L (**Figure 4C**) with a PHB content of 93.3 $\pm$ 0.9% (w/w) at 24 h after induction (**Figure 4D**). The effect of arabinose concentration on $Y_{P/S}$ (g-PHB/g-glucose) and $Y_{X/S}$ (g-RCM/g-glucose) was shown in **Figure 4B** and it was clearly found that 1% (w/v) L-arabinose provided the highest $Y_{P/S}$ of 0.28 g-PHB/g-glucose. Next, the inoculum size was varied from 4.5×10^8, 3.2×10^9, and 1.5×10^{10} CFU/mL. **Figure 5A** demonstrates the effect of inoculum size on the specific growth rate and specific production rate. In this study, using a low inoculum size of 4.5×10^8 CFU/mL gave the highest specific production rate compared with those obtained from a

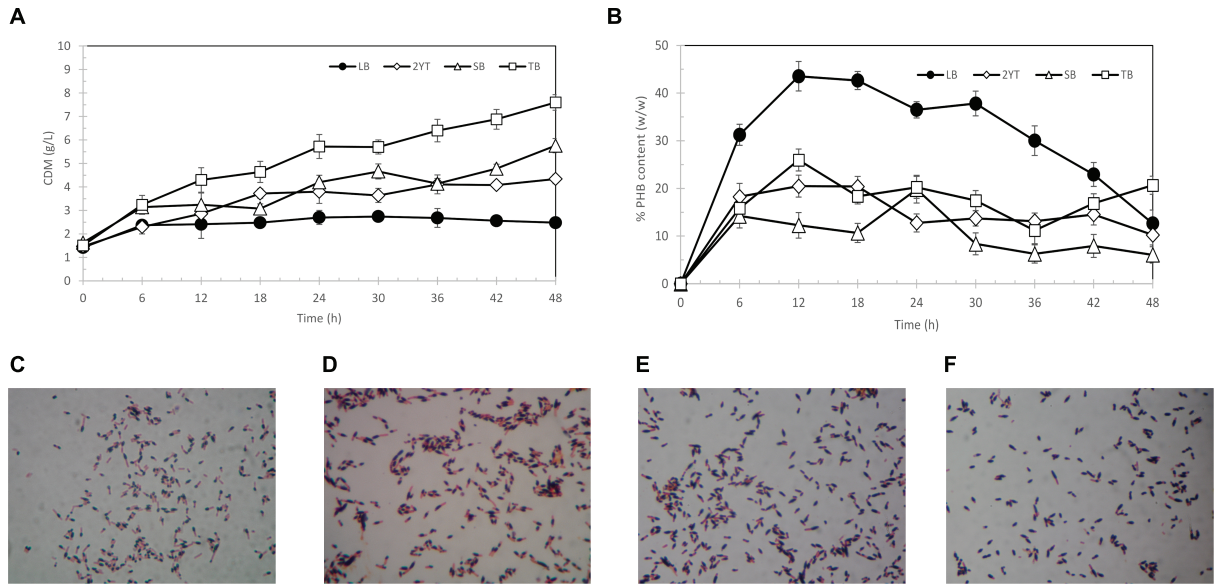

FIGURE 3 | Time courses of CDM (g/L) **(A)** % PHB content (w/w) **(B)** and Sudan black B-stained PHB granules (black section) in pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$ expressing *E. coli* TOP10 grown in LB medium at 6 h **(C)**, 12 h **(D)**, 18 h **(E)**, and 24 h **(F)**.

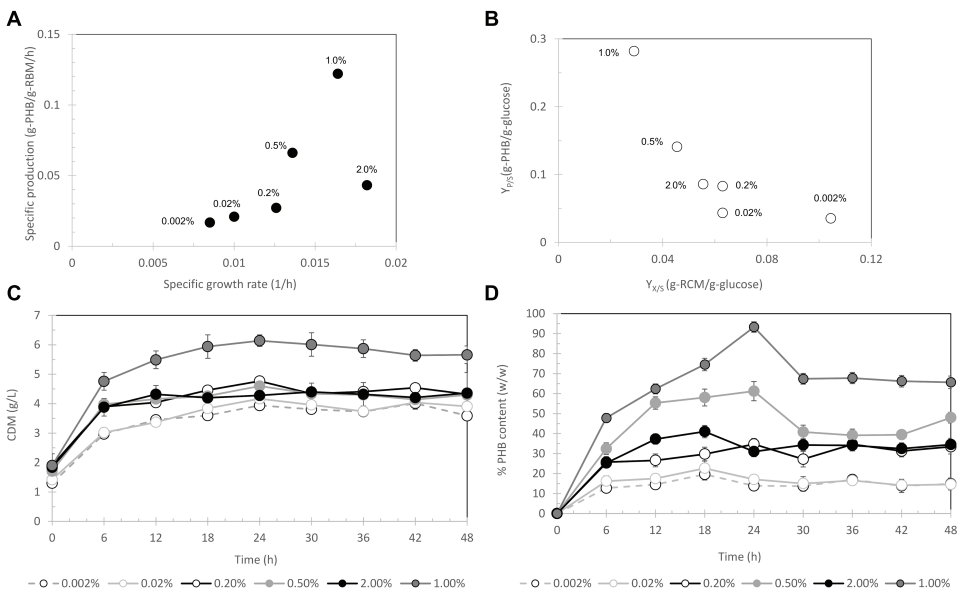

FIGURE 4 | Effect of L-arabinose concentrations of 0.002, 0.02, 0.2, 0.5, and 2% (w/v) on the relationship between the specific growth rate and specific production rate **(A)** and the relationship between $Y_{X/S}$ and $Y_{P/S}$ **(B)** time course of CDM (g/L) **(C)** and % PHB content (w/w) **(D)**.

high cell concentration of pre-culture. For the expression of pBAD/Thio-TOPO-*phaC*$_{A\text{-}04}$-expressing *E. coli* TOP10 using the above optimal conditions, PHB production was not observed. Next, the comparison of pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$ was compared between *E. coli* TOP10 and JM109. The optimal conditions of arabinose concentration, length of induction and inoculum size for pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$-expressing *E. coli* JM109 were similar but they gave lower amount of CDM and PHB concentration than those obtained from pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$-expressing *E. coli* TOP10. Finally, glucose concentration was varied from 0, 2, 5, 10, 20, 50 g/L for both pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$-expressing *E. coli* TOP10 and pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$-expressing *E. coli* JM109. The optimal glucose concentration was 20 g/L for both recombinant strains, but pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$-expressing *E. coli* TOP10 offered higher CDM (g/L), PHB (g/L),

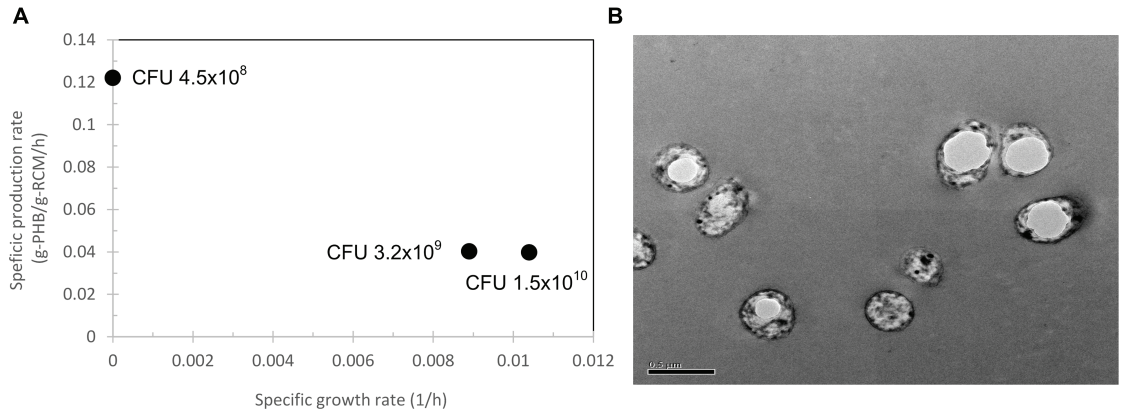

FIGURE 5 | Effect of inoculum size from 4.5×10^8, 3.2×10^9, and 1.5×10^{10} CFU/mL on the specific growth rate and specific production rate **(A)** and transmission electron micrograph of ultrathin sections of pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$ expressing *E. coli* TOP10 grown in LB medium. **(B)** Typical short rod cells containing one PHB granule (white fractions). Bar 0.5 μm.

TABLE 2 | Comparison of kinetic parameters obtained from flask cultivation and fed-batch cultivation for PHB production from glucose between pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$ and pColdTF-*phaCAB*$_{A\text{-}04}$ expressing *E. coli* strains JM109, TOP10, and XL1-Blue in LB medium and MR medium.

Kinetic parameters	pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$		pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$		pColdTF-*phaCAB*$_{A\text{-}04}$	
	Flask	Flask	Fermentor	Fermentor	Fermentor	Fermentor
Host strain	JM109	TOP10	XL1-Blue	XL1-Blue	XL1-Blue	XL1-Blue
Medium	LB	LB	MR	LB	LB	MR
Mode of operation	Batch	Batch	Fed-batch	Fed-batch	Fed-batch	Fed-batch
Glucose (g/L)	20	20	248	352	189	206
Maximum PHB concentration (g/L)	2.6 ± 0.2	5.6 ± 0.4	15.5 ± 0.5	29.0 ± 1.1	7.9 ± 0.6	31.4 ± 0.9
Maximum CDM (g/L)	3.9 ± 0.8	6.1 ± 1.1	46.7 ± 0.8	51.2 ± 1.0	28.2 ± 0.9	39.3 ± 1.2
% Maximum PHB content (w/w)	66.7 ± 2.2	93.3 ± 0.9	29.1 ± 1.8	60.2 ± 2.3	28.0 ± 1.5	83.0 ± 3.8
Specific growth rate (1/h)	0.08	0.02	0.06	0.04	0.06	0.05
Specific consumption rate (g-glucose/g-CDM/h)	0.31	0.38	0.38	0.21	0.11	0.22
Specific production rate (g-PHB/g-CDM/h)	0.02	0.12	0.06	0.09	0.01	0.09
$Y_{X/S}$ (g-CDM/g-glucose)	0.03	0.004	0.34	0.11	0.3	0.11
$Y_{P/S}$ (g-PHB/g-glucose)	0.1	0.28	0.16	0.21	0.10	0.39
Productivity (g/(L.h)	0.1	0.2	0.26	0.4	0.2	0.6
Time (h)	24	24	58	72	54	54

$Y_{P/S}$ (g-PHB/g-glucose), and PHB productivity (g/L·h) than those obtained from *E. coli* JM109 as shown in **Table 2**.

Transmission electron microscopy analysis (**Figure 5B**) revealed the morphology of PHB granules accumulating in pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$-expressing *E. coli* TOP10. Most recombinant cells contained only one single PHB granule varying in size. Finally, PHB was extracted from cells, purified and subjected to chemical structure analysis by 2D-^{1}H NMR and thermal analysis by DSC. The chromatogram of 2D-^{1}H NMR when compared with the PHA chemical structure in previous reports showed that the homopolymer PHB was produced (data not shown). The melting temperature, T_m, of all the PHB film samples produced in this study was in the range of 172–176°C (Owen et al., 1992), and the glass transition temperature, T_g, was in the normal range of 0–1.5°C (Shimamura et al., 1994; Doi et al., 1995; Chanprateep et al., 2010). Altogether, under shaken flask experiments, the optimal conditions consisting of a low inoculum size of 4.5×10^8 CFU/mL and LB medium containing 50 μg/ml carbenicillin supplemented with 1% (w/v) L-arabinose yielded 6.1 ± 1.1 g/L cell dry weight, a PHB content of 93.3 ± 0.9% (w/w) and a productivity of 0.2 g/(L·h), whereas the wild-type *C. necator* strain A-04 accumulated 78% (w/w) PHB with a productivity of 0.09 g/(L·h).

Comparison of PHB Production Between pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$ and pColdTF-*phaCAB*$_{A\text{-}04}$ in a 5-L Fermenter by the pH-Stat Strategy

However, to attain high cell density cultivation, *E. coli* TOP10 was not a good candidate because of the limitation to attain high cell density in fed-batch cultivation experiments (data not shown). As discussed with a well-known expert in the field, *E. coli* XL1-blue is highly recommended to be used as a

host strain (personal communication with Professor Takeharu Tsuge at Tokyo Institute of Technology). In addition, the optimal conditions for *phaCAB*$_{H16}$ expression by arabinose P_{BAD} promoter in *E. coli* XL1-blue have already been reported and were the same conditions as in our study. Thus, in fed-batch cultivation experiments, we changed the host strain from *E. coli* TOP10 to *E. coli* XL1-blue and compared its potential PHB production efficiency between pBAD/Thio-TOPO-*phaCAB*$_{A-04}$ and pColdTF-*phaCAB*$_{A-04}$ in fed-batch cultivation experiments by applying the pH-stat strategy that was previously reported by Kahar et al. (2005) with some modifications by comparing LB medium and MR medium. The objective of this study was to compare araBAD promoter (pBAD/Thio-TOPO, arabinose induction) with the cspA promoter (pColdTF, cold temperature and IPTG induction) because there is no data supporting the use of these promoters when expressing *phaCAB* in high cell density of recombinant *E. coli* in fed-batch cultivation. In addition, our research group have studied and compared pColdTF-*phaCAB*$_{A-04}$ with pGEX-6P-1, pColdI, pColdTF, pBAD/Thio-TOPO, and pUC19 (native promoter) in *E. coli* JM109 and found that pColdTF was the best among various promotors tested in shake flask cultivations (manuscript submitted for publication). Therefore, pColdTF-*phaCAB*$_{A-04}$ was chosen in this study and compared with pBAD/Thio-TOPO-*phaCAB*$_{A-04}$ using the same host strain.

The fed-batch culture with pH-stat feeding of glucose was carried out using *E. coli* XL1-blue and compared between pBAD/Thio-TOPO-*phaCAB*$_{A-04}$ and pColdTF-*phaCAB*$_{A-04}$. In addition, MR medium (Kahar et al., 2005), which is a synthetic medium, was also compared with LB medium, which is a complex medium. For pBAD/Thio-TOPO-*phaCAB*$_{A-04}$ expressing *E. coli* XL1-blue in LB and MR medium, the culture conditions were controlled for optimal cell growth and PHB production as obtained from the above experiments. At 24 h, expression of the *pha* operon was induced by the addition of IPTG to a final concentration of 0.5 mM to promote the expression of *phaCAB*$_{A-04}$ and obtain PHB production. For comparison, pColdTF-*phaCAB*$_{A-04}$ expressing *E. coli* XL1-blue in LB and MR medium was performed in the same manner.

Figure 6 shows the time courses of CDM (g/L) (**Figure 6C**), PHB (g/L) (**Figure 6A**), and PHB content (% w/w) (**Figure 6B**) during batch cultivation in a 5-L fermenter, comparing pBAD/Thio-TOPO-*phaCAB*$_{A-04}$ and pColdTF-*phaCAB*$_{A-04}$ expressing *E. coli* XL1-blue when cultivated in LB medium and MR medium. After 54 h, the CDM of 39.3 ± 1.2 g/L and PHB 31.4 ± 0.9 g/L with 83.0 ± 3.8% (w/w) of PHB content were obtained from pColdTF-*phaCAB*$_{A-04}$ expressing *E. coli* XL1-blue in MR medium whereas CDM of 51.2 ± 1.0 g/L and PHB 29.0 ± 1.1 g/L with 60.2 ± 2.3% (w/w) of PHB content were obtained from pBAD/Thio-TOPO-*phaCAB*$_{A-04}$ expressing *E. coli* XL1-blue in MR medium. In MR medium, the PHB yield from glucose, $Y_{P/S}$, was 0.21 for pBAD/Thio-TOPO-*phaCAB*$_{A-04}$ and 0.39 for pColdTF-*phaCAB*$_{A-04}$. However, in LB medium, $Y_{P/S}$ was 0.16 for *E. coli* XL1-blue harboring pBAD/Thio-TOPO-*phaCAB*$_{A-04}$ and 0.10 for *E. coli* XL1-blue harboring pColdTF-*phaCAB*$_{A-04}$ (**Table 2**). The highest productivity (PHB in g/L·h) was 1.9 g-PHB/L·h for pColdTF-*phaCAB*$_{A-04}$, which was slightly higher than the 1.4 g-PHB/L·h of pBAD/Thio-TOPO-*phaCAB*$_{A-04}$ expressing *E. coli* XL1-blue.

DISCUSSION

Cupriavidus necator strain A-04 was demonstrated in previous studies to synthesize various types of monomeric PHAs from a variety of carbon sources. It was morphologically and taxonomically identified, and 16S rRNA gene analysis revealed that the isolate was close to *C. necator* H16 with different cell morphologies, PHA yields, productivity and growth rates on the same carbon source. To date, a number of transcription-based inducible systems have been tested in *C. necator* H16 (Bhubalan et al., 2008; Fukui et al., 2011), and a number of recombinant *phaCAB*$_{H16}$-expressing *E. coli* have been constructed (Kahar et al., 2005; Rehm and Steinbüchel, 2005; Agus et al., 2006). Therefore, we would like to challenge the expression of *phaCAB*$_{A-04}$ in *E. coli* and investigate the optimal conditions that promoted high cell density with high PHB production. Among various types of promoters, there have been few reports on the application of arabinose-inducible expression systems (Fukui et al., 2011). Thus, in this study, *phaCAB*$_{A-04}$ was cloned and expressed in pBAD/Thio-TOPO (araBAD promoter, arabinose-inducible vector, N-terminal thioredoxin fusion protein and C-terminal 6His-fusion protein). First, four different media (LB, 2YT, SB, and TB media) were examined for PHB production in shake flask experiments. It was found that TB medium promoted the highest cell formation because it contained the highest amount of yeast extract (rich nitrogen source), but not suitable for promoting PHB production. In contrast, LB medium contained the lowest amount of yeast extract and tryptone with a slightly higher amount of sodium chloride and was found to promote PHB production better than TB, 2YT and SB media. This finding was in accordance with Wang et al. (2009) who investigated various amount of nitrogen (NH_4Cl, 0.2 g/L, 1 g/L, 2.5 g/L, 5 g/L) in M9 medium supplied with glucose as carbon source and who concluded that low nitrogen concentration led to the fast and high PHB accumulation in their engineered *E. coli*. The more NH_4Cl supplied in the medium, the less PHB accumulated in the cells that could support our results. From **Figure 3B**, % PHB content (w/w) went down for LB medium over time after 12 h whereas RCM remained constant and PHB production slightly decreased after 30 h. This phenomenon caused by the decrease of organic nitrogen source and Mg^{2+} in LB medium was not sufficient, which were discussed again in fed-batch cultivation as feeding of Mg^{2+} was performed in both LB medium and MR medium to improve this limitation (Nikaido, 2009). Next, basically, the expression level via the araBAD promoter could be tightly regulated by varying the arabinose concentration. We found that 1% L-arabinose was the optimum concentration, which was the same concentration that was previously reported (Horng et al., 2010). Horng et al. (2010) reported co-expression of *phaCAB*$_{H16}$ and *vgb* (bacterial hemoglobin from *Vitreoscilla stercoraria*) constructed and controlled by the same arabinose P_{BAD} promoter in *E. coli* XL1-blue. The optimal arabinose

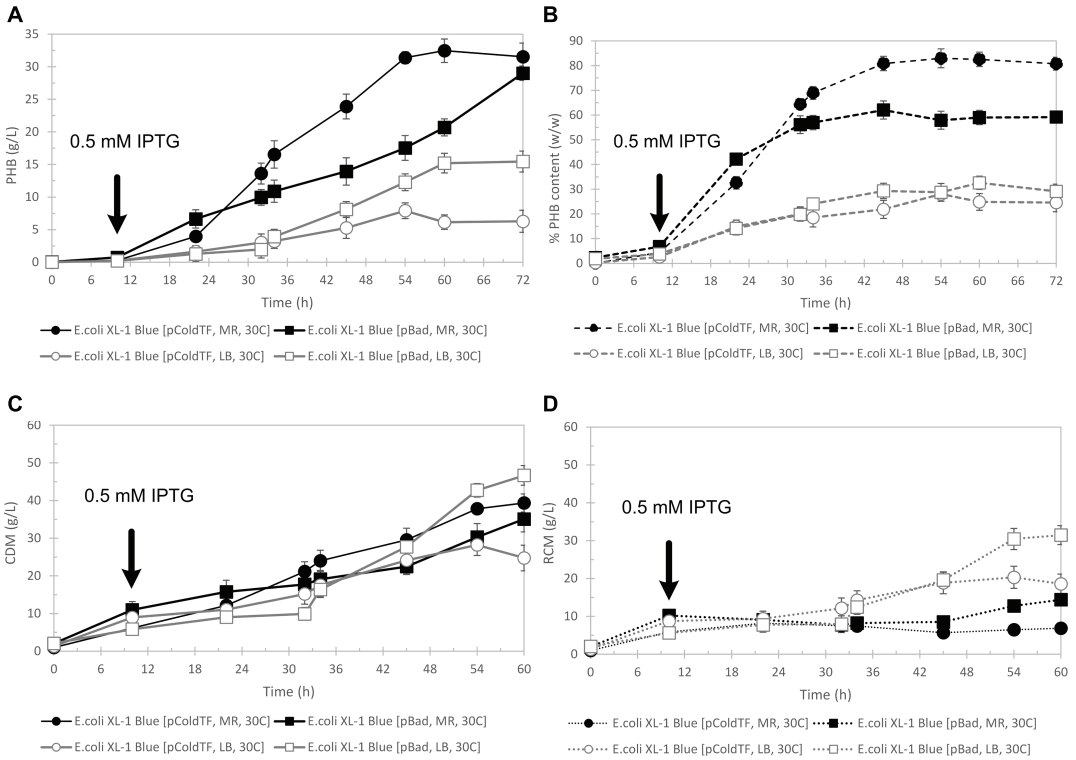

FIGURE 6 | Time courses of PHB g/L **(A)**, PHB content % (w/w) **(B)**, CDM (g/L) **(C)**, and RCM (g/L) **(D)** during batch cultivation in a 5-L fermenter in comparison between pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$ and pColdTF-*phaCAB*$_{A\text{-}04}$-expressing *E. coli* XL1 blue-cultured in LB medium and MR medium.

