SUSTAINABLE PRODUCTION OF BIOACTIVE PIGMENTS

EDITED BY: Wee Sim Choo, Laurent Dufossé and Lourdes Morales-Oyervides
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SUSTAINABLE PRODUCTION OF BIOACTIVE PIGMENTS

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Table of Contents

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EDITORIAL
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Editorial: Sustainable Production of Bioactive Pigments

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Editorial on the Research Topic

Sustainable Production of Bioactive Pigments

Pigments are compounds that are perceived by humans to have color. Natural pigments such as chlorophylls are the source of green in all plants and the most abundant pigments in nature. Natural pigments are also responsible for yellow, orange, red, blue, and purple colors found in nature (Choo, 2019). Anthocyanins, carotenoids, and betalains are the common plant pigments found in nature besides chlorophyll (Choo, 2018). Besides plant pigments, pigments are derived to a lesser extent from insects or animals. Emerging sources of pigments are from microalgae and microorganisms (Morales-Oyervides et al., 2020). Pigments are considered to be bioactive if they have an interaction with, or have an effect on, cell tissue in the human body. There is huge interest in bioactive pigments that render beneficial health effects on humans. Consumers are driving the demand for natural food compounds such as bioactive pigments. However, continual prospecting of terrestrial or aquatic natural resources for natural food compounds will impose a huge demand on these resources. Sustainable production of bioactive pigments is therefore needed to ensure continuity of use for future generations and allow feasible increase in the utilization of these bioactive pigments.

This Research Topic provides reviews and researches on the sustainable production of various bioactive pigments. This Research Topic comprises a collection of 12 articles, including 4 reviews, 2 mini-reviews, and 6 original research articles. The review by Yong et al. provides a comprehensive evaluation of the impact of high-pressure homogenization (HPH) on the extractability and stability of phytochemicals such as anthocyanins, carotenoids, and others. HPH technology is considered as green technology as it does not use polluting solvents, short processing duration, low consumption of energy, and emission of carbon dioxide (Mesa et al., 2020). Another review by Venil et al. highlights the significance of bacterial colorants and summarizes their application in food and pharma industries. The potential of bacterial pigments for mass production of diversified coloring properties was first prospective and is now getting the notable importance and attention of both the researchers and industries. Tactics in strain improvement, fermentation conditions, metabolic engineering, and easy extraction techniques are needed to produce high-end products. Likewise, Rana et al. reviewed the types of microbial pigments used as food additives; the authors emphasized the colorants associated biological activities, recent advancements and future challenges. Lopes and Ligabue-Braun summarized the information obtained during the last decade regarding the valorization of agro-industrial residues as an eco-friendly and low-cost alternative for the production of microbial pigments. Certainly, natural bioactive pigments are molecules with high added-value, which could be key to develop cost-effective processes using wastes toward a sustainable future.

The two mini-reviews investigate other aspects of microbial pigments. Pailliè-Jiménez et al. state that microorganisms, because of their genetic simplicity as compared with plants, may be a better

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source to understand biosynthetic mechanisms and to be engineered for producing high pigment yields. Moreover, despite the origin of the pigmented microorganism, it seems very important to develop protocols using organic industrial residues and agricultural by-products as substrates for pigment production and find novel green strategies for rapid pigment extraction. Despite the huge concern in secluding and collecting marine bacteria, microbial metabolites are progressively alluring to science due to their wide-ranging applications in various fields, particularly those with distinctive color pigments. The mini-review by Velmurugan et al. is an appraisal of the studies undertaken over the past 5 years on the bacterial pigments sourced from the marine environment.

The original research section starts with De Wit et al. who report the antioxidant properties of fresh and processed cactus pear fruit peels of different fruit-colored cultivars. Cactus pear fruit peels are usually discarded and considered as waste. In this work, authors found that the purple and orange-colored cultivars were the best choice in terms of antioxidant properties. Coffee (*Coffea arabica* L.) by-products are another source of carotenoids and phenolic compounds investigated by Esquivel et al.. Analysis of pulp and peels of Arabica coffee varieties with different external fruit color allowed the identification of a great number of compounds using high-performance liquid chromatography with diode array detection and electrospray ionization multi-stage mass spectrometry (HPLC-DAD-ESI-MSn).

Marine sponges have been known to harbor microorganisms that produce secondary metabolites like bioactive pigments. One red bacterium isolated from the sponge *Petrosia* sp. in Hawai'i by Sakai-Kawada et al. was identified as belonging to the *Pseudoalteromonas* genus. The draft genome was sequenced, assembled, and annotated. This revealed a prodiginine biosynthetic pathway and the first cited-incidence of a prodiginine-producing *Pseudoalteromonas* species isolated from a marine sponge host. Further, it has been well-documented that the fungal pigments produced by *Monascus* strains possess various biological activities. Accordingly, Mohankumari et al. investigated the effect of *Monascus* fermented rice (RMR, traditional Chinese food additive) on the cholesterol blood levels in rats fed with a high-fat diet. The authors reported satisfactory results concluding that RMR is a promising anti-atherogenic food supplement. Other biological activities displayed by the natural bioactive pigments which have called researchers attention are the antimicrobial properties. In this regard, Mekala et al. isolated and characterized a pigment producer bacterium (*Exiguobacterium* sp GM010) which pigments showed bactericidal effects against food-borne pathogens, both Gram positive and Gram negative. The antibacterial mode of action and non-toxic effect of the pigments toward *A. franciscana* were elucidated, suggesting the potential application of the pigments in food preservation. Indeed, the properties of the bioactive pigments make them promising for a wide range of industrial applications. However, one important aspect of ensuring any industrial application is the stability of the pigments, especially if they are compared with synthetic pigments. On this subject, Ghosh and Mishra studied the stability of C-phycoerythrin, a water-soluble pigment, which was produced by the microalgae *Lyngby*a sp. (CCNM 2053). The pigments were stable under acidic to neutral conditions (pH 3–8) and presented moderate stability under light exposure; however, the pigments were poorly stable under high temperatures (>50°C).