concentration for maximum inductions of pha*CAB*$_{H16}$ was 1%, and there was no significant increase in PHB production at more than 2% arabinose, which was similar to our finding. In shaken flask experiments, pBAD/Thio-TOPO-*phaCAB*$_{H16}$ and *vgb* expressing *E. coli* XL1-blue, CDM 5.13 ± 0.02 g/L with 3.06 ± 0.02 g/L (59.51 ± 0.17% w/w) were obtained which was slightly lower than our results, pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$ expressing *E. coli* TOP10, with 6.1 ± 1.1 g/L of CDM, PHB content of 93.3 ± 0.9% (w/w) and productivity of 0.2 g/(L·h). The reason may be from some differences, including the use of a low inoculum size of 4.5×10^8 CFU/mL and LB medium containing 2% glucose and 50 μg/ml carbenicillin at 37°C, while Horng et al. (2010) used LB medium containing 1% glycerol and 65 μg/ml ampicillin at 37°C. Nevertheless, Wu et al. (2016) also reported the use of the L-arabinose promoter to overexpress tubulin-like protein (*FtsZ*) and MinC, MinD and MinE, which regulate the formation of division sites. The optimal L-arabinose concentration was reported to be 0.2% for plasmid p15a-Pbad-ftsQLWN in *E. coli* JM109Δ*minCD*, resulting in 11.58 g/L CDM with a PHB content of 82.13% (w/w) in LB medium containing 20 g/L glucose at 30°C. The decrease of PHB production with 2% L-arabinose induction can be referred to the study of Khlebnikov et al. (2000) who reported that genes encoding arabinose catabolic genes (*araBAD*) are under arabinose-inducible control through AraC; arabinose binds to the AraC protein, which positively regulates the expression of transporters and negatively regulates its own expression. They investigated the effects of the arabinose concentration and arabinose-independent transport control on population homogeneity using flow cytometry. It was reported that arabinose concentration of 2% was a saturate concentration and a decline in the specific fluorescence was observed. This phenomenon might be ascribed to arabinose catabolism, resulting in a lower internal arabinose concentration (Khlebnikov et al., 2000) and lower PHB production in our case. The morphology of pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$-expressing *E. coli* TOP10 containing PHB granules was observed by TEM, and most cells contained a single PHB granule varying in size.

In fed-batch experiments, a pH stat strategy employing ammonium hydroxide solution to maintain glucose concentration (Kahar et al., 2005) was applied in this study, and it was found that the pH stat strategy effectively worked well for both pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$ expressing *E. coli* XL1-blue and pColdTF-*phaCAB*$_{A\text{-}04}$-expressing *E. coli* XL1-blue in LB medium and MR medium. The pH-stat strategy together with MR medium promoted PHB production by both pBAD/Thio-TOPO-*phaCAB*$_{A\text{-}04}$ expressing *E. coli* XL1-blue (CDM of 51.2 ± 1.0 g/L and PHB 29.0 ± 1.1 g/L with 60.2 ± 2.3% (w/w) PHB content) and pColdTF-*phaCAB*$_{A\text{-}04}$ expressing *E. coli* XL1-blue (CDM of 39.3 ± 1.2 g/L and PHB 31.4 ± 0.9 g/L with 83.0 ± 3.8% (w/w) PHB content). We found that LB medium promoted higher cell density than that obtained from MR medium, but LB medium resulted in lower PHB production than MR medium. **Figures 6C,D** shows that

pBAD/Thio-TOPO-*phaCAB*$_{A-04}$-expressing *E. coli* XL1-blue grown in LB medium attained a CDM of 46.7 ± 0.8 g/L and PHB 15.5 ± 0.5 g/L with 29.1 ± 1.8% (w/w) PHB content. The low content of PHB may have resulted from the LB medium containing insufficient mineral compounds to promote PHB biosynthesis. In addition, MR medium contained a high concentration of Mg^{2+}, in which *E. coli* required high amounts of Mg^{2+} and Ca^{2+} to bridge the highly negatively charged lipopolysaccharide (LPS) molecules in its outer membrane. The total amount of Mg^{2+} associated with *E. coli* cells is estimated to be approximately 100 mM, where LB broth contains only between 30 and 200 μM Mg^{2+}, so that the amount of Mg^{2+} in LB is not sufficient (Nikaido, 2009). During cultivation in LB medium, *E. coli* cells undergo alterations in carbon nutrition. Cells subsequently use easier-to-utilize amino acids until they are depleted and then switch to harder-to-use amino acids (Sezonov et al., 2007), resulting in a slow specific production rate and growth rate. During cultivation in MR medium, *E. coli* cells growing in glucose-minimal medium convert glucose into acetate and other organic acids during the early exponential phase; then, in the mid-exponential phase, they grow primarily by oxidizing these organic acids. It has been reported that MgATP is the substrate of numerous phosphorylating enzymes and the principal energy source of the cell. Indeed, any increase in metabolic activity increases the rate of MgATP use and, consequently, the rate of ADP and magnesium release, and vice versa (Gout et al., 2014). Therefore, because MR medium and the feeding solution contained a greater amount of Mg^{2+} than those in LB medium, they enhanced the metabolic advantage of *E. coli* cells cultured in this medium as well as supplemented Mg^{2+} together with glucose using fed-batch cultivation techniques. However, the same composition of glucose, $MgSO_4{\cdot}7H_2O$ and thiamine in the feeding solution was used for both MR medium and LB medium. We found that LB medium together with Mg^{2+} feeding promoted only cell growth but did not enhance PHB biosynthesis. Gahlawat and Srivastava (2012) studied the optimum concentration of medium components obtained from statistical design to identify the batch growth and product kinetics parameters of PHB fermentation in *Azohydromonas australica* using sucrose. They reported that mineral (essential) nutrients ($MgSO_4{\cdot}7H_2O$, KH_2PO_4, and Na_2HPO_4), which were similar to MR medium (nutrients ($MgSO_4{\cdot}7H_2O$, KH_2PO_4, and $(NH_4)_2PO_4$), play a very important role in the susceptibility of the buffering capacity of the nutrient medium, which is necessary to support the growth of microbial cells and PHB production.

CONCLUSION

This study demonstrates that the araBAD promoter and thioredoxin fusion proteins are able to yield high cell density and PHB production in fed-batch cultivation using a pH-stat feeding strategy. To achieve economical productivity, we found that MR medium with a pH stat feeding strategy (NH_4OH) coupled with a sufficient amount of Mg^{2+} in glucose feeding solution is an important key to attaining both a high cell density of CDM and PHB production. LB medium plus Mg^{2+} in glucose feeding solution promoted only high cell density. However, the advantage of the P_{BAD} expression system is that the cost of arabinose is cheaper than that of the IPTG induction system. In this study, we compared araBAD promoter (pBAD/Thio-TOPO, arabinose induction) with the cspA promoter (pColdTF, cold temperature and IPTG induction) and attained high cell density in both cases. Therefore, pBAD/Thio-TOPO-*phaCAB*$_{A-04}$ expressing *E. coli* XL1-blue and pColdTF-*phaCAB*$_{A-04}$ expressing *E. coli* XL1-blue constructed by our research group possess a high potential for PHB production.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, FJ897463.1; https://www.ncbi.nlm.nih.gov/genbank/, FJ897461.1; and https://www.ncbi.nlm.nih.gov/genbank/, FJ897462.1.

AUTHOR CONTRIBUTIONS

SCN and SV performed the experiments. OP provided guidance and suggestions for the experimental design and discussed the results. KH and KO provided guidance and suggestions. SCN provided guidance and suggestions for the experimental design, discussed the results, and wrote and edited the manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2021.661096/full#supplementary-material

Supplementary Figure 1 | Growth profile on various glycerol concentrations of *C. necator* strain A-04 (green line), *C. necator* H16 (red line) and variant V6C6 (blue line) that is an adaptive laboratory evolution of *C. necator* H16. This experiment was performed by Dr. Tuck Seng Wong (University of Sheffield, United Kingdom) under the research collaboration between National Center for Genetic Engineering and Biotechnology (BIOTEC) and Chulalongkorn University.

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Strategies for Poly(3-hydroxybutyrate) Production Using a Cold-Shock Promoter in *Escherichia coli*

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The present study attempted to increase poly(3-hydroxybutyrate) (PHB) production by improving expression of PHB biosynthesis operon derived from *Cupriavidus necator* strain A-04 using various types of promoters. The intact PHB biosynthesis operon of *C. necator* A-04, an alkaline tolerant strain isolated in Thailand with a high degree of 16S rRNA sequence similarity with *C. necator* H16, was subcloned into pGEX-6P-1, pColdI, pColdTF, pBAD/Thio-TOPO, and pUC19 (native promoter) and transformed into *Escherichia coli* JM109. While the *phaC*$_{A\text{-}04}$ gene was insoluble in most expression systems tested, it became soluble when it was expressed as a fusion protein with trigger factor (TF), a ribosome associated bacterial chaperone, under the control of a cold shock promoter. Careful optimization indicates that the cold-shock cspA promoter enhanced phaC$_{A\text{-}04}$ protein expression and the chaperone function of TF play critical roles in increasing soluble phaC$_{A\text{-}04}$ protein. Induction strategies and parameters in flask experiments were optimized to obtain high expression of soluble PhaC$_{A\text{-}04}$ protein with high $Y_{P/S}$ and PHB productivity. Soluble phaC$_{A\text{-}04}$ was purified through immobilized metal affinity chromatography (IMAC). The results demonstrated that the soluble phaC$_{A\text{-}04}$ from pColdTF-*phaCAB*$_{A\text{-}04}$ was expressed at a level of as high as 47.4 ± 2.4% of total protein and pColdTF-*phaCAB*$_{A\text{-}04}$ enhanced soluble protein formation to approximately 3.09−4.1 times higher than that from pColdI-*phaCAB*$_{A\text{-}04}$ by both conventional method and short induction method developed in this study. Cultivation in a 5-L fermenter led to PHB production of 89.8 ± 2.3% PHB content, a $Y_{P/S}$ value of 0.38 g PHB/g glucose and a productivity of 0.43 g PHB/(L.h) using pColdTF-*phaCAB*$_{A\text{-}04}$. The PHB film exhibited high optical transparency and possessed M_w 5.79 × 10^5 Da, M_n 1.86 × 10^5 Da, and PDI 3.11 with normal melting temperature and mechanical properties.

Keywords: polyhydroxybutyrate, pCold, cold shock, *Cupriavidus necator*, *E. coli* – *Escherichia coli*, cspA gene

INTRODUCTION

The global environmental concern regarding microplastics in the marine environment as contaminants with significant impacts on animal and human health has led to a call for national and international policies from more than 60 countries to ban or place a levy on single-use plastics (Steensgaard et al., 2017; Xanthos and Walker, 2017; Schnurr et al., 2018; Prata et al., 2019). Renowned global companies have also integrated regulations and policies to ban single-use plastics into their green marketing and corporate social responsibility policies. Bioplastics are becoming a popular alternative to single-use plastics to reduce the amount of microplastic waste. Recently, the role of compostable plastics within the circular economy has been highlighted. To establish a truly circular economy, the EU focuses on the contribution of biodegradable and compostable plastics to help the EU to meet its organic waste recovery targets by the end of 2023. The market for bioplastics is growing and the demand for bioplastics is rising. European Bioplastics reported that the global bioplastic production capacity will increase by 36 percent from 2.1 million tons in 2020 to approximately 2.8 million tons in 2025 (Bie, 2020). Among the various types of bioplastics, polyhydroxyalkanoates (PHAs) are an important biodegradable polymer family, as they are one hundred percent biobased and fully biodegradable in all environments, especially marine (ASTM 7081) and fresh water environments (Gross and Kalra, 2002; Volova et al., 2007).

To obtain both the environmental and economic benefits of PHAs over synthetic plastics and other bioplastics, microorganisms that exhibit efficient PHA production from inexpensive and renewable carbon sources are urgently required to develop a low-cost approach. Microbial cells typically accumulate PHA at approximately 30–50% of the cell dry mass (CDM). The best known industrial PHA producer, *Cupriavidus necator* H16 (formerly known as *Alcaligenes eutrophus*, *Ralstonia eutropha,* and *Wautersia eutropha*), is capable of accumulating polyhydroxybytyrate (PHB) at over 80% of the CDM. PHA accumulation is tightly regulated by imbalanced growth conditions with excess carbon but limited nitrogen (Kawaguchi and Doi, 1992). One of the major limitations in the production of PHAs in wild-type strains has been intracellular polymer degradation caused by endogenous PHA depolymerases, which is different from the behavior of exogenous PHA depolymerases (Gebauer and Jendrossek, 2006). Therefore, intracellular PHAs are often spontaneously degraded during cultivation when the bacteria require carbon, resulting in low PHA content and a wide range of molecular weight distributions in wild-type strains. Thus, many recombinant strains have been developed by metabolic engineering to obtain a high yield of PHB and a molecular weight that is high enough for polymer processing (Liu et al., 1998; Ahn et al., 2000; Kahar et al., 2005; Taguchi et al., 2005; Agus et al., 2006b; Hiroe et al., 2012). Ordinarily, the PHB biosynthesis pathway begins with acetyl-CoA and requires three major enzymes, namely, 3-ketothiolase (*phaA*), NADPH-dependent acetoacetyl-CoA reductase (*phaB*), and PHA synthase (*phaC*), and these three genes are sufficient for the production of PHB in non-PHA-producing bacteria at more than 90% of the CDM when heterologously expressed in *Escherichia coli* (Lee et al., 1994). It has been reported that PhaC plays a key role in obtaining the polymeric form, resulting in a high level and high molecular weight of PHB (Kahar et al., 2005; Agus et al., 2006b).

To date, PHA synthases have been categorized into four major classes based on their sequence, substrate specificity, and subunit composition (Rehm, 2003; Tsuge, 2016). It was reported that PhaC derived from *C. necator* H16 ($PhaC_{H16}$) is a Class I PhaC and is one of the most widely studied PHA synthases. It has a molecular weight of approximately 64 kDa (589 amino acids) and is located as the first gene in the PHA biosynthetic phaCAB operon, followed by PhaA and PhaB (Schubert et al., 1988; Peoples and Sinskey, 1989). It was demonstrated that the weight-average molecular weight (M_w) of PHB synthesized by wild-type bacteria is generally in the range of 0.1–2.0 $\times$ 10^6 Da. When recombinant $PhaC_{H16}$ was overexpressed in *E. coli*, most of the protein formed insoluble inclusion bodies due to its low aqueous solubility (Gerngross et al., 1994; Gerngross and Martin, 1995; Zhang et al., 2000; Yuan et al., 2001). To feasibly achieve industrial-scale production, PhaC would need to be produced in large quantities and its solubility would need to be improved (Thomson et al., 2013). There have been many reports that have attempted to resolve the problem mentioned above, including by modulating the concentration of the PhaC protein by varying the chemical inducer quantities (Agus et al., 2006a); expressing the protein at a reduced temperature (30°C) (Thomson et al., 2013); fusing the PhaC protein with a glutathione S-transferase (GST) tag, which is a hydrophilic tag, to improve its solubility (Harada et al., 2019); and coexpressing the protein with chaperones to obtain high total quantities of enzyme and a larger proportion in the soluble fraction than obtained without chaperones. In this study, we reported the use of pCold (cspA promoter) to improve PhaC expression as well as its combination with trigger factor (TF) chaperone and compared with the promoters mentioned above.

In a previous study, we reported the generation of the *C. necator* strain A-04, possessing 99.78% 16S RNA sequence similarity with *C. necator* H16 but differing in PHA production ability (Chanprateep et al., 2008). Designed using the gene walk technique, the PHA biosynthesis operon of *C. necator* strain A-04 consisted of three genes, encoding acetyl-CoA acetyltransferase ($phaA_{A\text{-}04}$, 1182 bp, 40.6 kDa, accession no. FJ897461), acetoacetyl-CoA reductase ($phaB_{A\text{-}04}$, 741 bp, 26.4 kDa, accession no. FJ897462) and PHB synthase ($phaC_{A\text{-}04}$, 1770 bp, 64.3 kDa, accession no. FJ897463). Sequence analysis of the $phaA_{A\text{-}04}$, $phaB_{A\text{-}04}$, and $phaC_{A\text{-}04}$ genes revealed that $phaC_{A\text{-}04}$ was 99% similar to $phaC_{H16}$ from *C. necator* H16. The difference was in the amino acid residue situated at position 122, which in $phaC_{A\text{-}04}$ was proline but in *C. necator* H16 was leucine. The total amino acid sequences of $phaA_{A\text{-}04}$ and $phaB_{A\text{-}04}$ were 100% matched with those of *C. necator* H16 (Napathorn et al., 2021). Notably, *C. necator* strain A-04 prefers fructose over glucose as a carbon source, accumulating PHB at 78% of the CDM under a C/N ratio of 200, whereas it could incorporate a high mole fraction of monomeric

4-hydroxybutyrate monomeric into the poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] copolymer under a C/N ratio of 20 (Chanprateep et al., 2010), as well as the poly(3-hydroxybutyrate-*co*-3-hydroxyvaterate-*co*-4-hydroxybutyrate) [P(3HB-*co*-3HV-*co*-4HB)] terpolymer (Chanprateep and Kulpreecha, 2006). In the prior study, the intact *phaCAB*$_{A-04}$ operon was cloned into the arabinose-inducible araBAD promoter and transformed into *E. coli* strains Top 10, JM109 and XL-1 blue. The results showed that optimal conditions obtained from shaken flask experiments yielded 6.1 ± 1.1 g/L cell dry mass (CDM), a PHB content of 93.3 ± 0.9% (w/w) and a productivity of 0.24 g/(L·h). Finally, fed-batch cultivations by pH-stat control in a 5-L fermenter of *E. coli* strains XL1-Blue harboring pBAD/Thio-TOPO-*phaCAB*$_{A-04}$, leading the PHB production of 29.0 ± 1.1 g/L with 60.2 ± 2.3% PHB content in the cell dry mass (CDM) of 53.1 ± 1.0 g/L, a $Y_{P/S}$ value of 0.21 g PHB/g glucose and a productivity of 0.4 g PHB/(L·h) in LB medium.

Thus, the objective of this work was to express *phaCAB*$_{A-04}$ genes from the isolated *C. necator* strain A-04 in pColdI (cspA promoter, cold- and IPTG-inducible vector, N-terminal 6His-fusion protein) and pColdTF (cspA promoter, cold- and IPTG-inducible vector, trigger factor (TF) chaperone, N-terminal 6His-fusion protein). The reason for choosing pColdI and pColdTF is that there have been few reports on utilizing cold inducible promoter for PHB production. Therefore, the obtained results will prove that if phaC solubility is enhanced, this outcome would finally result in enhancing PHB production. It has been well known that CspA was originally found as the major cold-shock protein in *E. coli*, consisting of 70-amino-acid residues. CspA forms a β-barrel structure with five anti-parallel β-strands and functions as an RNA chaperone. Its transient induction upon cold shock is regulated at the level of transcription, mRNA stability and translation (Yamanaka et al., 1998). Cold shock proteins are not only produced during cold stress, but in *E. coli*, CspA also forms 1% of all soluble proteins at the early exponential growth phase at 37°C, suggesting that CspA also functions at optimal growth temperature (Brandi et al., 1999). Furthermore, insolubility of protein overexpressed in *E. coli* is a common problem. Therefore, co-expression with solubility enhancers was utilized to resolve these issues, such as GST and TF. TF is a prokaryotic ribosome associated chaperone (MW 48 kDa), which facilitates co-translational protein folding, thus, reducing the chances of forming misfolded and insoluble proteins (Patzelt et al., 2002; Maier et al., 2005). As a native product from prokaryotes, TF is highly expressed in *E. coli*, which allowed for high yield of recombinant proteins (Baneyx, 1999). Another benefit of using the pCold system is that the induction was carried out at cold temperatures, which has been shown to significantly improve protein folding by decreasing the rate of transcription and translation, thus providing more time for the protein to be folded (Agashe et al., 2004; Baneyx and Mujacic, 2004). Compared with previous reports, the PHA biosynthesis operon of *C. necator* strain A-04 was also cloned into pGEX-6P-1 [tac promoter, isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible vector, N-terminal GST fusion protein], pBAD/Thio-TOPO (araBAD promoter, arabinose-inducible vector, N-terminal thioredoxin fusion protein and C-terminal 6His-fusion protein) (Napathorn et al., 2021) and pUC19 (control strain, *phaCAB*$_{A-04}$ biosynthesis genes with native promoter of *phaC*$_{A-04}$) and transformed into *E. coli* JM109 (**Table 1**). Next, to optimize phaC$_{A-04}$ overexpression in shake flask cultivation, three induction methods were tested and compared with conventional induction method (**Figure 1**, details are described in the section "Materials

TABLE 1 | Bacterial strains and plasmids used in this study.