This editorial summarizes the articles in this Research Topic. We hope that this collection of articles will contribute to the advancement of research on the sustainable production of bioactive pigments. Lastly, we would like to thank all authors, reviewers and Frontiers editorial team for their contributions in this Research Topic.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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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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ORIGINAL RESEARCH
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Studies on Extraction and Stability of C-Phycoerythrin From a Marine Cyanobacterium

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Being a naturally produced colorant with fluorescent properties, C-phycoerythrin (CPE) has important food industry applications. However, optimization of extraction and its stability under storage is an aspect which needs further attention. In this study, optimization of CPE extraction from *Lyngbya* sp. CCNM 2053 was undertaken using response surface methodology (RSM). Preliminary screening suggested a phosphate buffered system with freeze thaw for cell disruption as the most suitable method. 22.40 ± 1.31 mg g^{-1} CPE and 52.76 ± 0.07 mg g^{-1} total proteins were extracted using phosphate buffer while freeze thaw method yielded 19.87 ± 0.43 mg g^{-1} CPE and 58.92 ± 3.49 mg g^{-1} total proteins. RSM was used to optimize phosphate buffer pH (5.5–8), molarity (0.1–1 M) and number of freeze thaw cycles (2–7). Interaction between buffer molarity and number of freeze thaw cycles was most significant for modeling the responses. Extraction of CPE was highest with pH 8 and low molarity buffers. The predicted values from the equations agreed well with the experimental values, proving the robustness of the model. The stability of the extract was also evaluated at different temperatures, light exposure times and pH values. Results indicated that light exposure should be kept to a minimum while the stability was affected by extreme temperatures and pH. CPE was comparatively stable in a pH and temperature range of 3–8 and 4–40°C respectively.

Keywords: cyanobacteria, extraction optimization, food color, phycoerythrin, stability

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INTRODUCTION

C-phycoerythrin (CPE) is a water soluble, light harvesting accessory pigment found in some strains of cyanobacteria and is responsible for absorption of wavelengths inaccessible to chlorophyll. In addition to its role as a light harvesting protein, it is widely studied for its applications as a natural food colorant, anti-oxidant and anti-hyperglycemic agent, fluorescent tag, and small ion sensor (Sekar and Chandramohan, 2007; Ghosh et al., 2016, 2018, 2020; Ghosh and Mishra, 2020).

Due to its many practical applications, the extraction and purification procedures are generally multi-step and elaborate, to ensure quality and purity (Mishra et al., 2011; Ghosh et al., 2015). Although phycoerythrin, with its bright fluorescence, is widely regarded as a potential food colorant, most of the sources are limited to red seaweeds (Dufossé et al., 2005; Sekar and Chandramohan, 2007). A part of the reason is the lack of cyanobacterial sources; however, the other part is the problem of cyanotoxins produced by many members of the cyanobacteria family (van Apeldoorn et al., 2007; Bláha et al., 2009). Purification and stability of CPE is, thus, an area

which requires detailed studies. Extraction of CPE normally involves cell disruption through one of the many available techniques like repeatedly freezing and thawing the biomass, high pressure homogenization, ultrasonication or enzymatic digestion (Reis et al., 1998; Sun et al., 2009; Mishra et al., 2011; Dumay et al., 2013). A proper extraction protocol should extract the maximum CPE with minimal contamination. However, there is a lack of consensus among the procedures. For instance, enzymatic digestion, although useful for seaweeds owing to their cell walls, might be unnecessary for cyanobacteria.

Stability of the extracted CPE is another factor for its acceptance in the food industry. Natural origin molecules are often seen as very environment susceptible and unstable, thus bringing down their shelf lives. There are a few studies reporting the stability of phycoerythrin from red algae and cyanobacteria (Mishra et al., 2010; Senthilkumar et al., 2013a; Munier et al., 2014; Anwer et al., 2016; Patel et al., 2018). However, barring one study, all of them used purified forms of phycoerythrin for their studies.

This study is an attempt to simplify and standardize the conditions for CPE extraction through a 2-step approach. A preliminary screening of buffers and cell disruption methods was carried out for obtaining maximum CPE with minimum total proteins. The chosen buffer and disruption method were further optimized using response surface methodology to obtain mathematical relationships for CPE and total protein extraction. The relationships were experimentally verified using a given set of conditions. Further, the crude extract obtained was investigated for its stability at different pH, light exposure durations, and temperatures. We believe that the results can shed more light on the acceptability of CPE as a food ingredient in the near future.

MATERIALS AND METHODS

Microorganism, Culture Conditions, and Harvesting

Lyngbya sp. (CCNM 2053) was used from our laboratory's microalgal culture collection. It was cultured in a 500 ml shake flask culture containing 250 ml ASN III at 25 ± 2°C in a light intensity of 60 μmol m^{-2} s^{-1} and a photoperiod of 12:12 light: dark period. After 20 days, the filamentous biomass was skimmed off using a simple mesh filter and used as such. The moisture content was determined by drying a known amount of wet biomass at 110°C till constant weight. All the salts and chemicals were of analytical grade (HiMedia Chemicals, Mumbai, India) and were used without further purification.

Total Protein and CPE Estimation

The UV–visible spectral scans of the samples were recorded using a Cary 50 Bio UV–spectrophotometer (Agilent Inc., USA) in a range of 350–800 nm using a 1 cm pathlength quartz cuvette. The C-phycocyanin (CPC), allophycocyanin (APC) and CPE contents were determined using the equations given below (Bennett and Bogorad, 1973):

$$\text{CPC (mg ml}^{-1}) = (A_{615} - 0.474 * A_{652})/5.34 \quad (1)$$

$$\text{APC (mg ml}^{-1}) = (A_{652} - 0.208 * A_{615})/5.09 \quad (2)$$

$$\text{CPE (mg ml}^{-1}) = (A_{562} - 2.41*\text{CPC} - 0.849*\text{APC})/9.62 \quad (3)$$

The purity ratio was calculated from the A_{562}/A_{280} ratio.

The total protein was determined by a modified Bradford method (Bradford, 1976). Briefly, 100 μl of the protein sample was mixed with 100 μl of the respective buffer. One milliliter of Bradford reagent was added to this mixture and the absorbance of the sample was read at 595 nm. Bovine serum albumin (BSA) was used as a standard while the yields were calculated on a dry mass basis in all cases.

Experimental Design and Evaluation

The optimization of the extraction conditions was performed in two sequential steps. The efficiency of extraction under various different buffers and cell breakage methods were first analyzed in a preliminary screening. The buffer and cell disruption method with the maximum yields of CPE were further optimized through response surface methodology.

Preliminary Screening of Extraction Buffer

A preliminary screening of the various buffers methods was first investigated based on reported methods. We screened phosphate, tris chloride and piperazine-N,N′ bis (2-ethanesulphonic acid) (PIPES) buffers in comparison with de-ionized water (DW, MilliQ water purification system, Merck Millipore, USA). The total protein and CPE content were determined as detailed in section Total Protein and CPE Estimation.

Screening for Cell Disruption Method

Ultrasonication, freeze thaw and manual crushing of the biomass were carried out in phosphate buffer (0.1 M, pH value 7) to select the best suited method for cellular disintegration. A power input of 90 W at 100 V_{rms} and 0.9 A_{rms} was used to sonicate the biomass using 10 s pulses followed by a rest period of 5 min. The biomass was kept on ice during the process to minimize protein denaturation. Freeze thaw process was carried out by freezing the biomass in the respective buffer at −70°C followed by a thaw cycle at 27°C. Manual crushing of the wet biomass suspended in an appropriate buffer was done using a pestle–mortar.

Design for Response Surface Methodology

The optimized buffer and cell disruption method was further evaluated using response surface methodology (RSM) for maximizing CPE extraction (Design-Expert 8, Stat-Ease Inc., USA). We used a central composite design (CCD, 3 factor−3 level) with pH (A), molarity (B) and number of freeze thaw cycles (C) as independent variables to achieve maximum extraction (**Table S1**, Electronic Supplementary Information, ESI). The experiment consisted of 20 experimental runs at 3 coded levels (−1, 0 and +1) with all the points being run in triplicate as per the design to minimize error. The responses (Y) were evaluated using a quadratic model equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_{ij} + \varepsilon \quad (4)$$

where β_0 represents the intercept, β_i is the linear effect coefficient, β_{ii} is the quadratic effect coefficient and β_{ij} is the interaction

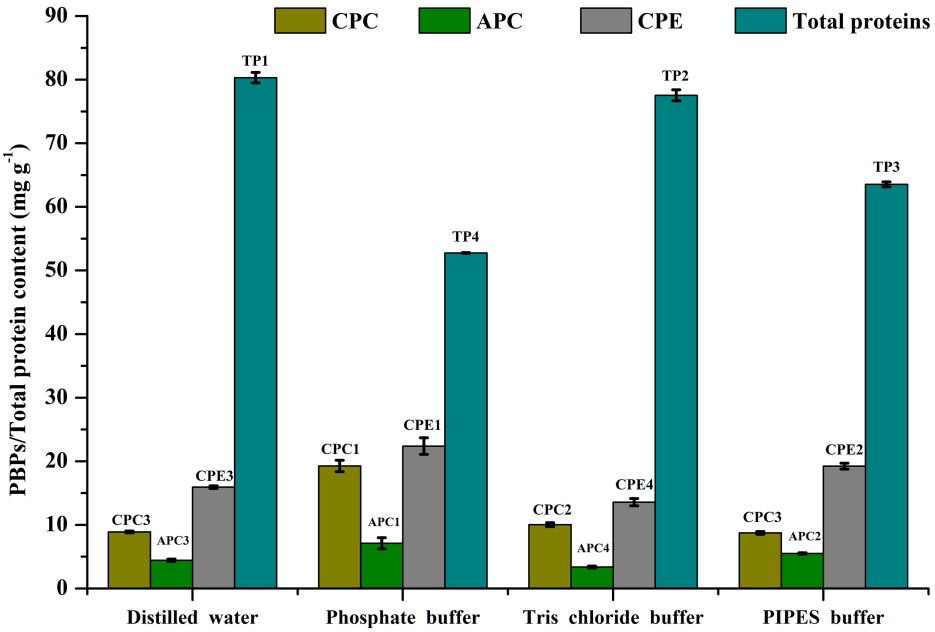

FIGURE 1 | Screening of different buffers for CPE and total protein extraction. pH of all buffers adjusted to 7. Significance of each extracted analyte denoted by its initials followed by significance number. A different number denotes statistical significance ($p < 0.05$, $n = 3$). TP, Total proteins.

effect coefficient, x_i and x_j are the independent variables and ε is the error.