Strains/plasmids	Relevant description	Reference/ source
Strain		
Cupriavidus necator strain A-04	Wild Type	
Escherichia coli JM109	F′*traD36 proA+B+ lacI*^{q}*(lacZ)*Δ*M15/*Δ*(lac-proAB) glnV44 e14- gyrA96 recA1 relA1 endA1 thi hsdR17*	Promega Corporation, Madison, WI, United States
Plasmid		
pUC19	Amp^r	Thermo Scientific, MA, United States
pColdI	Amp^r, lacI, cold-shock cspA promoter	Takara Bio Inc., Shiga, Japan
pColdIF	Amp^r, lacI, cold-shock cspA promoter and trigger factor	Takara Bio Inc., Shiga, Japan
pGEX-6P-1	Amp^r, lacI, tac promoter and glutathione S-transferase (GST)	Novagen, WI, United States
pBAD/Thio-TOPO	Amp^r, araBAD promoter and thioredoxin	Invitrogen, CA, United States
pUC19-nativeP-*phaCAB*$_{A-04}$	pUC19 derivative, carrying *phaCAB* with native promoter from *C. necator* strain A-04	This study
pColdI-*phaCAB*$_{A-04}$	pColdI derivative, carrying N-terminal 6His-fused *phaCAB* from *C. necator* strain A-04	This study
pColdTF-*phaCAB*$_{A-04}$	pColdTF derivative, carrying N-terminal 6His-fused *phaCAB* from *C. necator* strain A-04	This study
pGEX-6P-1-*phaCAB*$_{A-04}$	pGEX-6P-1 derivative, carrying N-terminal GST and 6His-fused *phaCAB* from *C. necator* strain A-04	This study
pBAD/Thio-TOPO-*phaCAB*$_{A-04}$	pBAD/Thio-TOPO® derivative, carrying C-terminal 6HIS- and N-terminal thioredoxin fused *phaCAB* from *C. necator* strain A-04	Napathorn et al., 2021
Primer		
pCold-F	5′-ATGGATCCCTCGAGATGGCGA CCGGCAAAG-3′	This study
pCold-R	5′-GTGAATTCAAGCTTTCAGCCCATAT GCAGGCC-3′	This study
pGEX-F	5′-GGCCCCTGGGATCCCCGGAAATG GCGACCGGCAA-3′	This study
pGEX-R	5′- GCACTCGACTCGAGTCAGCCCAT ATGCAGG-3′	This study
nativeP-*phaCAB*$_{A-04}$-F	5′-TGGTCCCTGA CTGGC-3′	This study
nativeP-*phaCAB*$_{A-04}$-R	5′-CGTCGACGACC TTGAAT-3′	This study

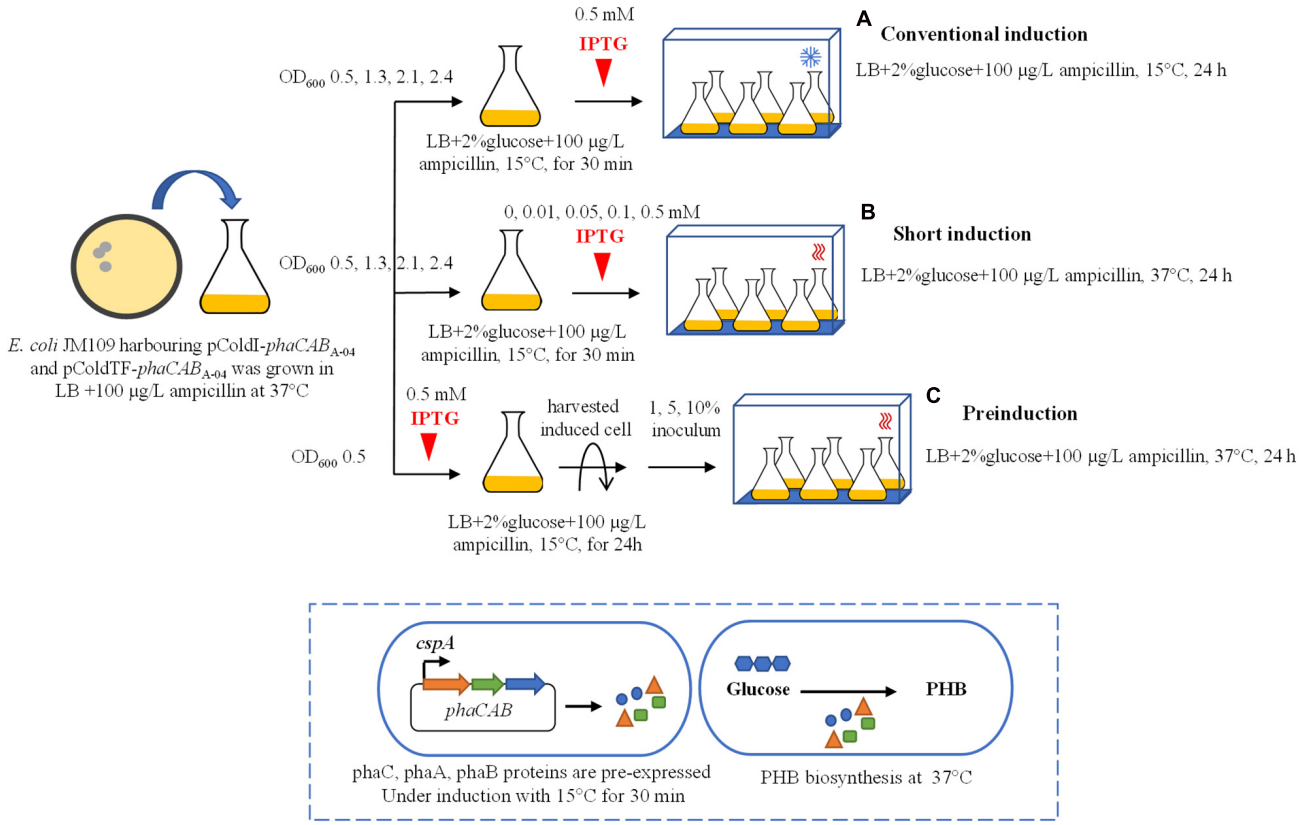

FIGURE 1 | Schematic of three different induction methods for heterologous expression of the *phaCAB*$_{A-04}$ biosynthesis operon in *E. coli* JM109 (pColdI-*phaCAB*$_{A-04}$) and *E. coli* JM109 (pColdTF-*phaCAB*$_{A-04}$). **(A)** Conventional induction method: the culture was incubated at 37°C and 200 rpm until the OD_{600} reached 0.5, 1.3, 2.1, and 2.4. Then, the cultivation temperature was decreased from 37 to 15°C for 30 min, and the expression of the *phaCAB*$_{A-04}$ operon was induced by the addition of 0.5 mM IPTG. The cultivation temperature was further maintained at 15°C for 24 h. **(B)** Short-induction method: the culture was incubated at 37°C and 200 rpm until the OD_{600} reached 0.5, 1.3, 2.1, and 2.4. Then, the temperatures were varied at 15, 25, 30, and 37°C for 30 min. Next, the expression of the *phaCAB*$_{A-04}$ operon was induced by adding various concentrations (0.01, 0.05, 0.1, 0.5, and 1.0 mM) of IPTG, and the cultivation temperature was maintained at 37°C for 24 h. **(C)** Preinduction method: the culture was incubated at 37°C and 200 rpm until the OD_{600} reached 0.5. Next, 0.5 mM IPTG was added into the culture when the temperature was decreased from 37°C to 15°C for 24 h. Then, the induced cells were harvested by centrifugation, the medium was discarded, and the cells were resuspended in an equal volume of fresh LB medium. Finally, the induced cells at 1, 5, or 10% (v/v) were transferred into fresh LB medium supplemented with 100 µg/L ampicillin and 20 g/L glucose and incubated at 37°C and 200 rpm for 24 h.

and Methods"). The effect of phaC$_{A-04}$ overexpression on PHB production in recombinant *E. coli* with respect to cell growth, glucose consumption, PHB production, and kinetic parameters in conditions ranging from flask culture to a 5-L fermenter. PhaC$_{A-04}$ was purified, quantified, carefully compared versus pColdI-*phaCAB*$_{A-04}$ and pColdTF-*phaCAB*$_{A-04}$, and also between short induction and conventional induction methods. Furthermore, to examine the effect of cspA promoter and TF chaperone on the polymer properties, the produced PHB was subjected to molecular weight determination, thermal analysis and mechanical property measurement.

MATERIALS AND METHODS

Strains and Plasmids

The *E. coli* strains and plasmids used in this study are listed in **Table 1**. The PHB-producing *C. necator* strain A-04 (Chanprateep et al., 2008) was used to isolate the *phaCAB*$_{A-04}$ gene operon. All bacterial strains were grown at 37°C in Luria-Bertani (LB) medium supplemented with 100 µg/L ampicillin. The LB medium contained (per liter) 10 g of tryptone (Himedia, Mumbai, India), 5 g of yeast extract (Himedia, Mumbai, India) and 10 g of NaCl (Merck KGaA, Darmstadt, Germany). Stock cultures were maintained at −80°C in a 15% glycerol solution. The experiments were performed in a biosafety level 1 laboratory and by researchers and investigators who had undergone biosafety training.

Construction of Recombinant Plasmids

The *phaCAB*$_{A-04}$ operon PHB biosynthetic genes from *C. necator* A-04 were PCR-amplified using the following pair of primers: forward primer 5′-ATGGATCC CTCGAGATGGCGACCGGCAAAG-3′ (the XhoI site is underlined) and reverse primer 5′-GTGAATTCAAGCTT TCAGCCCATATGCAGGCC-3′ (the HindIII site is underlined).

Primers were designed based on accession numbers FJ897463, FJ897461, and FJ897462. The blunted PCR product was purified and subcloned into pBluescript SK- (Stratagene, La Jolla, CA, United States) linearized by SmaI. The recombinant plasmid digested with XhoI and HindIII was cloned into cold-shock-inducible pColdI and pColdTF vectors (Takara Bio Inc., Shiga, Japan) at the XhoI and HindIII restriction sites, yielding pColdI-phaCAB$_{A-04}$ and pColdTF-phaCAB$_{A-04}$, respectively. For the plasmid pGEX-6P-1-phaCAB$_{A-04}$, the phaCAB$_{A-04}$ operon was amplified by the primers pGEX-F and pGEX-R (**Table 1**). The 3,885-bp DNA fragment was digested by BamHI and XhoI and cloned into BamHI-XhoI-digested pGEX-6P-1 to obtain pGEX-6P-1-*phaCAB*$_{A-04}$. To construct pUC19-nativeP-*phaCAB*$_{A-04}$, the primers nativeP-*phaCAB*$_{A-04}$-F and nativeP-*phaCAB*$_{A-04}$-R were used to amplify the *phaCAB*$_{A-04}$ operon, including its native promoter. The blunted PCR product was purified and cloned into SmaI-linearized pUC19 (Thermo Fisher Scientific, Inc., Waltham, MA, United States), yielding pUC19-nativeP-*phaCAB*$_{A-04}$. PCRs were performed using Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, United States). *E. coli* JM109 was used as a host for cloning and PHB production. The accuracy of the constructed plasmid was verified by the corresponding restriction enzyme and sequencing.

Optimization of Culture Conditions for PHB Production in Shaken Flask Cultivation

Expression vectors named pColdI-*phaCAB*$_{A-04}$ and pColdTF-*phaCAB*$_{A-04}$ with the entire phaCAB$_{A-04}$ operon were transformed into *E. coli* JM109 by the heat shock method (Sambrook and Russell, 2001). Shake flask experiments were performed in 250-mL Erlenmeyer flasks containing 50 mL of medium. *E. coli* JM109 cells transformed with pColdI-*phaCAB*$_{A-04}$ or pColdTF-*phaCAB*$_{A-04}$ were grown in LB medium containing ampicillin (100 μg/mL) on a rotary incubator shaker (Innova 4300, New Brunswick Scientific Co., Inc., Edison, NJ, United States) at 37°C and 200 rpm for 24 h. The overnight seed culture was inoculated into fresh LB medium (5% v/v inoculum) containing 100 μg/L ampicillin and 20 g/L glucose prior to induction with temperature and IPTG using three separate induction methods (**Figure 1**).

For the synthesis of PHB using the conventional induction method, the procedure was performed according to the user manual (Takara Bio Inc., Otsu, Shiga, Japan). The culture was incubated at 37°C and 200 rpm until the optical density at 600 nm (OD_{600}) reached 0.5, 1.3, 2.1, and 2.4. Next, the cultivation temperature was reduced from 37°C to 15°C for 30 min. The expression of the *phaCAB* operon was induced by the addition of 0.5 mM IPTG, and cultivation was continued at 15°C for an additional 24 h.

For the synthesis of PHB using the short-induction method developed in this study, the culture was incubated at 37°C and 200 rpm until the OD_{600} reached 0.5, 1.3, 2.1, and 2.4. Then, the temperatures were varied at 15, 25, 30, and 37°C for 30 min. Next, the expression of the *phaCAB* operon was induced by adding various concentrations (0.01, 0.05, 0.1, 0.5, and 1.0 mM) of IPTG, and the cultivation was maintained at 37°C for 24 h.

For the synthesis of PHB using the preinduction method developed in this study, the culture was incubated at 37°C and 200 rpm until the OD_{600} reached 0.5. Then, 0.5 mM IPTG was added to the culture and the temperature was reduced from 37°C to 15°C for 24 h. The induced cells were harvested by centrifugation, the medium was discarded, and the cells were resuspended in an equal volume of fresh LB medium. Then, the induced cells at 1, 5, or 10% (v/v) were transferred into fresh LB medium supplemented with 100 μg/L ampicillin and 20 g/L glucose and incubated at 37°C and 200 rpm for 24 h.

For comparison of the effect of phaC expression on PHB production under various types of promoters, fusion proteins and chaperones, shake flask experiments were performed in 250-mL Erlenmeyer flasks containing 50 mL of LB medium containing ampicillin (100 μg/mL) on a rotary incubator shaker at 37°C and 200 rpm for 24 h. For PHB production, overnight cultures in LB medium (1 mL) were transferred into fresh LB medium supplemented with glucose (20 g/L) and ampicillin (100 μg/mL). Recombinant *E. coli* JM109 (pColdI-*phaCAB*$_{A-04}$) and *E. coli* JM109 (pColdTF-*phaCAB*$_{A-04}$) were induced to produce PHB using the conventional induction method and short-induction method. The effect of GST (the hydrophilic fusion protein) and the tac promoter on PHB production was investigated using *E. coli* JM109 (pGEX-6P-1-*phaCAB*$_{A-04}$), which was induced by the addition of IPTG (0.5 mM). The effect of the araBAD promoter and N-terminal thioredoxin fusion protein together with the C-terminal 6His-fusion protein on PhaC and PHB production was examined by inducing *E. coli* JM109 (pBAD/Thio-TOPO-*phaCAB*$_{A-04}$) with arabinose (1% w/v). *E. coli* JM109 (pUC19-nativeP-*phaCAB*$_{A-04}$), which exhibits expression from native promoter without addition of IPTG, was used as a control strain. All of these comparison experiments were performed at 15 or 37°C for 48 h. The crude glycerol used in this study was obtained from biodiesel industries belong to Bangchak Corporation Public Company Limited, a petroleum and energy conglomerate in Thailand. The content of glycerol was 80% w/v.

Conditions for PHB Production in a 5-L Fermentor

A preculture was prepared in 500-mL Erlenmeyer flasks containing 100 mL of LB medium and grown on a rotary shaker at 37°C at 200 rpm for 24 h. The preculture was inoculated into a 5-L bioreactor (MDL500, B.E. Marubishi Co., Ltd., Tokyo, Japan) containing 2 L of LB medium supplemented with 100 μg/L ampicillin and 20 g/L glucose at an inoculation volume of 5% (v/v). The agitation speed and the air flow rate were 500 rpm and 1 mL/min, respectively. After an OD_{600} of 0.5 was obtained, the cultivation temperature was reduced from 37 to 15°C for 30 min. Next, IPTG was added to the culture at a final concentration of 0.5 mM. After IPTG addition, the cultivation temperature was shifted from 15 to 37°C and maintained at 37°C for 48 h. Culture samples were collected at 6 h intervals for 48 h.

Analytical Methods

Cell growth was monitored by the CDM, which was determined by filtering 5 mL of the culture broth through preweighed cellulose nitrate membrane filters (pore size = 0.22 μm; Sartorius, Goettingen, Germany). The filters were dried at 80°C for 2 days and stored in desiccators. The net biomass was defined as the residual cell mass (RCM), which was calculated by subtracting the amount of PHB from the CDM. The PHB in dried cells was methyl-esterified using a mixture of chloroform and 3% (v/v) methanol-sulfuric acid (1:1 v/v) (Braunegg et al., 1978). The resulting monomeric methyl esters were quantified by a gas chromatograph (model CP3800, Varian Inc., Walnut Creek, CA, United States) using a Carbowax-PEG capillary column (0.25-μm df, 0.25-mm ID, 60-m length, Varian Inc.). The internal standard was benzoic acid, and the external standard was PHB (Sigma-Aldrich Corp.). The total reducing sugar concentration was determined using a 3,5-dinitrosalicylic acid (DNS) assay (Miller, 1959). Glycerol and acetate concentrations in culture medium were analyzed using an HPLC system (1200 Infinity series, Agilent technologies, United States) equipped with 1260 RID (Agilent Technologies, United States) and X-bridge-BEH amide column (4.6 × 250 nm × 5 μm) (Water, United States), with an isocratic mobile phase of acetonitrile: water (70:30, v/v) at a flow rate 1.0 mL/min and 30°C (Dharmadi et al., 2006; Simonzadeh and Ronsen, 2012).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

Recombinant *E. coli* cells were cultured with and without induction. Cells were collected by centrifugation at 17,000 × *g* and 4°C for 30 min. Cell pellets were resuspended in 100 mM Tris-HCl (pH 8.0) and normalized to an OD_{600} of 2.0. Total proteins were extracted from cells by using a sonicator (Sonics Vibra Cell VCX 130, Sonics & Materials, Inc., Newtown, CT, United States). The lysis mixture was then centrifuged at 17,000 × *g* at 4°C for 30 min. The protein concentration in the supernatant (soluble protein) was estimated by the Bradford method using a Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, United States), and bovine serum albumin was used as a standard. Thirty micrograms of total protein from each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels under reducing conditions and electrophoresed at 80 V for 10 min followed by 140 V for 60 min. For the western blot analysis, the protein from SDS-PAGE was then transferred to a polyvinylidene difluoride (PVDF) membrane using a semi-dry blotting system (Trans-Blot SD Cell, Bio-Rad Laboratories Inc, Hercules, CA, United States) at 150 mA for 40 min. The 6His tag was detected by a mouse anti-His antibody (Aviva Systems Biology Corp., San Diego, CA, United States) and an HRP-conjugated goat anti-mouse IgG as the primary and secondary antibodies, respectively. Color development was performed using a Mouse IgG DAB Chromogenic Reagent Kit (Boster Biological Technology, Pleasanton CA, United States) according to the manufacturer's instructions.

Protein Purification by Immobilized Metal Affinity Chromatography (IMAC)

The cell pellet (from 50 mL culture) was resuspended in 1 ml of lysis-equilibration-wash buffer (1X LEW buffer, 50 mM NaH_2PO_4, 300 mM NaCl and pH 8.0). Lysozyme (USB Corporation, OH, United States) and Benzonase® endonuclease (Novagen Inc., WI, United States) were added to concentrations of 0.2 mg/mL and 20 U/mL, respectively. The cells were ruptured by ultrasonic homogenizer (Vibra-Cell™ Ultrasonic Liquid Processors VCX 130, Sonics & Materials, Inc., CT, United States). The amplitude was set to 40% (pulse interval at 30/15 s for 5 min). The lysate was clarified by centrifugation at 16, 100 × *g* for 20 min and the supernatant was collected. Protino@® Ni-IDA 1000 His-Tag Protein purification columns (Macherey-Nagel GmbH & Co. KG, Düren, Germany) were pre-equilibrated with four bed volumes of 1X LEW buffer and allowed to drain by gravity. The cleared supernatant with 2 mg of total protein was loaded onto a pre-equilibrated column and washed with four bed volumes of 1X LEW buffer containing 20 mM imidazole. Finally, the polyhistidine-tagged protein was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole and pH 8.0). Each fraction was analyzed by running 10 μL of eluate on SDS-PAGE and quantified by Bradford protein assay. To ensure accurate quantitation of yields, the lysate flow-through was collected for detection of unbound product by SDS-PAGE analysis.

Analysis of Polymer Molecular Weight

The molecular weight was determined by Gel Permeation Chromatography (GPC; Shimadzu 10A GPC system, Shimadzu Co., Ltd., Kyoto, Japan) with a 10A refractive index detector and two Shodex columns (a GPC K-806M column (8.0 mm ID × 300 mm L, Showa Denko K.K., Tokyo, Japan) belong to Associate Professor Takeharu Tsuge's laboratory at Department of Materials Science and Engineering, School of Materials and Chemical Technology, Tokyo Institute of Technology, Yokohama, Japan. Polymer was dissolved in 0.1% (w/v) chloroform and filtered through a 0.45 μm low protein binding Durapore® (PVDF) membrane filter (Millex®-HV, Merck Millipore Ltd., Tullagreen, Carrigtwohill Co., Cork, Ireland). The temperature was 40°C and the flow rate was 0.8 mL/min. A standard curve was determined for polystyrene with low polydispersity in the same conditions for the molecular weight 1.26×10^3, 3.39×10^3, 1.30×10^4, 5.22×10^4, 2.19×10^5, 7.29×10^5, 2.33×10^6, and 7.45×10^6. The weight-average molecular weight (M_w) and the number-average molecular weight (M_n) were determined by gel permeation chromatography (GPC) and the polydispersity index (PDI) was calculated as the ratio $\frac{M_w}{M_n}$.

Preparation of PHB Films

PHB films were prepared according to the ASTM: D882-91 protocol. The PHB films were prepared from chloroform

solutions of the polyesters using conventional solvent-casting techniques and a glass tray [Pyrex, Corning Incorporated, NY, United States) as the casting surface (modified from Yoshie et al. (1995)]. The thickness of the thin polyester films was regulated by controlling the concentration of the polymer in chloroform (1% w/v) and the volume of the polymer solution. The thickness of the PHB films was 0.05 mm, which was confirmed using a caliper (Model 500-175: CD-12C, Mitutoyo Corporation, Kawasaki-shi, Kanagawa, Japan). A film samples were aged for 1 month in desiccator at ambient temperature to allow them to reach crystallization equilibrium.

Analysis of the Mechanical Properties of PHB Films

The mechanical tests were conducted at the Scientific and Technological Research Equipment Center, Chulalongkorn University, using a universal testing machine (H10KM, Wuhan Huatian Electric Power Automation Co., Ltd., Wuhan, China) with a crosshead speed of 10 mm/min. The variables measured included the elongation at the break point (%), the stress at maximal load (MPa), and the Young's modulus (MPa). The data represent the mean values for ten samples tested under the same conditions.

Thermal Analysis by Differential Scanning Calorimetry (DSC) of PHB Films

A 10-mg sample of PHB was encapsulated in an aluminum sample vessel and placed in the sample holding chamber of the DSC apparatus (DSC7, PerkinElmer, Inc., Waltham, MA, United States). STARe software (version SW 10.00; Mettler-Toledo International Inc., Columbus, OH, United States) was used to operate the DSC apparatus at the Petroleum and Petrochemical College, Chulalongkorn University. The previous thermal history of the sample was removed before the thermal analysis by heating the sample from ambient temperature to 180°C at 10°C/min. Next, the sample was maintained at 180°C for 5 min before cooling at 10°C/min to −50°C. The sample was then thermally cycled at 10°C/min to 180°C. The melting peak temperature, denoted by Tm, was given by the intersection of the tangent with the furthest point of an endothermic peak and the extrapolated sample baseline. The glass transition temperature, denoted by Tg, could be estimated by extrapolating the midpoint of the heat capacity difference between glassy and viscous states after heating of the quenched sample.

Data Analysis

All the data presented in this manuscript are representative of the results of three independent experiments and are expressed as the mean values ± standard deviations (SDs). Analysis of variance (one-way ANOVA) followed by Duncan's test for testing differences among means was conducted using SPSS version 22 (IBM Corp., Armonk, NY, United States). Differences were considered significant at $P < 0.05$.

RESULTS

Effect of the Growth Phase on the Production of $PhaC_{A-04}$ and PHB by the Conventional Induction Method

In preliminary experiments, after the pColdI-$phaCAB_{A-04}$ and pColdTF-$phaCAB_{A-04}$ vectors were transformed into *E. coli* JM109, the heterologous expression of $phaCAB_{A-04}$ biosynthesis genes was performed by conventional induction method and by varying IPTG concentrations. It was found that 0.5 mM IPTG was the optimal concentration, which was the same as that recommended by the manufacturer's instructions (**Supplementary Figure 2**). Next, to optimize the conditions, expression was induced with 0.5 mM IPTG at different growth phases by varying OD_{600} based on cultivation time: 0.5 (2 h, early exponential phase), 1.3 (4 h, middle exponential phase), 2.1 (6 h, late exponential phase), and 2.4 (10 h, stationary phase). Concurrently, the temperature was shifted from 37°C to 15°C for 24 h. **Figure 2** shows the effect of the growth phase for gene induction on the CDM (g/L), PHB content (% w/w) and levels of insoluble and soluble $PhaC_{A-04}$ protein, comparing *E. coli* JM109 (pColdI-$phaCAB_{A-04}$) and *E. coli* JM109 (pColdTF-$phaCAB_{A-04}$). The $PhaC_{A-04}$ protein was detected by western blot analysis using an anti-His tag antibody as the primary antibody. A band appeared in the western blot at the position corresponding to that of the His-tagged $phaC_{A-04}$ protein (67 kDa) for pColdI-$phaCAB_{A-04}$ and the fusion protein of His-tagged $phaC_{A-04}$ and TF at 115 kDa. By varying the time courses of the growth phase, His-tagged $PhaC_{A-04}$ and the His-tagged $phaC_{A-04}$-TF fusion protein were successfully expressed, with the highest amount of total $phaC_{A-04}$ protein obtained when the $phaCAB_{A-04}$ operon was induced at an OD_{600} of 0.5. The content of soluble $PhaC_{A-04}$-TF fusion protein (**Figure 2B**, lane 3) in the sample after IPTG induction at an OD_{600} of 0.5 was much higher than that of the $phaC_{A-04}$ protein alone from pColdI-$phaCAB_{A-04}$ (**Figure 2A**, lane 3), suggesting that the TF chaperone facilitates the expression of highly soluble protein in *E. coli* JM109. The highest amount of soluble $PhaC_{A-04}$ and TF fusion protein was produced only at an OD_{600} of 0.5 and was not detected in other growth phases. Functional $PhaC_{A-04}$ protein production was confirmed by determining the amount of PHB produced; however, the value was only 46.2 ± 1.8% w/w with a productivity of 0.03 ± 0.01 g/(L·h) (**Table 2**). Furthermore, the quantification of purified soluble $PhaC_{A-04}$ protein and $PhaC_{A-04}$ and TF fusion protein was performed by IMAC affinity chromatography under native conditions and the results were shown in **Table 3** (the details of this experiment are given below).

Comparison of the Effect of phaC Expression on PHB Production Under Various Types of Promoters, Fusion Proteins and Chaperones

In $phaCAB_{A-04}$-overexpressing *E. coli* JM 109 (pColdI-$phaCAB_{A-04}$) under the conventional conditions, the formation

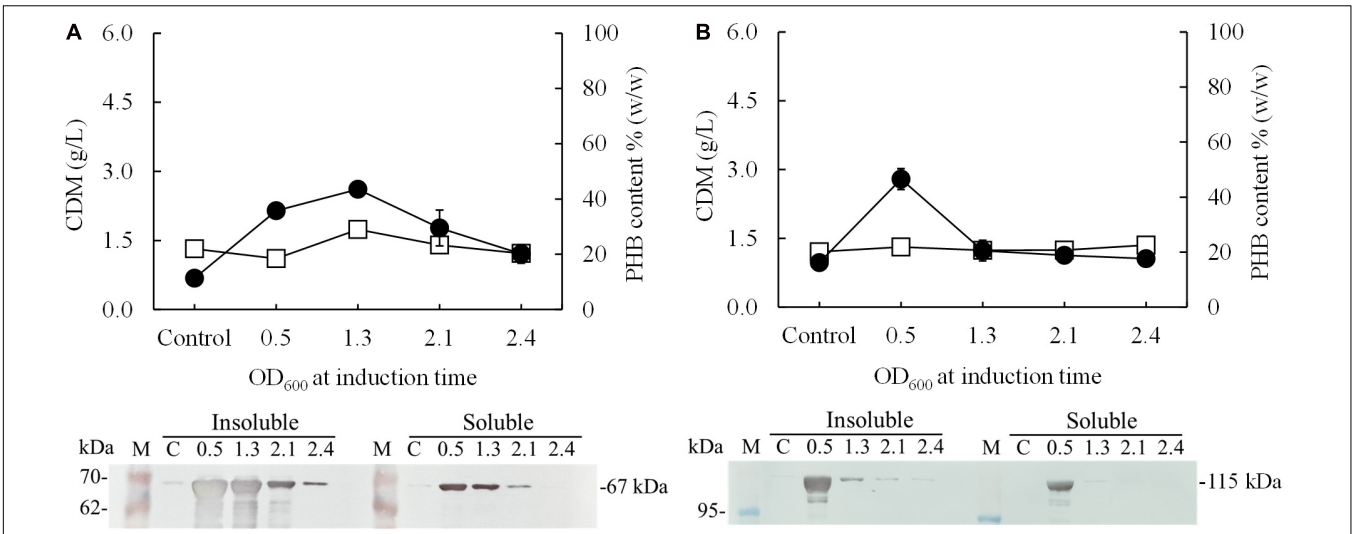

FIGURE 2 | Effect of the growth phase suitable for cold-shock induction on CDM and PHB content (% w/w) under the conventional induction method. The different growth phases were investigated by varying OD_{600} based on cultivation time [0.5 (2 h, early exponential phase), 1.3 (4 h, middle exponential phase), 2.1 (6 h, late exponential phase), and 2.4 (10 h, stationary phase)] for **(A)** *E. coli* JM109 (pColdI-*phaCAB*$_{A-04}$) and **(B)** *E. coli* JM109 (pColdTF-*phaCAB*$_{A-04}$). A control experiment was performed with 0.0 mM IPTG induction. All the data are representative of the results of three independent experiments and are expressed as the mean values ± standard deviations (SDs). The PhaC$_{A-04}$ protein was detected by western blot analysis using anti-His tag antibody as the primary antibody. The band appearing in the western blot at the position corresponding to that of the His-tagged phaC$_{A-04}$ protein was 67 kDa in size for pColdI-*phaCAB*$_{A-04}$, and the fusion protein of His-tagged phaC$_{A-04}$ and TF was 115 kDa in size. All the data are representative of the results of three independent experiments and are expressed as the mean values ± standard deviations (SDs). Symbols: open squares, CDM (g/L); closed circle, PHB (g/L).

TABLE 2 | Effect of IPTG concentration on CDM (g/L), PHB (g/L), % (w/w) PHB content and PHB productivity in a comparison between *E. coli* JM109 harboring pColdI-*phaCAB*$_{A-04}$ and *E. coli* JM109 harboring pColdTF-*phaCAB*$_{A-04}$.

Plasmid	Inoculum % (v/v)	IPTG (mM)	CDM (g/L)	RCM (g/L)	PHB (g/L)	PHB content (% w/w)	Productivity g/(L.h)
pColdI-*phaCAB*$_{A-04}$							
Short induction	5	0	2.8 ± 0.1	2.5 ± 0.1	0.3 ± 0.0	10.7 ± 1.1	0.01 ± 0.00
		0.01	2.8 ± 0.2	1.0 ± 0.2	1.8 ± 0.1	64.3 ± 3.1	0.07 ± 0.03
		0.05	2.6 ± 0.3	0.7 ± 0.2	1.9 ± 0.2	73.1 ± 3.5	0.08 ± 0.04
		0.1	2.6 ± 0.2	0.5 ± 0.1	2.1 ± 0.2	80.8 ± 0.7	0.08 ± 0.05
		0.5	4.5 ± 0.3	0.6 ± 0.1	3.9 ± 0.1	86.7 ± 2.6	0.16 ± 0.07
		1	2.6 ± 0.1	0.4 ± 0.0	2.2 ± 0.1	84.6 ± 0.6	0.09 ± 0.02
Conventional induction	5	0.5	1.3 ± 0.1	0.7 ± 0.0	0.6 ± 0.1	46.2 ± 1.8	0.03 ± 0.01
Preinduction	1	0.5	0.8 ± 0.1	0.7 ± 0.2	0.1 ± 0.0	12.5 ± 1.3	0.001 ± 0.00
	5	0.5	2.8 ± 0.6	0.9 ± 0.3	1.9 ± 0.6	67.9 ± 1.8	0.04 ± 0.01
	10	0.5	4.5 ± 1.1	1.0 ± 0.5	3.5 ± 1.1	77.8 ± 2.5	0.07 ± 0.02
pColdTF-*phaCAB*$_{A-04}$							
Short induction	5	0	2.5 ± 0.1	2.3 ± 0.2	0.2 ± 0.0	8.0 ± 0.8	0.01 ± 0.00
		0.01	2.5 ± 0.2	1.1 ± 0.2	1.4 ± 0.1	56.0 ± 1.7	0.06 ± 0.02
		0.05	2.7 ± 0.2	0.9 ± 0.1	1.8 ± 0.1	66.7 ± 1.5	0.07 ± 0.03
		0.1	2.8 ± 0.1	0.8 ± 0.2	2.0 ± 0.3	71.4 ± 2.2	0.08 ± 0.08
		0.5	3.5 ± 0.1	0.7 ± 0.2	2.8 ± 0.3	80.0 ± 2.9	0.12 ± 0.07
		1	2.9 ± 0.2	0.7 ± 0.0	2.2 ± 0.2	75.9 ± 0.8	0.09 ± 0.04

Conventional induction was performed with 0.5 mM IPTG at 15°C, and cultivation was performed at 15°C for 24 h.
Short induction was performed with 0.5 mM IPTG at 15°C for 30 min, and cultivation was performed at 37°C for 24 h.
Preinduction was performed with 0.5 mM IPTG at 15°C for 24 h, and cultivation was performed at 37°C for 24 h.

of inclusion bodies of PhaC$_{A-04}$ has been observed due to the low aqueous solubility of the protein, as described previously (Harada et al., 2019). To verify that the cold-shock cspA promoter works together with the TF chaperone to improve the solubility of PhaC$_{A-04}$, the hydrophilic GST tag was fused to the N-terminus of PhaC$_{A-04}$ (pGEX-6P-1- *phaCAB*$_{A-04}$), and the effect of the GST tag at 37°C on the polymerization reaction of phaC$_{A-04}$ based on the

TABLE 3 | Quantification of purified soluble phaC$_{A-04}$ produced by *E. coli* JM109 (pColdI-*phaCAB*$_{A-04}$) and *E. coli* JM109 (pColdTF-*phaCAB*$_{A-04}$) under short induction method compared with the conventional method in shake flask cultivation.

Strains	Induction method	Initial protein loading (μg)	Total protein obtained after purification (μg)	% recovery	Total soluble his-tagged phaC$_{A-04}$	Soluble his-tagged phaC$_{A-04}$ (%)
pColdI-*phaCAB*$_{A-04}$	Short induction	2,000	1,855 ± 75	93 ± 3.8	66 ± 2.8	3.6 ± 1.4
pColdTF-*phaCAB*$_{A-04}$	Short induction	2,000	1,933 ± 28	97 ± 1.4	287 ± 37	14.8 ± 1.7
pColdI-*phaCAB*$_{A-04}$	Conventional induction	2,000	1,795 ± 53	90 ± 2.7	274 ± 36	15.3 ± 2.3
pColdTF-*phaCAB*$_{A-04}$	Conventional induction	2,000	1,880 ± 106	94 ± 5.3	890 ± 95	47.4 ± 2.4

The short induction was performed at 15°C for 30 min with 0.5 mM IPTG, and then cultivation was performed at 37°C for 48 h.
The conventional induction was performed at 15°C with 0.5 mM IPTG at 15°C, and cultivation was performed at 15°C for 48 h.
The experiments were performed as n = 3 technical replicates, and the results are expressed as the mean values ± standard errors (SEs).

TABLE 4 | Comparison of the kinetics of cell growth, $Y_{\frac{P}{S}}$ (g PHB/g-glucose), and PHB production g/(L.h) by *C. necator* strain A-04, *E. coli* JM109 (pColdI-*phaCAB*$_{A-04}$), (pColdTF-*phaCAB*$_{A-04}$), (pGEX-6P-1- *phaCAB*$_{A-04}$), (pBAD/Thio-TOPO-*phaCAB*$_{A-04}$), and (pUC19-nativeP-*phaCAB*$_{A-04}$) in shake flask cultivation.

Kinetic parameters	Promoters						
	pColdI-*phaCAB*$_{A-04}$	pColdI-*phaCAB*$_{A-04}$	pColdTF-*phaCAB*$_{A-04}$	pColdTF-*phaCAB*$_{A-04}$	pGEX-6P-1-*phaCAB*$_{A-04}$	pBAD/Thio-TOPO-*phaCAB*$_{A-04}$	pUC19-nativeP-*phaCAB*$_{A-04}$
Induction method	A	B	A	B	B	B	B
Maximum PHB concentration (g/L)	1.4 ± 0.2	2.6 ± 0.2	1.3 ± 0.1	2.5 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	0.7 ± 0.1
Maximum CDM (g/L)	1.7 ± 0.1	2.9 ± 0.2	1.7 ± 0.2	2.8 ± 0.1	1.3 ± 0.1	1.2 ± 0.2	1.1 ± 0.2
Maximum PHB content (%w/w)	82.4 ± 2.5	89.7 ± 0.8	76.5 ± 3.3	89.3 ± 4.3	69.2 ± 2.6	66.7 ± 1.8	63.6 ± 2.2
Specific growth rate (1/h)	0.001	0.001	0.001	0.001	0.003	0.003	0.004
Specific consumption rate (g glucose/g CDM/h)	1.03	0.75	0.73	1.13	0.56	0.31	0.5
Specific production rate (g PHB/g CDM/h)	0.09	0.19	0.07	0.29	0.05	0.03	0.05
$Y_{X/S}$ (g CDM/g glucose)	0.001	0.002	0.01	0.001	0.008	0.026	0.01
$Y_{P/a}$ (g PHB/g glucose)	0.07	0.18	0.08	0.18	0.08	0.10	0.06
Productivity (g/(L.h)	0.03	0.09	0.03	0.10	0.02	0.03	0.02
Time (h)	48	30	48	24	48	30	30

A: *The conventional induction was performed with 0.5 mM IPTG at 15°C, and cultivation was performed at 15°C for 48 h.*
B: *The short induction was performed with 0.5 mM IPTG at 15°C for 30 min, and then, cultivation was performed at 37°C for 48 h.*

amount of PHB production was investigated. In addition, pBAD/Thio-TOPO- *phaCAB*$_{A-04}$, encoding a hydrophilic N-terminal thioredoxin fusion protein and C-terminal 6His-fusion protein induced by arabinose, was also used for comparison (Napathorn et al., 2021). The control strain, harboring pUC19-nativeP-*phaCAB*$_{A-04}$, was under the control of the native promoter derived from *C. necator* strain A-04, and no induction agent was required under the same conditions. The amounts of PHB are shown in **Table 4**. The expressed phaC$_{A-04}$ protein was also verified, purified and quantified and shown in **Figures 3A,B** and **Table 3**. From **Table 4**, it was clearly found that pColdI-*phaCAB*$_{A-04}$ and pColdTF-*phaCAB*$_{A-04}$ yielded significantly higher amounts of PHB under the short-induction (15°C for 30 min and then 37°C) conditions than under the conventional induction (15°C) conditions (pColdI-*phaCAB*$_{A-04}$, pColdTF-*phaCAB*$_{A-04}$, pGEX-6P-1-*phaCAB*$_{A-04}$, pBAD/Thio-TOPO-*phaCAB*$_{A-04}$ and pUC19-nativeP-*phaCAB*$_{A-04}$). Next, the expression level of PhaC$_{A-04}$ protein was investigated by SDS-PAGE analysis. **Figures 3A,B** showed insoluble and soluble PhaC$_{A-04}$ expressed at 24 h of cultivation, respectively. The thioredoxin-tagged PhaC$_{A-04}$ fusion protein (lane 2 at 77 kDa) showed the highest amount of insoluble form, corresponding with a low amount of PHB production. Interestingly, pColdI-*phaCAB*$_{A-04}$ and pColdTF-*phaCAB*$_{A-04}$ produced significantly high amounts of phaC$_{A-04}$ under both the short and conventional induction methods (**Figure 3A**, lanes 4−7).