Model Validation

The optimized model was evaluated for its prediction accuracy by setting up an extraction system with known inputs while the predicted value of CPE and total proteins were calculated from their respective model equations.

Stability of the Crude CPE Extract

Stability Under Different pH Values

The stability of the CPE crude extract was investigated within a pH range of 2–12 using different buffers. The spectral scans were recorded from 350 to 800 nm.

Stability Under Different Light Exposure Duration

The stability of the crude extract was studied after exposure of the samples to a light intensity of 120 μmol m^{-2} s^{-1} using cool white fluorescent lamps. The samples were exposed for up to 2 days and the spectral scans were recorded in the 350–800 nm range at appropriate intervals.

Stability Under Different Temperatures

The thermal stability of the crude extract was studied in a temperature range of 4–100°C after 2 h of exposure. To study the long-term stability, the extract was stored at different temperatures (−60, 4 and 10°C) for up to a week with the spectral readings being collected at appropriate intervals. Samples at 4 and 10°C were kept in refrigerators (Samsung, South Korea), while the samples at 20 and 30°C were kept in an incubator shaker (Jeio Tech Co. Ltd., South Korea). Samples at −64°C were kept in a deep freezer (Eppendorf, Germany) while for heating the samples at 40, 50, 60, and 100°C, we used a heating block (Bionic Scientific, India).

Statistical Analysis

All the values have been presented as mean ± SD of 3 triplicate values. Fisher LSD test was used for the statistical analysis for the screening of buffer and cell disruption methods using InfoStat v2013d (Di Rienzo et al., 2011). The difference was considered significant at $p < 0.05$. The analysis of variance (ANOVA, partial sum of squares type III) and statistical analysis for the optimization data were done using Design-Expert v8.

RESULTS

Effect of Buffer Choice on Extraction of Total Protein and CPE

We chose to study the effect of three different buffers on the extractability of CPE from a cyanobacterium, *Lyngbya* sp. (CCNM 2053) while deionized water (DW) was taken as the control. The pH values of all the 3 buffers was adjusted to 7.0 to mirror the pH of deionized water while the ionic strength of each was 0.1 M. Phosphate buffer was the ideal solvent for extracting CPE (22.40 ± 1.31 mg g^{-1}) (**Figure 1**) followed by PIPES as the second-best alternative (19.24 ± 0.46 mg g^{-1}). However, the main difference is the amount of total proteins PIPES extracts out of the cells (63.54 ± 0.4 mg g^{-1}) compared to phosphate (52.76 ± 0.07 mg g^{-1}). DW also is a good candidate for CPE; however, it extracts the highest amount