To clarify and carefully compare the amounts of soluble phaC$_{A-04}$ expressed by pColdI-*phaCAB*$_{A-04}$ and pColdTF-*phaCAB*$_{A-04}$ under short induction and conventional

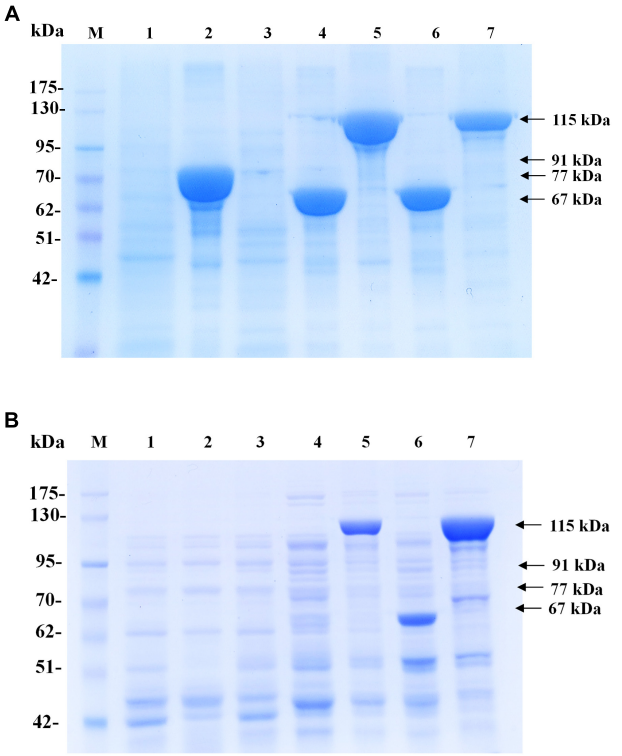

FIGURE 3 | Time courses of PHB production (g/L). **(A)** The insoluble $PhaC_{A-04}$ protein was confirmed by SDS-PAGE analysis (20 μg of total protein was loaded in each lane). **(B)** The soluble $PhaC_{A-04}$ protein was confirmed by SDS-PAGE analysis (20 μg of total protein was loaded in each lane). Lane M, Protein molecular weight marker; lane 1, *E. coli* JM109 (pUC19-nativeP-*phaCAB*$_{A-04}$) under short induction temperature profile, but without addition of IPTG; lane 2, *E. coli* JM109 pBAD/Thio-TOPO-*phaCAB*$_{A-04}$ under short induction method; lane 3, *E. coli* JM109 (pGEX-6P-1- *phaCAB*$_{A-04}$) under short induction method; lane 4, *E. coli* JM109 (pColdI-*phaCAB*$_{A-04}$) under short induction method; lane 5, *E. coli* JM109 (pColdTF-*phaCAB*$_{A-04}$) under short induction method; lane 6, *E. coli* JM109 (pColdI-*phaCAB*$_{A-04}$) under conventional induction method; lane 7, *E. coli* JM109 (pColdTF-*phaCAB*$_{A-04}$) under conventional induction method. The band appearing in the SDS-PAGE at the position corresponding to that of the $phaC_{A-04}$ protein was 64 kDa in size for pUC19-nativeP-*phaCAB*$_{A-04}$, His-tagged $phaC_{A-04}$ fusion protein was 67 kDa in size for pColdI-*phaCAB*$_{A-04}$, thioredoxin-tagged $phaC_{A-04}$ fusion protein was 77 kDa in size for pBAD/Thio-TOPO-*phaCAB*$_{A-04}$, GST-tagged $phaC_{A-04}$ fusion protein was 91 kDa in size for pGEX-6P-1-*phaCAB*$_{A-04}$, and the fusion protein of His-tagged $phaC_{A-04}$ and TF was 115 kDa in size for pColdTF-*phaCAB*$_{A-04}$.

induction, the $phaC_{A-04}$ protein was purified by IMAC affinity chromatography under native conditions. The eluted fractions were quantified by Bradford protein assay. The results are summarized in **Table 3**. The figures of SDS-PAGE and Western blot analysis were represented as **Supplementary Results**. The initial protein loading was adjusted to 2,000 μg and the maximum capacity of the IMAC column was 3,000 μg. The protein recovery was within the range of 90–97%. The conventional method induced soluble $phaC_{A-04}$ from pColdTF-*phaCAB*$_{A-04}$ at a level of as high as 47.4% of total protein and pColdTF-*phaCAB*$_{A-04}$ enhanced soluble protein formation to approximately 3.09−4.1 times higher than that from pColdI-*phaCAB*$_{A-04}$ by both conventional method and short induction method. Based on our observations, the cold-shock cspA promoter enhanced $phaC_{A-04}$ protein expression and TF promoted soluble $phaC_{A-04}$ protein (**Figure 3B** and **Table 3**). The PHB production from pGEX-6P-1-*phaCAB*$_{A-04}$, pBAD/Thio-TOPO-*phaCAB*$_{A-04}$ and pUC19-nativeP-*phaCAB*$_{A-04}$ was not different, which may be attributed to the host strain and induction method used in this study. Therefore, pColdTF-*phaCAB*$_{A-04}$ and pColdI-*phaCAB*$_{A-04}$ were chosen to validate their effectiveness of PHB production in the 5 L fermenter.

Development of Short-Induction Method and Pre-induction Method

Next, a short-induction method was investigated in this study with the aim of accelerating growth and PHB production and attaining higher productivity than that afforded by the conventional induction method. First, conditions were optimized by varying the OD_{600} based on cultivation time (0.5, 1.3, 2.1, and 2.4 h) and inducing expression with 0.5 mM IPTG at 15°C for 30 min. Then, the temperature was shifted from 15°C to 37°C for 24 h to enhance growth and PHB production. The effect of the growth phase (OD_{600}) on CDM (g/L) and PHB content (% w/w) is illustrated in **Figure 4**. Again, it was clearly observed that cells of both *E. coli* JM109 (pColdI-*phaCAB*$_{A-04}$) and *E. coli* JM109 (pColdTF-*phaCAB*$_{A-04}$) in the 2-h early exponential phase (OD_{600} of 0.5) exhibited higher CDM and PHB production than those in other growth phases. After induction with 0.5 mM IPTG at 15°C for 30 min and cultivation at 37°C for 24 h, *E. coli* JM109 (pColdI-*phaCAB*$_{A-04}$) attained 4.5 ± 0.3 g/L CDM, 3.9 ± 0.1 g/L PHB and 86.70 ± 2.6% (w/w) PHB content with a productivity of 0.16 ± 0.07 g PHB/(L.h), whereas *E. coli* JM109 (pColdTF-*phaCAB*$_{A-04}$) attained 3.5 ± 0.1 g/L CDM, 2.8 ± 0.3 g/L PHB and 80.0 ± 2.9% (w/w) PHB content with a productivity of 0.12 ± 0.07g PHB/(L.h). Thus, the short-induction method enhanced the PHB content and productivity more than the conventional method. Next, an OD_{600} of 0.5 was used to investigate the optimal concentration of IPTG (0, 0.01, 0.05, 0.1, 0.5, and 1.0 mM) under the short-induction conditions. The effects of various IPTG concentrations on CDM (g/L), PHB (g/L), PHB content (% w/w) and PHB productivity [g PHB/(L.h)], comparing *E. coli* JM109 (pColdI-*phaCAB*$_{A-04}$) and *E. coli* JM109 (pColdTF-*phaCAB*$_{A-04}$), are summarized in **Table 2**. It can be concluded that the optimal concentration of IPTG was 0.5 mM in both cases. The PHB content (% w/w) increased in accordance with the IPTG concentration, but the amount of PHB (g/L) produced was maximum under induction with 0.5 mM IPTG. The PHB content (% w/w) increased approximately 8-fold, and the productivity [g PHB/(L.h)] increased 16-fold, compared with those under the control condition in the case of pColdI-*phaCAB*$_{A-04}$.

The optimal short-induction temperature was investigated in a range between 15°C and 37°C for 30 min before increasing the temperature to 37°C for 24 h to confirm that the high PHB productivity resulting in this study is a result of the cold-shock cspA promoter and that 15°C is the optimal induction

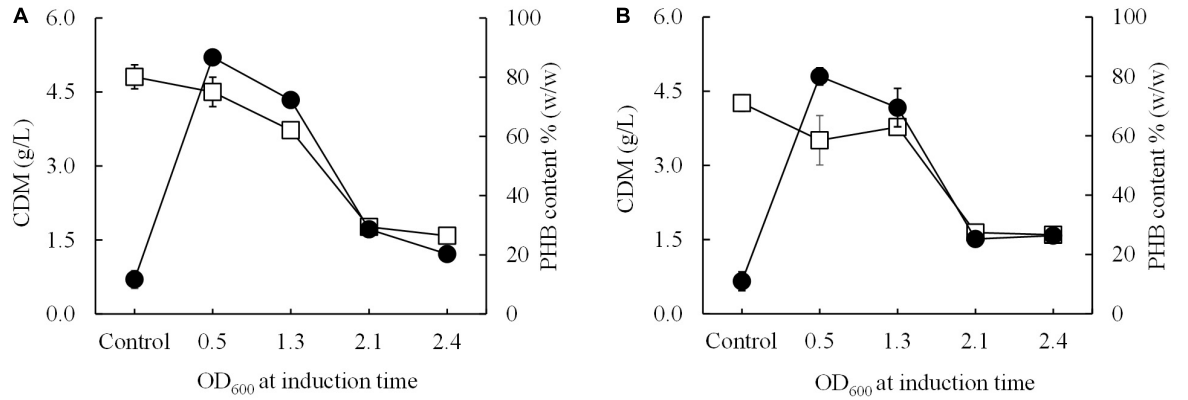

FIGURE 4 | Effect of the growth phase suitable for cold-shock induction on CDM and PHB content (% w/w) under the short-induction method. The different growth phases were investigated by varying OD_{600} based on cultivation time [0.5 (2 h, early exponential phase), 1.3 (4 h, middle exponential phase), 2.1 (6 h, late exponential phase), and 2.4 (10 h, stationary phase)] for **(A)** *E. coli* JM109 (pColdI-*phaCAB*$_{A\text{-}04}$) and **(B)** *E. coli* JM109 (pColdTF-*phaCAB*$_{A\text{-}04}$). A control experiment was performed with 0.0 mM IPTG induction. All the data are representative of the results of three independent experiments and are expressed as the mean values ± standard deviations (SDs). Symbols: open squares, CDM (g/L); closed circle, PHB (g/L).

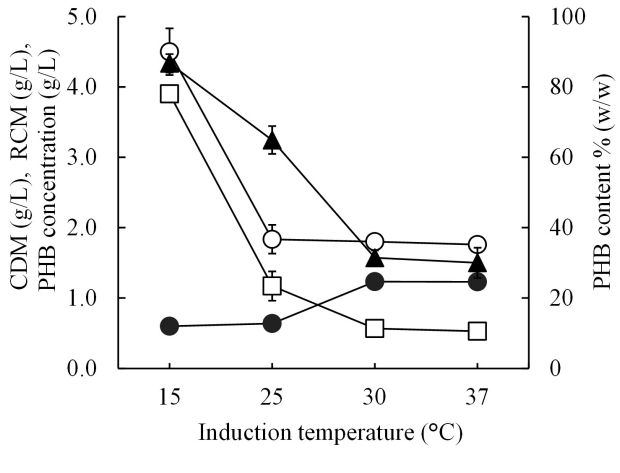

FIGURE 5 | Effect of different short-induction temperatures (15, 25, 30, and 37 °C) on the CDM (g/L), RCM (g/L), PHB (g/L), and PHB content (% w/w) of *E. coli* JM109 (pColdI-*phaCAB*$_{A\text{-}04}$). All the data are representative of the results of three independent experiments and are expressed as the mean values ± standard deviations (SDs). Symbols: open circle, CDM (g/L); closed circle, RCM (g/L); open square, PHB (g/L); closed triangle, PHB content (% w/w).

temperature. **Figure 5** shows the results of the effect of the short-induction temperature (15, 25, 30, and 37°C) on cell growth and PHB production. It was clear that 15°C was the optimal induction temperature for enhancing the amount of PHB produced, which resulted in a maximum PHB content of 86.7 ± 2.6% (w/w). The amount of PHB produced decreased as the induction temperature increased. The PHB productivity at 15°C was sevenfold higher than that obtained with an induction temperature of 37°C.

We also investigated a preinduction strategy to enhance PHB productivity by extending the PHB production phase at 37°C for an additional 24 h after conventional induction. When the OD_{600} reached 0.5, IPTG was added at 0.5 mM into the culture, and the temperature was reduced from 37 to 15°C. Then, cultivation was performed for 24 h to allow full expression of the phaCAB$_{A\text{-}04}$ protein. Concurrently, the effect of inoculum size [1, 5, and 10% (v/v)] of induced cells was investigated under the preinduction conditions. The results are shown in comparison with those of the conventional induction and short-induction methods (**Table 2**). The preinduction method with a 5% (v/v) inoculum gave a higher amount of PHB (1.9 ± 0.6 g/L) than conventional induction with an inoculum size of 5% (v/v) (0.6 ± 0.1 g/L) and could extend the productivity of 0.039 ± 0.01 g PHB/(L·h) for 48 h so that the PHB content increased from 46.2 ± 1.8% (w/w) to 67.9 ± 1.8% (w/w). The increase in PHB content and PHB productivity occurred with an increase in the inoculum size. Nevertheless, the short-induction method with an inoculum size of 5% (v/v) gave the highest levels of PHB content and productivity. Therefore, the short-induction method using *E. coli* JM109 (pColdI-*phaCAB*$_{A\text{-}04}$) with an inoculum size of 0.5% (v/v) and cultivated until the OD_{600} reached 0.5 (2 h) before induction with 0.5 mM IPTG was selected to investigate the effect of induction temperature in the subsequent experiment.

Comparison of PHB Production Between pColdI-*phaCAB*$_{A\text{-}04}$ and pColdTF-*phaCAB*$_{A\text{-}04}$ in a 5-L Fermentor by the Short Induction Method

Altogether, for flask cultivation, the optimal conditions were the short-induction method using an inoculum of 0.5% (v/v) in a culture with an OD_{600} of 0.5, cold shock induced with 15°C for a short time, 30 min, and the addition of 0.5 mM IPTG. These conditions were selected as optimal parameters for scaling up production in a 5-L fermenter. The comparison between pColdI-*phaCAB*$_{A\text{-}04}$ and pColdTF-*phaCAB*$_{A\text{-}04}$ in a 5-L fermenter by the short-induction method was performed because the ratio of the soluble fraction and inclusion bodies of the phaC$_{A\text{-}04}$ protein

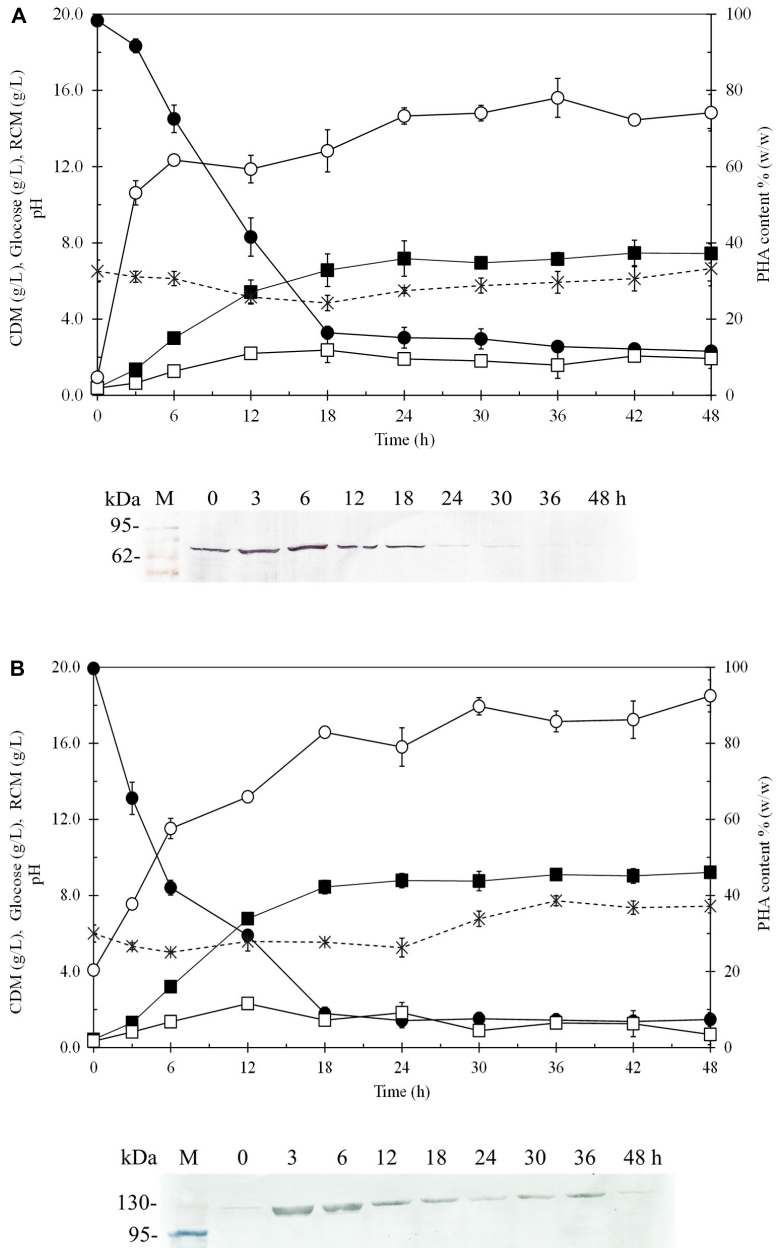

FIGURE 6 | Time courses of CDM (g/L), RCM (g/L), PHB (g/L), PHB content (% w/w), and glucose (g/L) and pH during batch cultivation in a 5-L fermenter under the short-induction method in a comparison between **(A)** *E. coli* JM109 (pColdI-*phaCAB*$_{A\text{-}04}$) and **(B)** *E. coli* JM109 (pColdTF-*phaCAB*$_{A\text{-}04}$). The band appearing in the western blot at the position corresponding to that of the His-tagged phaC$_{A\text{-}04}$ protein was 67 kDa in size for pColdI-*phaCAB*$_{A\text{-}04}$, and the fusion protein of His-tagged phaC$_{A\text{-}04}$ and TF was 115 kDa in size. All the data are representative of the results of three independent experiments and are expressed as the mean values ± standard deviations (SDs). Symbols: closed square, CDM (g/L); closed circle, glucose (g/L); asterisks, pH; open square, RCM (g/L); open circle, PHB content (% w/w).

may affect PHB productivity and molecular weight distribution as reported by Harada et al. (2019).

Figure 6 shows the time courses of CDM (g/L), RCM (g/L), PHB (g/L), PHB content (% w/w), glucose (g/L), dissolved oxygen (%) and pH during batch cultivation in a 5-L fermenter, comparing *E. coli* JM109 (pColdI-*phaCAB*$_{A\text{-}04}$) (**Figure 6A**) and *E. coli* JM109 (pColdTF-*phaCAB*$_{A\text{-}04}$) (**Figure 6B**). The soluble PhaC$_{A\text{-}04}$ protein detected by western blot analysis was also monitored at 6-h intervals over 48 h. The results shown in **Table 5** demonstrated that *E. coli* JM109 (pColdTF-*phaCAB*$_{A\text{-}04}$) was a more effective PHB producer than the other strain. A PHB content of 89.8 ± 2.3% (w/w), PHB production of 7.9 ± 0.7 g/L, CDM production of 8.8 ± 0.5 g/L, $Y_{P/S}$ value of 0.38 g PHB/g glucose and productivity of

FIGURE 7 | Morphology of PHB films produced by **(A)** *C. necator* strain A-04, **(B)** *E. coli* JM109 (pColdI-*phaCAB*$_{A-04}$), and **(C)** *E. coli* JM109 (pColdTF-*phaCAB*$_{A-04}$).

0.43 g PHB/(L.h) were the maximum values obtained using pColdTF-*phaCAB*$_{A-04}$, whereas a PHB content of 80.6 ± 2.1% (w/w), PHB production of 5.8 ± 0.1 g/L, CDM production of 7.2 ± 0.3 g/L, $Y_{P/S}$ value of 0.32 g PHB/g glucose and productivity of 0.24 g PHB/(L.h) were attained using pColdI-*phaCAB*$_{A-04}$. The phaC$_{A-04}$ protein produced by pColdTF-*phaCAB*$_{A-04}$ was more stable and longer lasting (**Figure 6B**) than that obtained from pColdI-*phaCAB*$_{A-04}$, which was no longer detectable after 30 h of cultivation (**Figure 6A**). Therefore, we report here that the short-induction strategy facilitates cold shock cspA and chaperone TF proteins to act synergistically to improve the stabilization of PhaC$_{A-04}$ and enhance productivity and the $Y_{P/S}$ value in comparison with the use of cspA alone in pColdI.

The PHB thin films were subjected to thermal analysis by DSC, molecular weight determination by GPC and mechanical property analysis by a universal testing machine as per the ASTM: D882-91 protocol (**Table 5**). The PHB from *E. coli* JM109 (pColdI-*phaCAB*$_{A-04}$) had an M_w of 8.17 × 10^5 Da, an M_n of 1.97 × 10^5 Da and a PDI of 4.1, whereas the PHB from E. coli JM109 (pColdTF-*phaCAB*$_{A-04}$) had an M_w of 2.6 × 10^5 Da, an M_n of 0.95 × 10^5 Da and a PDI 2.8, when glucose was used as a carbon source. However, the PHB from *E. coli* JM109 (pColdI-*phaCAB*$_{A-04}$) obtained using crude glycerol had the lowest M_w of 2.42 × 10^5 Da, an M_n of 0.89 × 10^5 Da and a PDI of 2.92. Interestingly, the PHB from *E. coli* JM109 (pUC19-nativeP-*phaCAB*$_{A-04}$) obtained using crude glycerol showed the highest M_w of 1.1 × 10^6 Da, an M_n of 2.6 × 10^5 Da and a PDI of 4.1. The melting temperature, T_m, of all the PHB film samples produced in this study was in the range of 165−178°C (Owen et al., 1992), and the glass transition temperature, T_g, was in the normal range of 1−4°C (Shimamura et al., 1994; Doi et al., 1995; Chanprateep et al., 2010). The Young's modulus and tensile strength of the PHB from *E. coli* JM109 (pColdTF-*phaCAB*$_{A-04}$) possessed the highest values of 5465 and 56.2 MPa, respectively. **Figure 7** shows the morphology and transparency of PHB films produced by *C. necator* strain A-04, *E. coli* JM109 (pColdI-*phaCAB*$_{A-04}$) and *E. coli* JM109 (pColdTF-*phaCAB*$_{A-04}$). The PHB films prepared by the film casting technique and produced from *E. coli* JM109 (pColdTF-*phaCAB*$_{A-04}$) showed a soft morphology with high transparency, which was different from the properties of the other PHB films. The PHB films were also subjected to ^{1}H-NMR and ^{13}C-NMR analyses and showed only chemical shifts of the PHB structure.

DISCUSSION

It has been reported that PhaC$_{H16}$, as a type I synthase and the most intensively studied of these proteins, preferentially catalyzes the polymerization of short-chain (R)-hydroxyalkanoic acids (4 to 6 carbon atoms), particularly the conversion of (R)-3-hydroxybutyrate-coenzyme A (3HBCoA) to poly(3-hydroxybutyrate) (PHB) (Schubert et al., 1988; Gerngross et al., 1994). In fact, a high concentration of PHB (157 g/L) has been achieved from glucose in high-cell-density cultures of recombinant *E. coli* harboring *phaCAB*$_{H16}$ and the additional cell division protein ftsZ gene (Wang and Lee, 1997; Choi and Lee, 2004). Ultrahigh-molecular-weight PHB and its applications have also been reported by many research groups (Kusaka et al., 1998; Iwata, 2005; Hiroe et al., 2012; Kabe et al., 2012). Beyond these previous reports, there have been few reports on the application of cold-shock systems for PHB production to address the challenges of soluble phaC expression in *E. coli*.

TABLE 5 | Comparison of kinetic parameters, molecular weight, and thermal and mechanical properties of PHB produced by *C. necator* strain A-04, *E. coli* JM109 (pColdI-*phaCAB*$_{A-04}$), *E. coli* JM109 (pColdTF-*phaCAB*$_{A-04}$), and *E. coli* JM109 (pUC19-nativeP-*phaCAB*$_{A-04}$) in 5-L fermenter.

Kinetic parameters and polymer properties of PHB	*C. necator* A-04		pColdTF-*phaCAB*$_{A-04}$	pColdI-*phaCAB*$_{A-04}$		pUC19-nativeP-*phaCAB*$_{A-04}$
	Fructose	Raw sugar	Glucose	Glucose	Crude glycerol	Crude glycerol
Carbon source (g/L)	20	30	20	20	20	20
Maximum PHB concentration (g/L)	5.8 ± 0.5	4.7 ± 0.2	7.9 ± 0.7	5.8 ± 0.1	2.0 ± 0.1	2.1 ± 0.1
Maximum cell dry weight (g/L)	7.4 ± 1.5	7.3 ± 1.2	8.8 ± 0.5	7.2 ± 0.3	4.0 ± 0.2	3.9 ± 0.3
Maximum PHB content (%wt)	78.4 ± 1.9	64.4 ± 2.8	89.8 ± 2.3	80.6 ± 2.1	50.0 ± 3.0	53.8 ± 2.2
Specific growth rate (1/h)	0.003	0.001	0.07	0.06	0.08	0.11
Specific consumption rate (g carbon source/g CDM/h)	0.14	0.05	0.52	0.35	0.20	0.19
Specific production rate (g PHB/g CDM/h)	0.012	0.019	0.20	0.11	0.02	0.016
$Y_{X/S}$ (g CDM/g-carbon source)	0.08	0.03	0.07	0.10	0.19	0.16
$Y_{P/S}$ (g PHB/g carbon source)	0.29	0.35	0.38	0.32	0.13	0.09
Productivity [g/(L.h)]	0.10	0.07	0.43	0.24	0.11	0.07
M_w ($\times 10^5$)	6.51	3.30	5.79	8.41	2.42	10.68
M_n ($\times 10^5$)	3.61	1.46	1.86	2.03	0.89	2.60
PDI	1.80	2.15	3.11	4.14	2.92	4.10
Young's modulus (MPa)	1497	1734	5465	2156	1980	2262
Tensile strength (MPa)	17.4	11.9	56.2	21.5	19.3	17.4
Elongation at break (%)	0.4	1.1	1.2	1.7	2.0	1.1
T_m (°C)	178	173	168	176	170	174
T_g (°C)	2.4	3.5	1.6	3.0	1.9	2.8
Time (h)	60	60	18	30	18	30

From our previous reports, *C. necator* strain A-04 exhibits 99.78% similarity of 16S rRNA, 99.9% similarity of *phaC*$_{A-04}$ and 100% similarity of *phaA*$_{A-04}$ and *phaB*$_{A-04}$ with those of *C. necator* H16. However, we observed differences in PHB productivity as well as the monomeric composition of the copolymers and terpolymers when we used the same carbon source (Chanprateep and Kulpreecha, 2006; Chanprateep et al., 2008, 2010). Interestingly, *C. necator* strain A-04 also exhibited growth abilities on pure glycerol as well as crude glycerol from a biodiesel plant in Thailand as compared with *C. necator* strain H16 (unpublished observations from personal communication with Tuck Seng Wong, University of Sheffield, United Kingdom). Thus, *C. necator* strain A-04 can be considered as one of the promising candidates for PHA production. However, the PHA productivity of *C. necator* strain A-04 still does not meet the requirement for industrial production. Thus, our objective was to investigate the ability of the phaCAB$_{A-04}$ gene operon when heterologously expressed in recombinant *E. coli*. We initially aimed to use the pColdI and pColdTF expression systems to address the challenges of soluble and functional phaC$_{A-04}$ expression in *E. coli* JM109 from glucose as a carbon source for PHB production in a 5-L fermenter and evaluate its ability to use crude glycerol to attain low cost PHB production.

First, this study aimed to increase soluble PhaC$_{A-04}$ expression in *E. coli* JM109 by using the cold-shock cspA promoter and TF. His-tagged phaC$_{A-04}$ was overexpressed by pColdI, but most of the protein was present in insoluble form, with significant aggregation resulting in smear bands (**Figures 2A**, **5** and **Table 3**), whereas the His-tagged phaC$_{A-04}$-TF fusion protein was expressed from pColdTF at lower levels than the protein from pColdI, but most of this protein was present in soluble form.

To examine the optimal induction temperature, the cold shock temperature was varied at 15, 25, 30, and 37°C for 30 min and then the cultivation temperature was shifted to 37°C for 24 h. It is noted that the optimal growth temperature of *E. coli* is 37°C where the optimal growth temperature for *C. necator* strain A-04 is 30°C. Our results demonstrated that the PHB content decreased when the temperature increased although the optimal growth temperature for *C. necator* strain A-04 was 30°C. The appropriate temperature for enzymatic activity of phaC$_{A-04}$ activity can be considered between 30 to 37°C similar to Sheu et al. (2012) who reported the effects of temperature on the activity of PHA synthases and who revealed that mesophilic (PhaC$_{H16}$) PHA synthases were differentiated distinctly based on the highest level of PHB accumulation at different temperatures. They demonstrated that the parental enzymes PhaC$_{H16}$ had optimal temperature of 37°C (Sheu et al., 2012). Thus, our strategy (short induction) for PHB production using a cold-shock promoter in *E. coli* is lowering the temperature at 15°C for 30 min and then shifting the temperature to 37°C which is the optimal temperature for phaC activity.

We also performed parallel experiments using different hydrophilic tags, including expression via the native promoter

of *C. necator* strain A-04, N-terminal GST-fused *phaCAB*$_{A-04}$, and N-terminal thioredoxin-fused and C-terminal 6His-fused *phaCAB*$_{A-04}$, to confirm that the high efficiency of PHB production was contributed by the cold-shock cspA promoter. It was found that the GST-PhaCAB$_{A-04}$, Thioredoxin-PhaCAB$_{A-04}$, 6His-phaCAB$_{A-04}$ and nativeP-pha*CAB*$_{A-04}$ gave similar values for PHB production and PHB content, which were 2.5 times lower than the values obtained with pCold and pColdTF (**Figure 3A** and **Table 4**). In our study, the GST-PhaC$_{A-04}$ did not improve PHB production and exhibited lower PHB productivity than the control pUC19-nativeP-*phaCAB*$_{A-04}$, same as previously mentioned by Harada et al. (2019). The *araBAD* promoter showed the highest level of PhaC$_{A-04}$ protein production, but most of them were in insoluble form. However, this study was performed using the short induction strategies that could limit the expression of PhaCAB$_{A-04}$ under different promoters.

In addition, the ratio of soluble fraction to the total phaC$_{A-04}$ proteins from pColdTF-*phaCAB*$_{A-04}$ and pColdI-*phaCAB*$_{A-04}$ under conventional induction and short induction method was carefully analyzed by IMAC affinity chromatography under native conditions protein (**Table 3**). The ratio of soluble fraction to the total phaC$_{A-04}$ proteins from pColdTF-*phaCAB*$_{A-04}$ was about 3−4 times higher than that from pColdI-*phaCAB*$_{A-04}$ both under short induction and conventional induction method. Thus, it can be concluded that the TF chaperone helped solubilize phaC$_{A-04}$ in our investigation. Finally, the production of PHB of pColdI-*phaCAB*$_{A-04}$ and pColdTF-*phaCAB*$_{A-04}$ was compared in the 5-L fermenter using the short induction method and yielded $Y_{P/S}$ of 0.38 g-PHB/g-glucose whereas the theoretical yield $Y_{P/S}$ was reported as 0.48 of *g*-PHB/*g*-glucose (Yamane, 1993). This phenomenon can be explained that the growth of *E. coli* on glucose causes acidic by-products formation, mainly acetate, under both aerobic and anaerobic conditions (Lee et al., 1994). In this study, it was observed that the pH decreased from 7.0 (initial pH) to 4.9–5.0 at 18 h as acetate accumulated and then increased back to 6.6–7.4 (**Figure 6**) as acetate was consumed (Chen et al., 2018). Therefore, the $Y_{P/S}$ obtained in these experiments was lower than the theoretical yield. In this study, the pH and dissolved oxygen were not controlled because Lee et al. reported that controlled pH and/or dissolved oxygen of the recombinant *E. coli* strains resulted in higher RCM with low PHB content of less than 40% due to better growth condition, where much acetyl-CoA can flow into the tricarboxylic acid (TCA) cycle (Lee et al., 1994). Acetyl-CoA is a fundamental metabolite in central metabolic pathways of *E. coli*, and also served as a precursor for biosynthesis of a large number of industrial chemicals and natural products including PHB (Martin et al., 2003; Meng et al., 2012; Liu et al., 2016; Sun et al., 2020). The main metabolic route for acetyl-CoA synthesis in *E. coli* is the glycolysis pathway coupled with decarboxylation of pyruvate by pyruvate dehydrogenase (Bates et al., 1977). Through this pathway, each mol of glucose is converted into 2 mol of acetyl-CoA with generation of 4 mol of NADH, 2 mol of ATP and 2 mol of CO_2. The release of CO_2 lowers the atomic economy of targeted chemical biosynthetic pathway, leading to the decrease of theoretical production yield, titer and productivity (Chae et al., 2017). In addition, glucose is catabolized through glycolysis pathway to pyruvate, which is converted into acetyl-CoA under catalysis of pyruvate dehydrogenase, with reduction of NAD+ to NADH in both glycolysis pathway and pyruvate dehydrogenation. As a result, NADH could not be oxidized through respiratory chain sufficiently, the build-up of NADH rapidly inactivated the pyruvate dehydrogenase (Hansen and Henning, 1966), leading to accumulation of pyruvate in cells, and the recycling of NAD+ must be achieved through the reduction of some metabolites (Bunch et al., 1997). Therefore, acetate is accumulated in medium in large quantity. Acetate overflow is caused by an imbalance between the pathways of glycolysis and TCA cycle in rapidly growing cells, and severely decreases the yield of target chemicals from glucose (Farmer and Liao, 1997; Wong et al., 2008). It has been known that *E. coli* cells grown on glucose produce acetate and consume acetate after glucose exhaustion, but do not grow on acetate due to the decoupling of acetate anabolism and acetate catabolism, and the growth restores only after prolonged exposure to acetate (Sun et al., 2020). With the same glucose concentration, Lee et al. (1994) performed a comparative study of recombinant *E. coli* strains (K12, B, W, XL1-Blue, JM109, DH5α, and HB101) for PHB production from glucose. They reported that $Y_{P/S}$ of *E. coli* strain XL1-Blue (pSYL105) grown in LB medium containing 20 g/L glucose was as high as 0.369 g PHB/g glucose whereas JM109 provided 0.299 g PHB/g glucose. Thus, the $Y_{P/S}$ obtained in this study was comparable. In the fed-batch cultivation studies, Wang and Lee (1997) performed a fed-batch culture of *E. coli* XL1-Blue (pSYL107) in a 50-l fermentor and attained $Y_{P/S}$ of 0.28 g of PHB/g of glucose (3,849 g of PHB produced from 13,900 g of glucose). They mentioned that a slightly lower PHB yield on glucose in a defined medium than in LB medium (0.37 g of PHB/g of glucose) because glucose was converted to CO_2 in a defined medium more than in LB medium (Wang and Lee, 1997). Next, they applied the feeding solution contained 700 g/L glucose, 20 $MgSO_4 \cdot 7H_2O$, and 250 mg/L of thiamine in fed-batch cultivation. As a result, cell concentration of 149 g/L and PHB concentration of 104 g/L (PHB content 69.5%) were obtained in 51 h, resulting in the PHB productivity of 2.0 g/L/h. This is the highest PHB concentration obtained by employing recombinant *E. coli* containing the *phaCAB*$_{H16}$ biosynthesis genes and the *E. coli* ftsZ gene. In our previous report, we performed fed-batch cultivations by pH-stat control in a 5-L fermenter using *E. coli* strain XL1-Blue harboring pColdTF-*phaCAB*$_{A-04}$, leading to a PHB production of 31.4 ± 0.9 g/L at 54 h with a PHB content of 83.0 ± 3.8% (w/w), a CDM of 37.8 ± 1.2 g/L, a $Y_{P/S}$ value of 0.39 g PHB/g glucose and a productivity of 0.6 g PHB/(L·h) in define medium. To investigate the possibility of improving the PHB yield and productivity, we are now carrying out fed-batch cultures under various conditions including oxygen limitation during PHB synthesis phase as it may be possible to enhance the PHB synthesis rate by increasing the acetyl-CoA flux into the PHB biosynthetic pathway and reducing its flux into the tricarboxylic acid cycle (Wang and Lee, 1997).

The produced PHB were characterized and it was found that the M_w of PHB produced from pColdTF-*phaCAB*$_{A-04}$, for which soluble phaC$_{A-04}$ was 4.1 times higher than pColdI-*phaCAB*$_{A-04}$ was lower than that from pColdI-*phaCAB*$_{A-04}$.

Hiroe et al. (2012) have reported that that the concentration of active PHA synthase had a negative correlation with PHB molecular weight and a positive correlation with cellular PHB content, similar to our observation. The M_w and M_n of PHB produced by pColdTF were lower than those of pColdI-*phaCAB*$_{A\text{-}04}$ and pUC19-nativeP-pha*CAB*$_{A\text{-}04}$. In this case, TF increases PhaC production and its Mw decreases due to the presence of more active PhaC. To achieve low-cost production, crude glycerol containing 80% glycerol provided by Bangchak Corporation Public Company Limited as a byproduct from biodiesel production, was used as a carbon source to produce PHB using pColdI-*phaCAB*$_{A\text{-}04}$ and pUC19-nativeP-pha*CAB*$_{A\text{-}04}$. With *E. coli* JM109 (pColdI-*phaCAB*$_{A\text{-}04}$), PHB produced from crude glycerol had an M_w of 2.42×10^5 Da and an M_n of 0.89×10^5 Da with a PDI of 2.92, whereas those results from glucose were M_w of 8.41×10^5 Da and an M_n of 2.03×10^5 Da with a PDI of 4.14. The crude glycerol caused the termination step involves a chain transfer (CT) reaction in which the polymer chain is transferred to the crude glycerol (CT agent) in this case (Madden et al., 1999). However, in our study, when pUC19-nativeP-*phaCAB*$_{A\text{-}04}$-expressing *E. coli* was used to produce PHB from crude glycerol, an M_w of 1.1×10^6 Da, an M_n of 2.6×10^5 Da and a PDI of 4.1 were obtained, indicating that slow and low phaC$_{A\text{-}04}$ expression prolonged and maintained the phaC$_{A\text{-}04}$ polymerization activity, which in turn resulted in a low amount of PHB with a high molecular weight (Hiroe et al., 2012).

CONCLUSION

This study revealed that the cspA promoter in a cold-inducible vector can enhance total PhaC$_{A\text{-}04}$ expression and TF chaperones showed obvious effects on enhancing PhaC solubility. The short induction strategies developed in this study did not affect on molecular weight distribution and polymer properties. Cultivation in a 5-L fermenter led to PHB production of 7.9 ± 0.7 g/L with 89.8 ± 2.3% PHB content in the cell dry mass (CDM), a $Y_{P/S}$ value of 0.38 g PHB/g glucose and a productivity of 0.43 g PHB/(L·h) using pColdTF-*phaCAB*$_{A\text{-}04}$. The PHB from pColdTF-*phaCAB*$_{A\text{-}04}$ had Mw 5.79×10^5 Da, Mn 1.86×10^5 Da and PDI 3.11 and the film exhibited high optical transparency with typical melting temperature and mechanical properties.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, FJ897463; https://www.ncbi.nlm.nih.gov/genbank/, FJ897461; and https://www.ncbi.nlm.nih.gov/genbank/, FJ897462.

AUTHOR CONTRIBUTIONS

TB performed the experiments and discussed the results. RW-S provided guidance for the experimental design and discussed the results. KH provided suggestions for the experimental design and discussed the results. SCN provided guidance and suggestions for the experimental design, discussed the results, and wrote and revised the manuscript. All the authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2021.666036/full#supplementary-material

Supplementary Figure 1 | Construction of PHA biosynthesis genes in cold-shock inducible expression vectors **(A)** pColdI-*phaCAB*$_{A\text{-}04}$ and **(B)** pColdTF-*phaCAB*$_{A\text{-}04}$.

Supplementary Figure 2 | Effect of IPTG concentrations (0.01, 0.05, 0.1, 0.5, and 1.0 mM) on the expression of His-tagged phaC$_{A\text{-}04}$ protein **(A)** (pColdI-*phaCAB*$_{A\text{-}04}$) and **(B)** the fusion protein of His-tagged phaC$_{A\text{-}04}$ and TF (pColdTF-*phaCAB*$_{A\text{-}04}$) under conventional induction method. The band appearing in the SDS PAGE at the position corresponding to that of the His-tagged phaC$_{A\text{-}04}$ protein was 67 kDa in size and the fusion protein of His-tagged phaC$_{A\text{-}04}$ and TF was 115 kDa in size.

Supplementary Figure 3 | Purification of His-tagged PhaC$_{A\text{-}04}$ protein of *E. coli* JM109 (pColdI-*phaCAB*$_{A\text{-}04}$) at 15°C **(A)** and the fusion protein of His-tagged PhaC$_{A\text{-}04}$ protein and TF of *E. coli* JM109 (pColdTF-*phaCAB*$_{A\text{-}04}$) at 15°C by IMAC affinity chromatography under native condition **(B)**. The extracted protein was normalized to 2 mg and loaded on Protino® Ni-IDA 1000 packed column. Ten microliters of each fraction eluted from IMAC column were loaded onto 10% w/v acrylamide gel for SDS-PAGE and Western blot analysis. M, Protein molecular weight marker; Ly, Bacterial lysate, soluble proteins; FI, Flow-though lysate; W1 and W2, Wash with 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole and pH 8.0; E1–E3, Eluted with 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole and pH 8.0. His-tagged phaC$_{A\text{-}04}$ fusion protein was 67 kDa in size for pColdI-*phaCAB*$_{A\text{-}04}$ and the fusion protein of His-tagged phaC$_{A\text{-}04}$ and TF was 115 kDa in size for pColdTF-*phaCAB*$_{A\text{-}04}$. The soluble fractions were quantified by Bradford protein assay. All IMAC purifications were performed as $n = 3$ technical replicates.