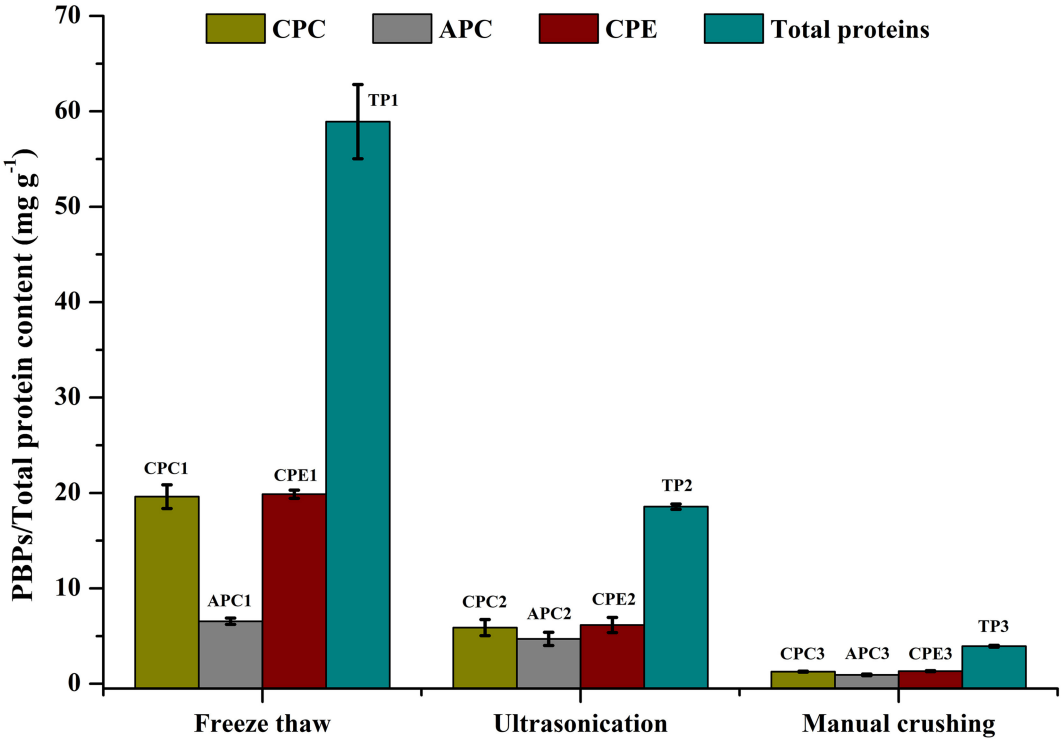

FIGURE 2 | Screening of different cell disruption methods for CPE and total protein extraction. Significance of each extracted analyte denoted by its initials followed by significance number. A different number denotes statistical significance ($p < 0.05$, $n = 3$). TP, Total proteins.

of other proteins from the cells. The probable reason is the absence of salts which can lead to osmolysis. On the other hand, tris chloride has the second highest amount of total proteins with the lowest CPE, thus making it ineffective for our purposes.

Alternate buffers like tris chloride have also been reported in literature for the extraction of phycobiliproteins. Soni et al. (2010) have utilized tris chloride for the extraction of CPE for crystallization studies. Mubarak Ali (2013) has reported yields of 85.84 ± 0.63 mg g^{-1} CPE and 45.51 ± 1.21 mg g^{-1} CPC using tris chloride. Good's buffers are another series of buffering salts that have been designed specifically for biological systems (Good et al., 1966). We used PIPES as a buffer system through which we have achieved a good extraction ratio for CPE. Although these buffers were developed as an alternate to phosphate or acetate buffers for routine biological work, little work has been reported on phycobiliproteins till date.

Another important observation was that the CPE purities in the crude extract were the highest when phosphate buffer was used as an extractant, which is an advantage during their downstream purification and processing. The CPE content was 42.46% of the total protein content. The next best buffer in this criterion was PIPES with CPE content of 30.28% of the total protein. The non-selective extraction of all soluble proteins in tris chloride and DW affected the purity of CPE to a great extent. A higher yield led us to choose phosphate buffer for further optimization studies.

Effect of Cell Disruption Method on Total Protein and CPE

Cell disruption is an essential parameter for extraction of metabolites. There are different methods that have been followed for maximizing cellular disruption leading to a higher product yield. We have chosen 3 different methods—ultrasonication, freeze thaw, and grinding—to compare their effects on CPE recovery from intact cells. Phosphate buffer (0.1 M, pH 7) was used for all the methods. The maximum recoveries as well as the highest purity of CPE were obtained through the freeze thaw method. The total protein recovery was also the highest in this method (**Figure 2**). The CPE content was 19.87 ± 0.43 mg g^{-1} while the protein content was 58.92 ± 3.49 mg g^{-1}. On a weight basis, 33.72% of the total protein content was CPE. The corresponding purity ratio was 1.92.

We were able to achieve yield of 6.16 ± 0.79 mg g^{-1} CPE using a 25 kHz sonicator (**Figure 2**). 33.17% (w/w) of the total protein content was CPE with a purity ratio of 0.58. The purity ratio was very low compared to the freeze thaw method, which points out denaturation during the process. Manual crushing of the wet biomass in an appropriate buffer was carried out using a pestle-mortar. We were able to extract 1.33 ± 0.05 mg g^{-1} CPE (**Figure 2**). On a weight basis, 33.76% of the total protein content was CPE. The total protein yield was 3.94 ± 0.10 mg g^{-1} while the purity ratio of CPE was 0.34. The low purity ratios underline the non-selective extraction of soluble proteins from the cells.

TABLE 1 | Experimental design and responses for optimizing CPE and total protein extraction.

Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2
	A:pH	B:Molarity	C:FT cycle	CPE (mg g^{-1})	Total protein (mg g^{-1})
1	6.8	0.55	2	1.38 ± 0.30	9.20 ± 0.33
2	6.8	0.55	5	4.82 ± 0.04	16.37 ± 1.15
3	5.5	0.1	7	11.49 ± 0.10	56.59 ± 0.95
4	6.8	0.55	5	3.48 ± 0.02	13.16 ± 0.65
5	6.8	0.55	5	5.01 ± 0.06	14.11 ± 0.28
6	5.5	1	2	0.42 ± 0.04	3.69 ± 0.51
7	6.8	0.55	7	7.52 ± 0.08	22.14 ± 0.69
8	5.5	1	7	8.71 ± 0.05	17.26 ± 1.51
9	6.8	1	5	0.51 ± 0.10	8.16 ± 0.35
10	8	0.1	7	22.99 ± 0.52	46.29 ± 0.93
11	5.5	0.1	2	10.11 ± 0.19	17.14 ± 0.57
12	6.8	0.1	5	11.54 ± 0.02	31.04 ± 2.52
13	8	1	2	0.23 ± 0.03	3.24 ± 0.38
14	8	1	7	0.33 ± 0.06	9.03 ± 0.70
15	8	0.1	2	8.63 ± 0.06	18.67 ± 0.29
16	5.5	0.55	5	13.03 ± 1.98	29.65 ± 0.21
17	6.8	0.55	5	8.77 ± 0.07	23.79 ± 0.83
18	6.8	0.55	5	5.75 ± 0.09	17.26 ± 0.22
19	6.8	0.55	5	3.43 ± 0.08	12.46 ± 0.51
20	8	0.55	5	2.64 ± 0.06	12.41 ± 0.21

Comparing all the three methods, higher purities and high yields led us to choose the freeze thaw method of cell disruption for further optimization.