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Efficient Production of Polyhydroxyalkanoate Through Halophilic Bacteria Utilizing Algal Biodiesel Waste Residue

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The objective of the current work was to investigate the potential of halophilic bacterial isolates for efficient utilization of crude glycerol from algal biodiesel waste into polyhydroxyalkanoates (PHAs) a green plastic. Screening of the isolates was directly done in algal biodiesel waste residue containing solid agar plates supplemented with Nile red. Crude glycerol is a biodiesel waste whose bioconversion into value-added products provides an alternative for efficient management with dual benefit. For the scale-up studies of PHAs, *Halomonas spp.* especially *H. daqingensis* was observed as a potential candidate growing well in 3% Algal biodiesel waste residue (ABWR), 5% NaCl supplementation at 35°C within 48 h of incubation. Maximum Cell dry weight (CDW) of 0.362 ± 0.001 g and 0.236 ± 0.003 g PHA was obtained with *H. daqingensis* when grown in the fermentor with 0.5 vvm air flow rate and 200 rpm containing 3% ABWR supplemented with 5% NaCl at 35°C incubation temperature for 48 h. ABWR can serve as a sole substrate for PHA production at an industrial scale serving two approaches: getting rid of the biodiesel industrial waste containing high amount of glycerol besides using waste replacing commercial substrate thereby reducing the cost of the product.

Keywords: algal biodiesel waste residue, *Halomonas daqingensis, Halomonas ventosae,* Polyhydroxyalkanoates, optimization, characterization

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INTRODUCTION

Petroleum-based plastics are essential for widespread applicability in routine life. The major drawback of these synthetic plastics is its non-biodegradability and its toxic nature during incineration with the release of harmful gases into the environment. This has led to serious damage to the environment. In order to avoid these problems, the prerequisite is to produce biodegradable plastics and look for renewable resources as nutrient feed for its commercialization. Polyhydroxyalkanoates (PHAs) have gained interest amongst researchers due to its characteristic properties which are appropriate for its wide applicability.

Bioplastic production may increase from 2 Mt/y in 2017 to 2.4 Mt/y in 2022 as per the reports (Bioplastics Market Data, 2017 2017-2022). The present scenario affecting the cost of PHAs in the world market is the use of expensive substrates and downstream processing methodology (Kumar and Kim, 2018). Several substrates are reported in the literature which are exploited for PHA production *viz.* sugars, fatty acids, vinasses-molasses mixture, waste sludge, waste water, etc.

(Frison et al., 2015; Huang et al., 2016; Koller et al., 2017; Liao et al., 2018; García et al., 2019). Amongst the cheap substrates explored so far, crude glycerol is the potential feedstock for PHA production also enhancing the sustainability of biodiesel industry (Shrivastav et al., 2010; Ghosh et al., 2015a,b). Biodiesel production may reach 41 Mm^3 by 2022 (Monteiro et al., 2018). The major byproduct of biodiesel industry is glycerol which needs to be dealt with for waste management systems. It is thus of great importance that crude glycerol is used as feed material for the production of polyhydroxyalkanoates exploiting wild strain of halophilic bacteria. Value added utilization of crude glycerol obtained during biodiesel production is essential as it is important for various industrial processes and products. Glycerol is therefore exploited as carbon substrate for the production of PHAs by several researchers (Freches and Lemos, 2017; Ntaikou et al., 2018; Kumar and Kim, 2018). The ever increasing price for synthetic nutrient media has led us use crude substrates e.g., glycerol generated as a waste stream during biodiesel production (Shrivastav et al., 2010; Bera et al., 2015). This could make the production of PHA from renewable resources more competitive with common plastics with almost zero effluent for biodiesel industries thus, lowering down the overall processing cost. Many fermentable and non-fermentable ingredients present in the crude glycerol generated through various methods could hinder the overall bio-process (Bhattacharya et al., 2016; Gahlawat and Soni, 2017; Fauzi et al., 2019). Thus, a suitable strain with proper growth optimized conditions utilizing the biodiesel co-product for fermentation is essential. Several researchers have worked upon reducing the input of synthetic substrates for PHA productivity. In few studies, they have investigated the glycerol as a suitable substrate for bacterial growth (Garlapati et al., 2016). Crude glycerol contains mainly fatty acids, methanol and salts which if accumulated in higher concentration during the growth of bacteria may hinder the fermentation process Rahman et al. (2015) (Bhattacharya et al., 2016) utilized wastewater microalgae-based media for the production of PHA from recombinant *E.coli*. Many halophilic bacteria are reported to accumulate PHAs (Kucera et al., 2018; Kumar and Kim, 2018; Hong et al., 2019). Halophilic bacteria have gradually emerged as an interesting candidate of research due to its ease of cultivation with less chances of contamination owing to its extremophilic nature. Several methodologies were adapted by researchers for the exploitation of halophilic bacteria for PHA production (Cervantes-Uc et al., 2014).

Extensive use of synthetic plastics as packaging materials has found applicability in several areas. Their cheap, light weight and material properties are appealing behind their widespread demand. The problem in disposing these plastics raises harsh environmental issues. Biodegradable plastics like polyhydroxyalkanoates (PHAs) with similar material properties and affordability would suitably replace such hazardous synthetic plastics. Biodegradation of PHA was reported by Madbouly et al. (2014) to be increased with the addition of distiller's dried grains with solubles. Torri et al. (2017) reported the conversion of PHA-containing sludge into alkenes and CO_2 through hydrothermal treatment. Scaling up the PHA production requires lowering down the production cost (Choi and Lee, 1999). Factors affecting the cost of the product are the fermentation substrate required, fermentation time, raw materials, type of bacteria downstream processing, efficient recovery process, purity, etc. with thermal and mechanical properties of polymer (Rodriguez-Perez et al., 2018). On the basis of its physicochemical and mechanical properties its applications could be decided. Few researchers explored the use of sugars from crops as bacterial feed, but it is not feasible during scaling up of the process as these crops are also consumed by humans as primary food. One of the important factors affecting the process is exploring potential wild bacterial strain capable of growing in open saline system. This reduces the chance of contamination and also the harvesting time which in turn saves energy during scale up of process.

Herein, we evaluated the growth and polymer productivity in halophilic bacteria. Taking into consideration the bottleneck concerning the commercial media utilization for PHA production, algal biodiesel waste residue (ABWR) was used as the sole nutrient source. To the best of our knowledge this is the first report on PHB production by *Halomonas ventosae* and *Halomonas daqingensis* isolated from the Experimental Salt Farm (ESF) (21°47′26.4″N 72°07′19.2″E) utilizing algal biodiesel waste residue as the sole production media without addition of any external nutrient sources.

MATERIALS AND METHODS

Direct Screening

Direct screening of PHA accumulating halophilic bacteria on Nile red dye agar plates helped us avoid extensive use of costly commercial media for screening purpose. To check the capability of bacteria to utilize glycerol as the carbon source for PHA production, the samples (0.1 mL) were spread on the minimal salt medium plates with varying NaCl concentration ranging from 3-25%. Samples collected were salt, soil and water samples from different salt pans (with varying salinity) Kumbharwada, Bhavnagar, Gujarat (21°47′26.4″N 72°07′19.2″E). The minimal salt medium (MSM) consists of Ammonium sulfate, 0.5 g/l; magnesium sulfate, 0.4 g/l; disodium hydrogen phosphate, 9.65 g/l; potassium dihydrogen phosphate, 2.65. Micronutrient solution composition: ferrous sulfate, 2.78 g/l; manganese chloride, 1.98 g/l; cobalt sulfate, 2.81 g/l; copper chloride, 0.17 g/l; zinc sulfate, 0.29 g/l containing synthetic glycerol and supplemented with a solution of 0.25 mg Nile red in DMSO. The plates were incubated for 48–120 h at 30°C to check the bacterial growth which was further checked for fluorescence under UV light in a Gel doc (Biorad, United States) **(Supplementary Figure 2)**. These PHA positive isolates were picked up and restreaked onto respective ZMA plates supplemented with NaCl and maintained further for secondary screening.

The isolates showing better fluorescence were selected for shake flask batch experiments. The media used as inoculum was Zobell marine broth supplemented with NaCl ranging from 5–25%. The experiments were setup in 500 ml Erlenmeyer flask with 100 ml production media containing algal biodiesel waste residue. The experiment was setup till 96 h of incubation and maximum biomass and polymer productivity was monitored

every 24 h interval. The cells were harvested by centrifugation at 10,000 rpm for 10 min. The cell pellets were further dried at 60°C for 24 h. The cell dry weight was noted gravimetrically and further PHA was extracted through sodium hypochlorite (4%) treatment from the dried biomass. The cell pellets and sodium hypochlorite was vortexed thoroughly until a homogeneous mixture is obtained followed by centrifugation. The pellets were washed with water and further with methanol and allowed to dry. The obtained polymer was dissolved in chloroform and films were prepared and measured gravimetrically.

Bacterial Strains

Two potential bacterial isolates *Halomonas ventosae* (GenBank KY953158) and *Halomonas daqingensis* (GenBank KY962964) were selected on algal biodiesel waste residue containing agar plates **(Supplementary Figure 3).** These isolates were further maintained on Zobell Marine agar (ZMA) supplemented with 3% NaCl containing slants at 4°C. The cultures were maintained in glycerol stocks at −80°C.

The Seed Culture

The seed culture was raised by growing the bacterial culture on Zobell marine broth/dry sea mix (DSM) (Ghosh et al., 2015b; Mishra et al., 2014; Dhangdhariya et al., 2015) supplemented with 3% (w/v) sodium chloride, pH 7.6 ± 0.2 at 30°C for 18 h and absorbance of the culture broth was noted at 600 nm in UV-visible spectrophotometer (Varian Cary Bio 50).

Optimization of Process Parameters for Maximum Polymer Productivity

Factors affecting bacterial growth and polymer accumulation in the promising bacterial isolates capable of utilizing algal biodiesel waste residue consisting of crude glycerol; additional sodium chloride concentrations; agitation/static; temperature; size of inoculum and pH. All the experiments were carried out in triplicates.

Optimization of Growth Parameters Using Algal Biodiesel Waste Residue

In this study, varying concentrations of algal biodiesel waste residues (ABWR) containing crude glycerol on growth and PHA accumulation by isolates *Halomonas daqingensis* and *Halomonas ventosae* were studied. The crude glycerol was prepared by the process mentioned in detail in supporting information **(Supplementary Figure 1).** Both the isolates were inoculated in medium containing algal biodiesel waste residue with varying concentrations *viz.* 1, 2, 3, 4, and 5%. Both biomass and polymer productivity were recorded.

Effect of NaCl Concentration Supplemented in Production Media

Time course study of NaCl concentration on growth and PHA accumulation by *H. daqingensis* and *H. ventosae* was checked by inoculating the overnight grown culture (Optical density = 1) in production media containing varying concentration of NaCl *viz.* 5, 10, 15, 20, and 25%.

Effect of pH in Production Media

The pH plays a crucial role for bacterial metabolism. Thus, we tried to optimize this condition for its better growth and polymer accumulation. The production medium prepared was adjusted to varying pH values ranging from 5.0, 6.0, 7.0, 8.0, and 9.0 with 0.1 N HCL or 5 M NaOH solutions prior to sterilization.

Effect of Temperature

Optimization of temperature conditions were carried out at various temperatures *viz.* 30°C, 32°C, 35°C, 37°C, 40°C, and 50°C. Overnight grown culture was inoculated in the production media and incubated further at various temperatures. Biomass and PHA productivity were recorded.

Effect of Agitation and Static Condition

The role of agitation and static condition on bacterial growth and PHA production was checked by inoculating the culture in one set of production media kept in static condition while the other set was incubated at 120 rpm shaking condition in an Orbitek shaker at 35°C. The production media consisted of 3% and 4% ABWR with pH 7 for *H. daqingensis* and *H. ventosae* respectively.

Effect of Inoculum Size

Inoculum size plays a critical role in the PHA productivity experiments. *H. daqingensis* and *H. ventosae* were inoculated with varying concentrations ranging from 5% v/v, 10% v/v, 15% v/v and 20% v/v in 3% and 4% ABWR medium respectively.

Fermentor Studies

Halomonas daqingensis was further selected for PHA productivity study at fermentor level due to its rapid growth and PHA accumulation which saves energy during scale-up process. The overnight grown culture of *H. daqingensis* (15%) was inoculated in 5 L fermentor for batch fermentation process with a working volume of 2 L using the optimized medium.

The media components were 3% ABWR and 5% NaCl and made up to 1700 ml with distilled water and pH was set to pH 7 using 5M NaOH. The media was further sterilized and 300 mL of overnight grown culture was seeded aseptically. The temperature was maintained at 35°C. No antifoaming agents were incorporated into the production media. The air inflow rate and agitation speed were initially adjusted to 0.5 vvm and 200 rpm, respectively, during the fermentation process. Samples (ca. 50 ml) were withdrawn after every 24 h interval and were checked for growth and PHA productivity.

Statistical Optimization

Statistical optimization was carried out through Design Expert software 8.0 to ascertain the interactive effects of different variables through response surface methodology (RSM). Based on the optimum results, central composite design was prepared and the most significant variables ABWR, NaCl and pH were checked.

The three distinct variables used were ABWR, NaCl and pH denoted as A, B and C respectively employed to optimize the fermentation conditions and thereby to obtain maximum PHB yield. An experimental design of 20 runs (experiments) was

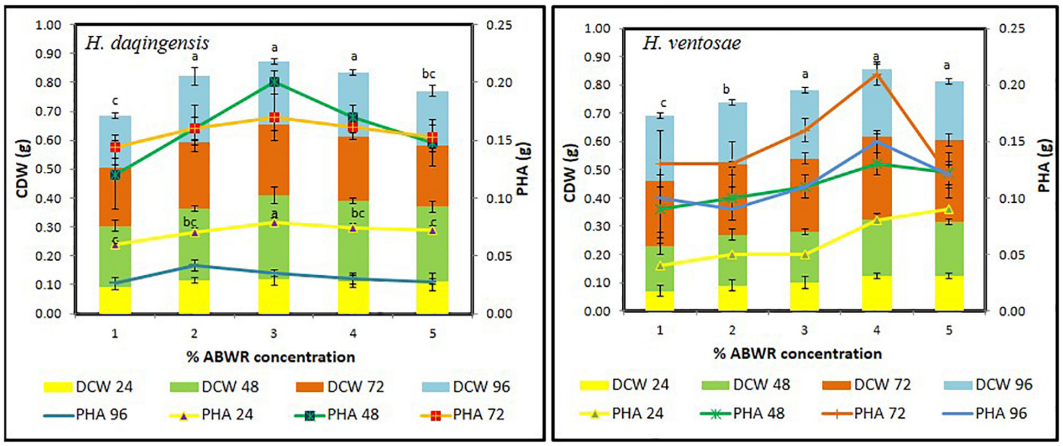

FIGURE 1 | Growth kinetics and PHA accumulation of *Halomonas daqingensis* and *Halomonas ventosae* respectively in varying concentration of ABWR.

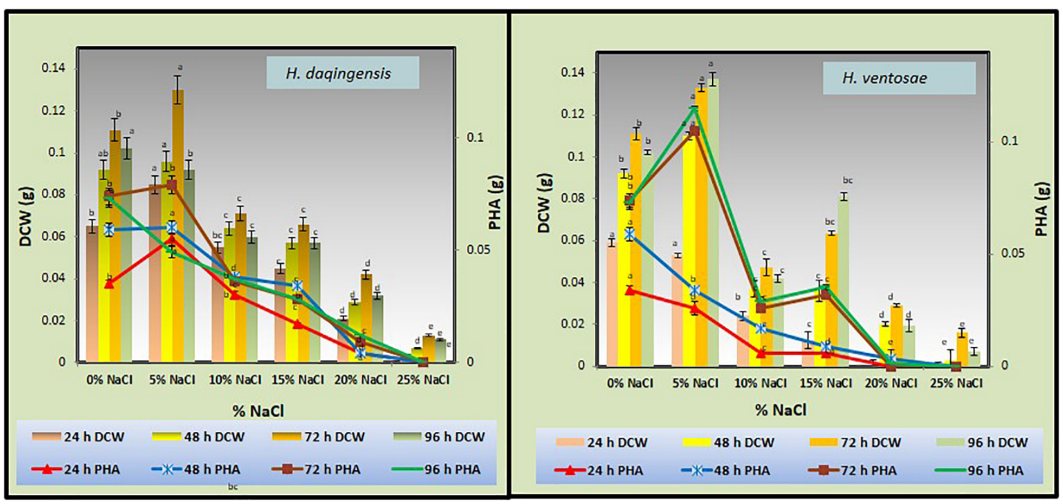

FIGURE 2 | Effect of NaCl on biomass and PHA productivity in both *Halomonas daqingensis* (D) and *Halomonas ventosae* (V).

formulated using the Design Expert software. All experiments were conducted in 500 ml Erlenmeyer flasks with 100 mL total working volume prepared according to the design and inoculated with 15 ml of seed culture. The production flasks were kept on a rotary shaker maintained at 35°C and 120 rpm. Response calculated was PHA (g) at the end of 48 h for *H. daqingensis.* The contour surface plots were created to understand the interaction of different factors, and the graphs were used to evaluate the optimized components and conditions of the medium, which influences the responses. The point prediction is a unique feature of this software which was used to confirm the obtained optimum value.

Extraction of Polyhydroxyalkanoate From Bacterial Biomass

The culture was harvested through centrifugation which was dried at 60°C overnight and treated with 4% sodium hypochlorite solution. The biomass was thoroughly vortexed until a uniform mixture is formed followed by centrifugation at 10000 rpm for 10 min. The obtained pellets were washed with water and methanol consecutively. The obtained pellets were dissolved in chloroform and the solvent was allowed to evaporate and PHA film was obtained.

Chemical Analysis of Extracted Polymer

Thermal Gravimetric Analysis (TGA)

The thermal properties of the extracted polymer were checked using thermogravimetric analysis using TG-DTA system in TG 209 F1 instrument. The sample was analyzed over a temperature range of 500°C at a heating rate of 10°C min^{-1} under nitrogen atmosphere.

Fourier Transform Infrared Spectroscopy (FT-IR)

The extracted polymer was mixed with KBr spectra was noted in 4000 – 400 cm^{-1} range with PerkinElmer

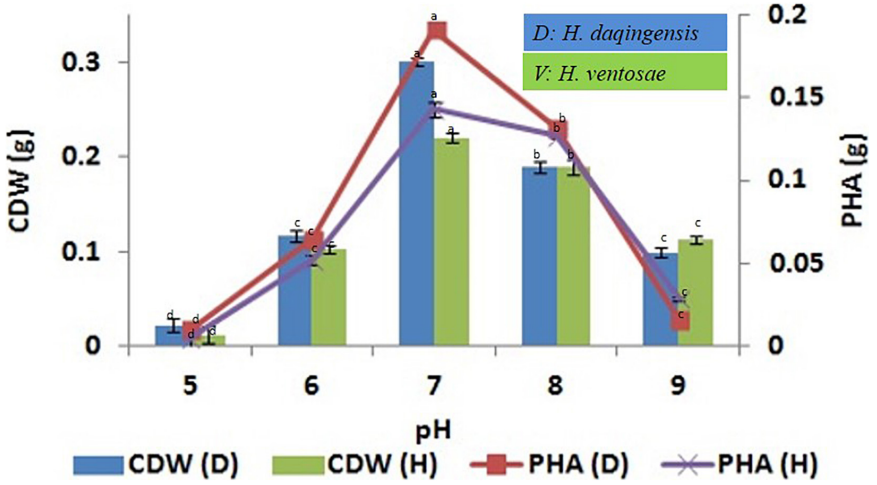

FIGURE 3 | Effect of pH on biomass and PHA productivity in both *Halomonas daqingensis* and *Halomonas ventosae*.

spectrum GX FTIR spectrometer in by mixing the extracted polymer with.

Nuclear Magnetic Resonance (NMR)

The extracted polymer was dissolved in deuterated chloroform for ^{1}H NMR analysis at 500 MHz and compared with standard PHB (Sigma Aldrich).

GPC Analysis

The Column used was 2 PL Gel Mixed D (300 mm × 7 mm) with Guard column in series. Mobile Phase CHCl3 stabilized with 1% Ethanol and polystyrene was used as calibration standard.

RESULTS AND DISCUSSION

Exploitation of waste substrates like crude glycerol from biodiesel for PHA production helps in getting rid of waste management problems and thereby decreasing environmental concerns too (Ibrahim and Steinbüchel, 2009; Zhu et al., 2013). Many researchers have worked on the utilization of synthetic substrates which will add to the cost of the product. A wide range of raw substrates are explored for its potential use for bacterial growth (Raza et al., 2018; Volova et al., 2019). The experiments were performed in triplicate and Tukey's test was applied for data interpretation in the data analyzed by applying the Tukey's test using the MStat-C software.

Optimization Studies

Optimization of Algal Biodiesel Waste Residue Concentration

Increasing the algal biodiesel waste residue concentration decreases the total organic carbon utilization, which shows that, both isolates do not utilize the organic carbon present in the medium completely. The optimum concentration for growth and PHA production is 30 g/l and 40 g/l respectively for both the isolates *Halomonas daqingensis* (**Figure 1**) and *Halomonas ventosae* (**Figure 1**) respectively.

The cell dry weight (CDW) obtained was significantly high with 0.29 ± 0.03 g/100 mL at 30 g/L ABWR concentration at 48 h incubation for *Halomonas daqingensis* and 0.29 ± 0.01 g/100 mL for *Halomonas ventosae* at 40 g/L ABWR concentration after 72 h of incubation. PHA content of 0.20 ± 0.02 g/100 mL and 0.21 ± 0.01 g/100 mL was obtained in *Halomonas daqingensis* and *Halomonas ventosae* respectively. Increase in the concentration above 3% ABWR in *H. daqingensis* decreased both the bacterial growth and PHA accumulation which clearly indicates that high concentration of ABWR may be detrimental for bacterial growth whereas, concentrations above 4% ABWR does not affect much of *H. ventosae* growth. A similar growth trend was observed at different time intervals with varying concentration which depicts the optimum time of harvesting to be 48 h and 72 h for *H. daqingensis* and *H. ventosae* respectively. Whole genome sequenced *Halomonas*

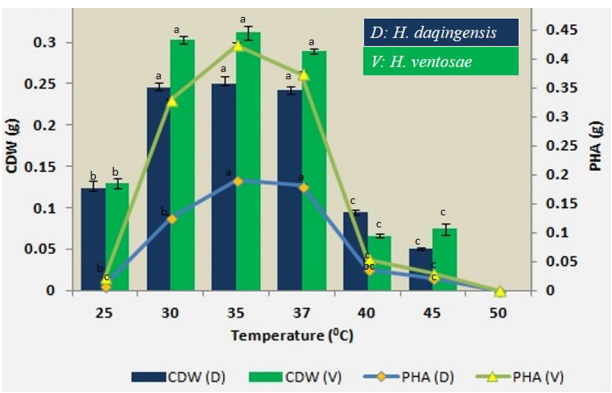

FIGURE 4 | Effect of temperature on biomass and PHA productivity in both *Halomonas daqingensis* and *Halomonas ventosae*.

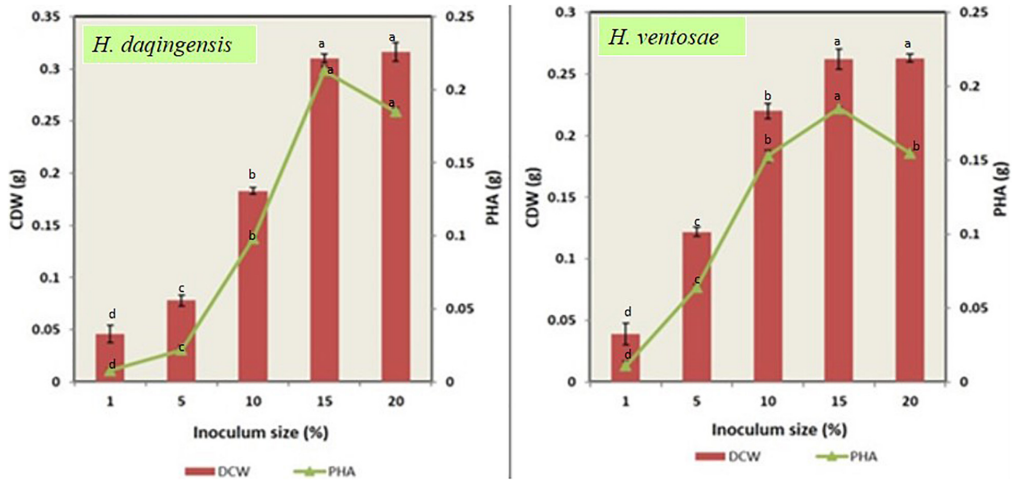

FIGURE 5 | Role of inoculum size on biomass and PHA productivity in both *Halomonas daqingensis* and *Halomonas ventosae*.

hydrothermalis is reported to accumulate co-polymer when fed with crude levulinic acid and *Jatropha* biodiesel wastes (Bera et al., 2015; Bharadwaj et al., 2015).

Effect of Sodium Chloride on Cell Growth and PHA Production

Sodium chloride is one of the constituent of crude glycerol derived from biodiesel during alkaline catalyzed transesterification of the algal oil. Thus, the effect of additionally supplemented sodium chloride on growth and PHA accumulation in the isolates was estimated by inoculating the cultures in medium containing different concentrations of sodium chloride *viz.* 5, 10, 15, 20, and 25% and no additional NaCl (denoted as 0%) in algal biodiesel waste residue. It was observed from the graph that NaCl content affects the rate of bacterial growth affecting its lag phase. In case of *Halomonas daqingensis* with no additional NaCl highest CDW was observed at 48 h of incubation while increasing the NaCl concentration to 5% increased the lag phase shifting the optimum growth at 72 h of incubation **(Figure 2)**.

Highest PHA productivity was observed at 72 h of incubation with additional 5% NaCl concentration. In *Halomonas ventosae* the growth increased at 96 h of incubation with 5% NaCl concentration depicting the large lag phase **(Figure 2)**.

Moderately halophilic bacteria grow in the presence of 3 to 15% salt concentration while, extremely halophilic bacteria require more than 15% of salt concentration. Several ions such as NaCl is essential for the growth and stability of these micro-organisms both growth and metabolism of these bacteria (Gibbons, 1969).

Effect of pH on the Biomass and PHA Accumulation

The influence of initial pH on biomass and PHA productivity by *H. daqingensis* and *H. ventosae* was checked using 3% ABWR and 4% ABWR production media respectively. The optimum pH for PHA productivity for both the isolates was pH 7 with 0.191 ± 0.002 g and 0.143 ± 0.005 g PHA in *H. daqingensis* and *H. ventosae* respectively. Increasing the pH above 7 decreases PHA accumulation in both the isolates as observed from the **Figure 3**.

TABLE 1 | Role of agitation and static condition on bacterial growth and polymer accumulation.

Incubation	*H. daqingensis*		*H. ventosae*	
	CDW (g)	PHA (g)	CDW (g)	PHA (g)
Static	0.12	0.06	0.11	0.05
Agitation	0.33	0.24	0.26	0.18

TABLE 2 | ANOVA for response surface quadratic model.

Analysis of variance table

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	0.048	9	5.32	21.38	< 0.0001
A-ABWR	6.28	1	6.28	25.24	0.0005
B-NaCl	0.016	1	0.016	65	< 0.0001
C-pH	1.25	1	1.256	5.04	0.0485
AB	4.51	1	4.51	18.12	0.0017
AC	4.05	1	4.05	0.16	0.6952
BC	3.28	1	3.28	13.18	0.0046
A^2	0.012	1	0.012	48.66	< 0.0001
B^2	1.03	1	1.03	4.17	0.0684
C^2	5.45	1	5.45	21.89	0.0009
Residual	2.49	10	2.49		
Lack of Fit	2.20	5	4.41	7.79	0.0209
Pure Error	2.83	5	5.66		
Cor Total	0.050	19			

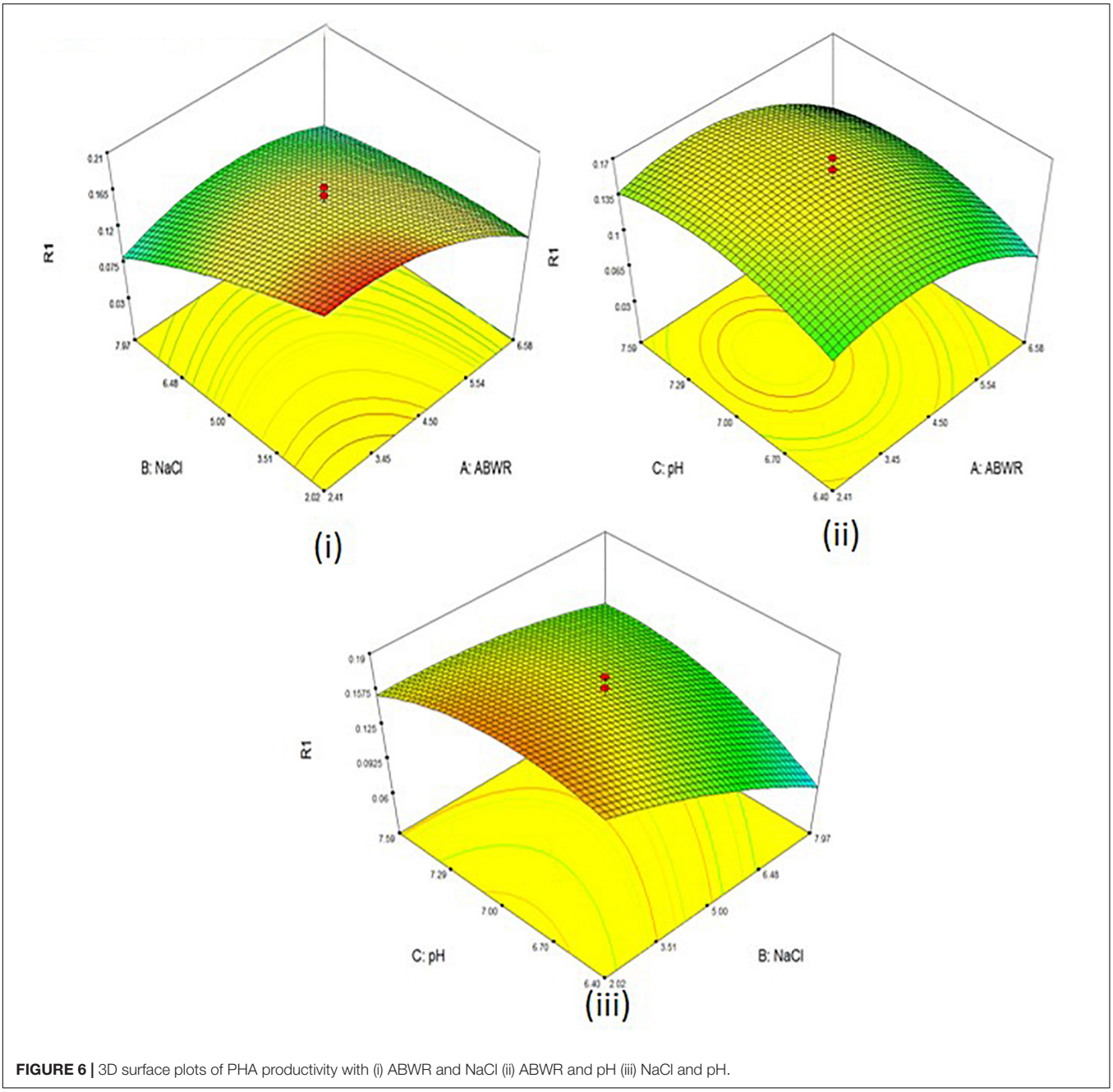

FIGURE 6 | 3D surface plots of PHA productivity with (i) ABWR and NaCl (ii) ABWR and pH (iii) NaCl and pH.

Effect of Temperature on the Biomass and PHA Productivity

The role of temperature on biomass and PHA productivity was checked in *H. daqingensis* and *H. ventosae* at different incubation temperatures *viz.* 25°C, 30°C, 35°C, 40°C, 45°C and 50°C. The maximum PHA accumulation was obtained at 35 °C temperature for both the isolates as indicated in **Figure 4.**

Effect of Static and Agitation on the Growth and Polymer Accumulation

The effect of agitation and static condition on growth and PHA accumulation was estimated by incubating one set of the inoculated medium in agitated condition in a shaker at 120 rpm and incubating the other set of inoculated medium in static condition. The medium constituent was 3% and 4% algal biodiesel waste residue for *H. daqingensis* and *H. ventosae* respectively incubated at 35°C. *H. daqingensis* showed 0.12g of CDW with 0.06g PHA under static condition while 0.33g CDW and 0.24g PHA under agitation condition. In case of *H. ventosae* 0.11 g CDW with 0.05 g PHA accumulation under static condition while 0.26 g CDW and 0.18 g PHA accumulation was found to be under agitation condition. As observed from these results, shaking condition is an important parameter for growth and PHA accumulation due to maximum oxygen transfer

rate in the culture medium enhancing the bacterial metabolism for efficient accumulation of PHAs.

Effect of Inoculum Size on the Biomass and PHA Accumulation

Inoculum size is also one of the important factor responsible in the optimization process. Both the isolates *Halomonas daqingensis* and *Halomonas ventosae* were inoculated into the production medium with varying inoculum size of 1% v/v, 5% v/v, 10% v/v, 15% v/v, 20% v/v in 30 g/L and 40 g/l of algal biodiesel waste. **Figure 5** indicates bacterial growth along with polymer accumulation.

An increase in CDW and polymer content was observed in both the isolates from the graph till 15%v/v inoculum. Thus, maximum growth with 0.310 ± 0.04 g and polymer accumulation with 0.213 ± 0.03 g was recorded at 15% v/v concentration in *H. daqingensis* whereas 0.262 ± 0.08 g biomass and 0.185 ± 0.01 PHA productivity in *H. ventosae.* Increasing the inoculum size to 20% v/v decreased both CDW and polymer content.

Scaling Up of the Optimized Process in Bioreactor

Fermentor studies was carried out under optimized conditions utilizing *H. daqingensis.* In the present experiment, the inexpensive ABWR was used as production media with additional 5% NaCl. Samples were withdrawn every 24 h time interval to check possible bacterial growth and PHA production. The centrifuged pellets were collected for PHA. PHA extraction and supernatant was filter sterilized (0.45 μ) and stored at 4°C until used for total organic carbon analysis. The results obtained indicated potential utilization of cheap substrates as nutrient source for bacterial growth and PHA production at industrial scale. The un-optimized conditions resulted in 0.362 ± 0.001 g of dried bacterial biomass and 0.236 ± 0.003 g of PHA within 48 h of incubation.

TABLE 3 | CCD for PHA optimization ABWR (1-8%); NaCl (0-10%); pH (6-8).

Std	ABWR (%)	NaCl (%)	pH	PHA (g)
1	2.41	2.02	6.40	0.21
2	6.58	2.02	6.40	0.09
3	2.41	7.97	6.40	0.024
4	6.58	7.97	6.40	0.06
5	2.41	2.02	7.59	0.15
6	6.58	2.02	7.59	0.1
7	2.41	7.97	7.59	0.106
8	6.58	7.97	7.59	0.09
9	0.99	5	7	0.115
10	8	5	7	0.03
11	4.5	0	7	0.19
12	4.5	10	7	0.071
13	4.5	5	5.99	0.079
14	4.5	5	8	0.12
15	4.5	5	7	0.16
16	4.5	5	7	0.15
17	4.5	5	7	0.17
18	4.5	5	7	0.16
19	4.5	5	7	0.16
20	4.5	5	7	0.15

Statistical Optimization

The study was conducted using Design Expert software version 8. Three different variables: ABWR (1-8%), NaCl (0-10%) and pH (6-8) was considered for the experiment. The experiment was set up at 100 mL scale in 500 mL Erlenmeyer flasks containing 85 mL of production medium and 15 mL of inoculum, 35°C incubation temperature, 48 h production age, 120 rpm agitation in an Orbitek shaker. The above mentioned medium was considered for seed culture medium. To estimate the effect of different factors on PHA as response surface, central composite design was used. Based on the results obtained by optimizing different factors through one factor at a time approach, the ranges of factors were selected. The experimental data derived from the design were analyzed by multi regressions through the least square method to fit the below second order polynomial equation. A quadratic model was obtained in this study.

Y is the measured response variable, coefficients βo, βi, βij, and βii are constant which represent either synergistic or antagonistic effects for different combinations and regression coefficient of the model and xi and xj represents the independent variables in coded values. The predictive power was verified using Fischer (F) through analysis of variance (ANOVA) determining the level of significance. Also, *p*-values (Prob. > F) less than 0.05 is preferable. **(Tables 1, 2)**

A three- factor, five level central composite design (CCD) was employed to check the effects of different combinations on polymer accumulation **(Figure 6 and Table 3)**.

The concentrations of the different factors were set as per our preliminary experiments conducted at the same level. The experiment consisted of 20 runs. The Model F-value of 21.38 suggests the model is significant. There is only a 0.01% chance that a "Model F-value" this large might be due to noise. Final equation for PHA derived in terms of coded factors:

$0.16 + -0.021 * A + -0.034 * B + 9.589E - 03 * C + 0.024 * A * B +$

$2.250E + -003 * A * C + 0020 * B * C + -0.029 * A^2 + -8.489E-003 * B^2 + -0.019 * C^2$

The maximum productivity with 0.21 g was observed at 2.41% ABWR; 2.02% NaCl and 6.40 pH.

Extraction and Characterization of the Extracted Polymer

The supernatant after centrifugation of the bacterial biomass was filter sterilized (0.45μ) and stored at 4 °C until use. This filter sterilized supernatant was analyzed for organic carbon content which was 14.45% at 48 h of incubation. Extraction was carried out using conventional technique utilizing 4% sodium hypochlorite. Dubey et al., 2017 reported the extraction of PHA utilizing green solvents. Also, the dissolution of PHA

TABLE 4 | Comparative analysis of PHA production.

Sr No.	Strain	Feedstock	Working volume	Crude glycerol (%)	Temperature	PHA productivity	References
1.	Mixed culture	Crude glycerol; volatile fatty acids;1,3-propanedio	1.7 L	1	37 ^{0}C	-	Burniol-Figols et al., 2018
2.	*P. putida* KT2440	Crude glycerol	1.5 L	3%	30 ^{0}C	47%	Poblete-Castro et al., 2014
3.	*Novosphingobium sp.*	Crude glycerol	-	2%	-	45%	Teeka et al., 2012
4.	*Bacillus thuringiensis*	Crude glycerol; Nutrient broth	125mL	2%	37 ^{0}C	74.8%	Kumar et al., 2015
4.	*Pannonibacter phragmitetus ERC8*	Crude glycerol	200 mL	0.8%	30 ^{0}C	17.41%	Ray et al., 2016
5.	*Bacillus sp. ISTVK1*	Pure glycerol	-	5%	30 ^{0}C	85.19%	Morya et al., 2018
6.	*Paracoccus sp. strain LL1*	Glycerol	150 mL	2%	30 ^{0}C	39.3%	Kumar and Kim, 2018
7.	*Halomonas daqingensis*	Crude glycerol	100 mL	3%	35 ^{0}C	68.96%	This study
8.	*Halomonas ventosae*	Crude glycerol	100 mL	4%	35^{0}C	72.41%	This study

was reported in green solvent diethanol ammonium acetate with maximum solubility of 3% w/v at 70°C (Sequeira et al., 2020). Herein, the extracted polymer showed good thermal and mechanical properties compared to standard PHB from Sigma Aldrich. The detailed description of the characterization experiments are mentioned in Supporting information file (**Supplementary Figures 2, 3**) as **Table 4** above indicates the comparative PHA productivity in different bacterial strains compared with our present study.

Comparative TGA Profiles of PHA Extracted at Different Time Intervals With Standard PHB (Sigma Aldrich)

The thermal degradation temperature of extracted PHA from *H. daqingensis* and *H. ventosae* were observed to be 290°C and 296°C which is higher than detected for commercial PHB (Sigma Aldrich). The increase in degradation temperature for observed biomass can be due to trace amount of impurities which was further checked through elemental C,H,N,S analysis. Extracted PHA sample was checked for impurities left after extraction process through Elemental analysis. The extracted sample contains 55.95% and 56.56% carbon which is similar to that of standard PHB obtained from Sigma Aldrich.

Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectra of PHB (Sigma Aldrich) displayed characteristic peaks, ν-OH str. at 3436 cm-1, ν-CH2 and ν-CH3 str. at 2880 to 2977 cm-1, ν-C = O str.at 1724 cm-1 and ν-C-O str. at 1000-1300 cm-1. The recovered polymer (both from *H. daqingensis* and *H. ventosae*) exhibited all specific peaks, consistent to that detected for PHB (Sigma Aldrich). Hence, it was definite that the recovered polymer is PHB

Nuclear Magnetic Resonance (NMR) Analysis

The ^{1}H-NMR spectra of PHB indicated doublet at δ = 1.28 and 1.27 ppm due to methyl group, doublet of quadruplet at δ = 2.58 and 2.49 ppm, ascribed to the methylene group, multiplet at δ = 5.25 ppm is characteristic due to methine group.

Dynamic Mechanical Analysis (DMA)

The robustness of the PHA film reduced with the rise in temperature as depicted in the graph. The E′ became constant at around 125°C which designates the glass transition temperature though, the glass transition recorded through DSC was around 175°C which can be due to the sensitivity of instruments.

Gas Permeation Chromatography (GPC) Analysis

The extracted polymer has molecular weight of 309 KDa extracted from *H. daqingensis* with 1.82 PDI.

Comparative Production Analysis of PHA Utilizing Glycerol

Polyhydroxyalkanoates accumulation in halophilic bacteria in the presence of high salts is beneficial not requiring sterile conditions thus preventing growth of wild bacterial strains.

Novelty of the Current Study and Its Significant Contribution

The novelty of the current study is utilization of ABWR as sole substrate for PHA production in both the halophilic bacterial strains i.e., *H. daqingensis* and *H. ventosae*. These two strains are capable of accumulating PHA within 48–72 h of incubation which decreases the overall production cost.

CONCLUSION

The grail of the current work was to reconnoiter and formulate the process for the exploitation of algal biofuel wastes containing crude glycerol for the production of polyhydroxyalkanoate that can replace commercial substrates. The priority in our current study for selecting salt pans as sample collection site was its extreme environment nurturing several bacteria adapted in such harsh environment. This wonder niche is a focal point for potential microbes *viz. Halomonas daqingensis* and *Halomonas ventosae,* as an efficient PHA producer with maximum PHA productivity in presence of 3% and 4% ABWR concentration respectively at 35°C temperature.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SD performed the conceptualization, methodology, conducting experiments and writing. SM did the conceptualization, editing and reviewing. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2021.624859/full#supplementary-material

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