Optimization of CPE and Total Protein Yields Using Response Surface Methodology

The actual experimental runs as designed by the software and the corresponding responses have been presented in **Table 1** while ANOVA and significance of the models have been presented in **Table S2** (ESI). The coded levels were selected on the basis of the screening experiments. Based on the preliminary findings, freeze thaw method in potassium phosphate buffer (0.1 M, pH 7) had yielded the maximum CPE content as well as the highest purities. Optimization of the pH (A), molar strength of the buffering system (B) and the number of freeze thaw cycles (C) was carried out to accurately predict the behavior of the system. The predicted responses reasonably agreed with the experimental values, emphasizing the reasonable accuracy while using a quadratic model. The positive terms in each equation denote a synergistic and the negative terms denote an antagonistic effect of the variables on the respective responses.

Extraction of CPE

The extraction of CPE was modeled using the following quadratic equation:

$$\begin{aligned}\text{CPE}\ \left(\text{mg g}^{-1}\right) &= 68.6803 - 22.00429 * \text{A} + 4.98545 * \text{B} \\ &+ 5.63342 * \text{C} - 1.59657 * \text{A} * \text{B} - 0.24782 \\ &* \text{A} * \text{C} - 2.56489 * \text{B} * \text{C} + 1.70387 * \text{A}_2 \\ &+ 3.76373 * \text{B}_2 - 0.11592 * \text{C}_2 \end{aligned} \quad (5)$$

The three-dimensional plot has been shown in **Figure 3** while the ANOVA has been presented in **Table S2** (ESI). The adjusted R^2 value (0.8975) agrees with the predicted R^2 (0.8794) while the model equation explains 89.75% of the variations in CPE content. The adequate precision ratio value of 28.16 is able to ignore the noise generated effectively. We observed higher CPE contents when buffers of low molarity were used for extraction. We were interested in the relationship between pH and buffer molarity on CPE extraction, whether any conditions could give us higher yields. We observed that low molarity buffers with the pH 8 resulted in more CPE. It is quite understandable if the extraction efficiency increases with the number of times a batch of biomass is frozen and thawed repeatedly; however, the low salt concentration in the buffers tended to increase the "salting in" effect. The maximum CPE was observed with 0.1 M, pH 8 buffer and 7 freeze thaw cycles (22.99 ± 0.52 mg g^{-1}) (**Table 1**). It was interesting to note that, individually, pH was more influential (p-value 0.0019) compared to when it was analyzed as part of an interaction with other factors (**Table S2**, ESI). It signifies the role that it plays in the stability of CPE in the crude extract. On the other hand, freeze thaw cycles and molarity, taken together, had more significance in CPE extraction (p-value < 0.0001), which supports our argument of "salting in" in low molarity buffers with more freeze thaw cycles per biomass batch.

Extraction of Total Proteins

The total protein content was modeled using the following quadratic equation:

$$\begin{aligned}\text{Total protein}\ \left(\text{mg g}^{-1}\right) &= 109.76057 - 33.40631 * \text{A} \\ &- 16.29643 * \text{B} + 13.92529 \\ &* \text{C}0.016665 * \text{A} * \text{B} - 0.78453 * \text{A} \\ &* \text{C} - 5.02168 * \text{B} * \text{C} + 2.52969 \\ &* \text{A}_2 + 11.20019 * \text{B}_2 - 0.22433 \\ &* \text{C}_2 \end{aligned} \quad (6)$$

The ANOVA values have been presented in **Table S2** (ESI) while the three-dimensional graph has been shown in **Figure 4**. The adjusted R^2 (0.93) agreed with the predicted R^2-value (0.9211) while the signal—to—noise ratio was 36.88, sufficient for the model to ignore the noise generated. The model was capable of explaining 93% of the variation in total protein content. The highest protein yields have been found with 0.1 M buffer and 7 freeze thaw cycles (56.59 ± 0.95 mg g^{-1}, **Table 1**) that again supports the "salting in" hypothesis as proposed with

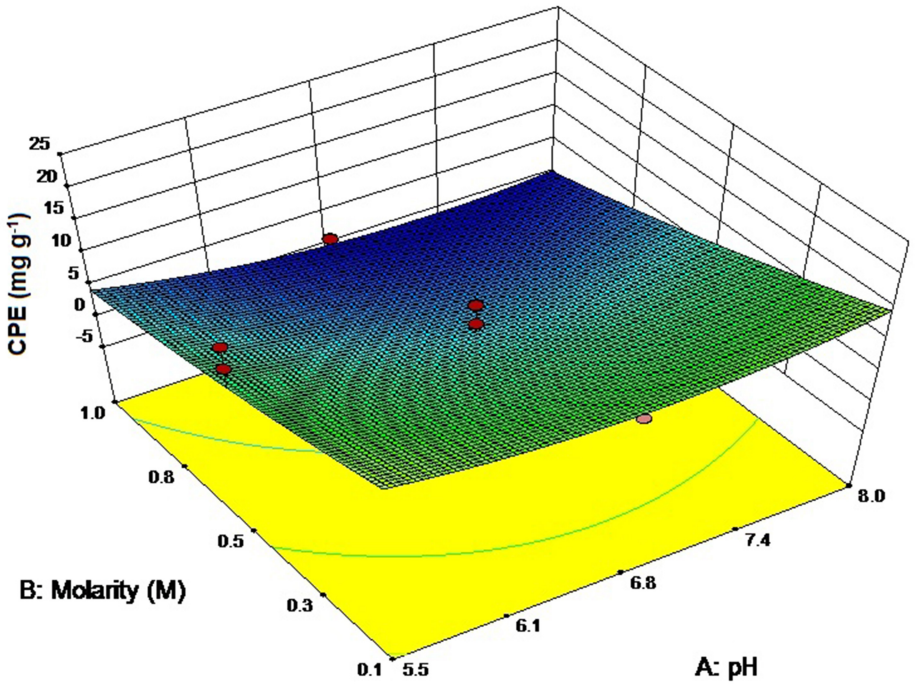

FIGURE 3 | Three-dimensional plot of interaction between pH and buffer molarity for CPE extraction.

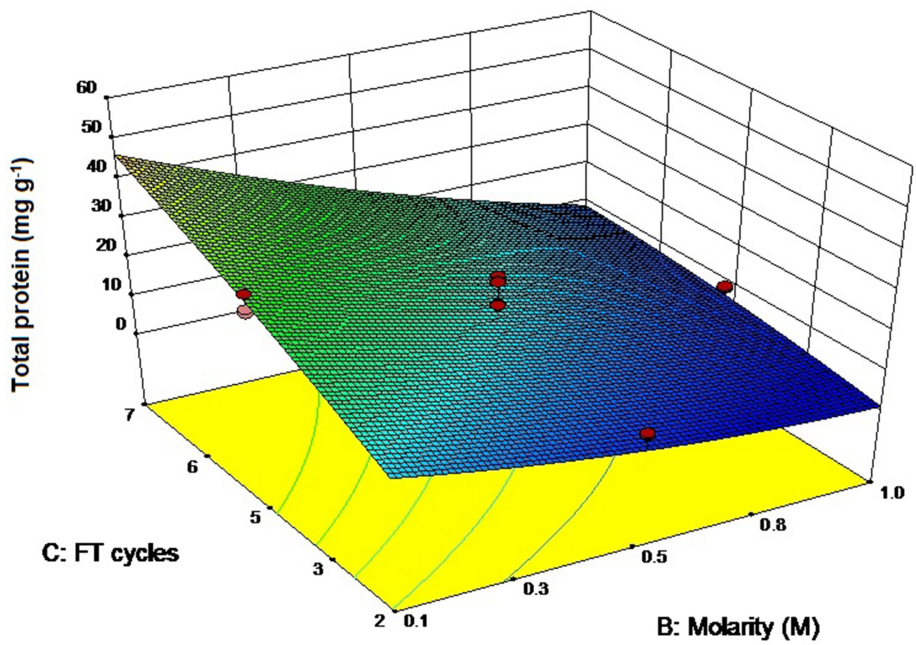

FIGURE 4 | Three-dimensional plot of interaction between freeze thaw cycles and buffer molarity for total protein extraction.

the extraction of CPE. Soni et al. (2008) have reported total protein values of 4.3 mg g^{-1} from *Phormidium fragile* using the freeze thaw extraction method. In comparison, we were able to achieve protein yields as high as 56.59 ± 0.95 mg g^{-1} when the cell mass was freeze thawed in 0.1 M phosphate buffer 7 times. Compared to CPE, pH and molarity together

had a lesser role to play here (p-value 0.9890), presumably because we were interested in all the proteins instead of a specific one. However, the combination of buffer molarity and number of freeze thaw cycles again had a significant role to play in the extraction process (p-value < 0.0001) (**Table S2**, ESI).

Verification of Optimized Models for Extraction of CPE and Total Proteins

The optimized equations were used to predict the CPE and total protein content using 0.1 M phosphate buffer (pH value 6.8) and 5 cycles of freeze thaw for validation of the model. The values have been presented in **Table S3** (ESI). The observed values agreed with the predicted values for all responses, which signify the robustness of the model and the relevance of prediction equations.

Stability of the Crude CPE Extract

Stability in Different pH Values

CPE was found to be pre-dominantly stable under slightly acidic conditions (**Figures 5A,B**). Under a pH range of 3–8, there was not any appreciable difference in the color of CPE extract (data not shown). The spectral differences were also minimal (**Figure 5A**). However, after being stored at pH 9 and upwards, there was a marked difference between the control and the stored samples with significant decrease in absorbance of the chromophore while there was almost complete denaturation under pH 11 and 12. If we look at the trace intensity plot (**Figure 5B**), we can see that the region of stability extends from pH 3–8 while the values at pH 9 and 10 could also be treated being fairly stable. However, the source organism of this CPE is not an alkaliphilic, nor does it come from a region of elevated pH ranges or fluctuations. Hence, its metabolites cannot be safely considered as stable for prolonged durations under extreme pH values.

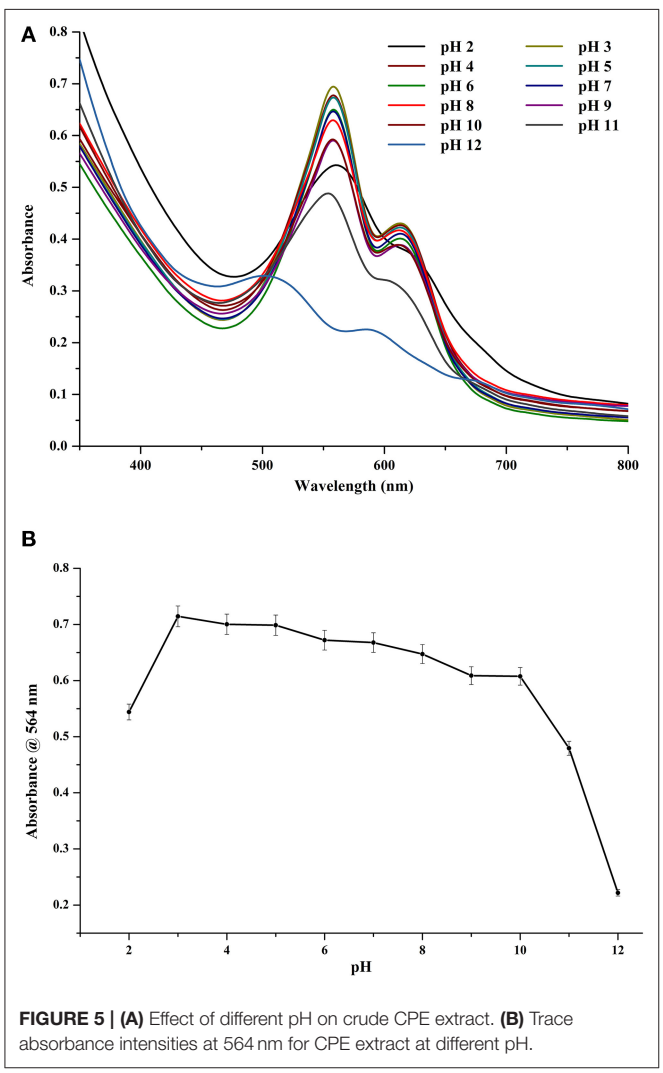

FIGURE 5 | (A) Effect of different pH on crude CPE extract. **(B)** Trace absorbance intensities at 564 nm for CPE extract at different pH.

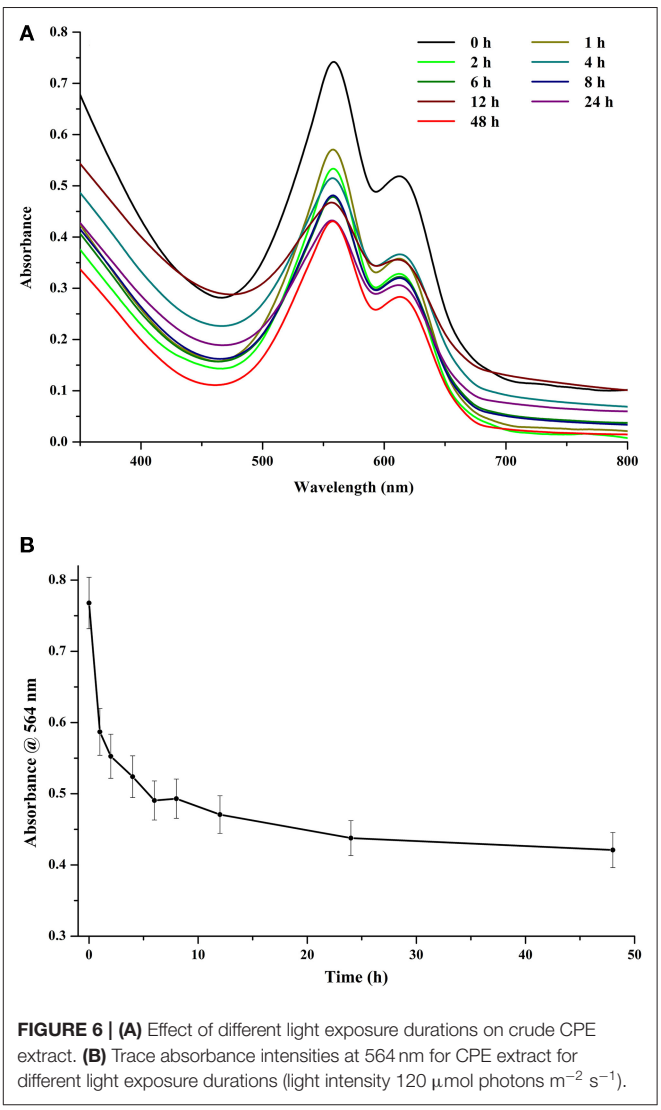

FIGURE 6 | (A) Effect of different light exposure durations on crude CPE extract. **(B)** Trace absorbance intensities at 564 nm for CPE extract for different light exposure durations (light intensity 120 μmol photons m^{-2} s^{-1}).

Stability Under Different Light Exposure Durations

Light played a major role in the stability of chromophore and, consequently, that of CPE. At an exposure of 120 μmol photons m^{-2} s^{-1}, even a time period of 1 h had a detrimental effect on the stability of the crude extract. However, increasing the exposure time further did not have very significant effect on the stability of the chromophore (**Figures 6A,B**). After 12 h of continuous exposure, CPE began to show signs of denaturation (**Figure 6A**). The denaturation profile was noticeable when the control was compared with the sample exposed to 48 h of light. If we look

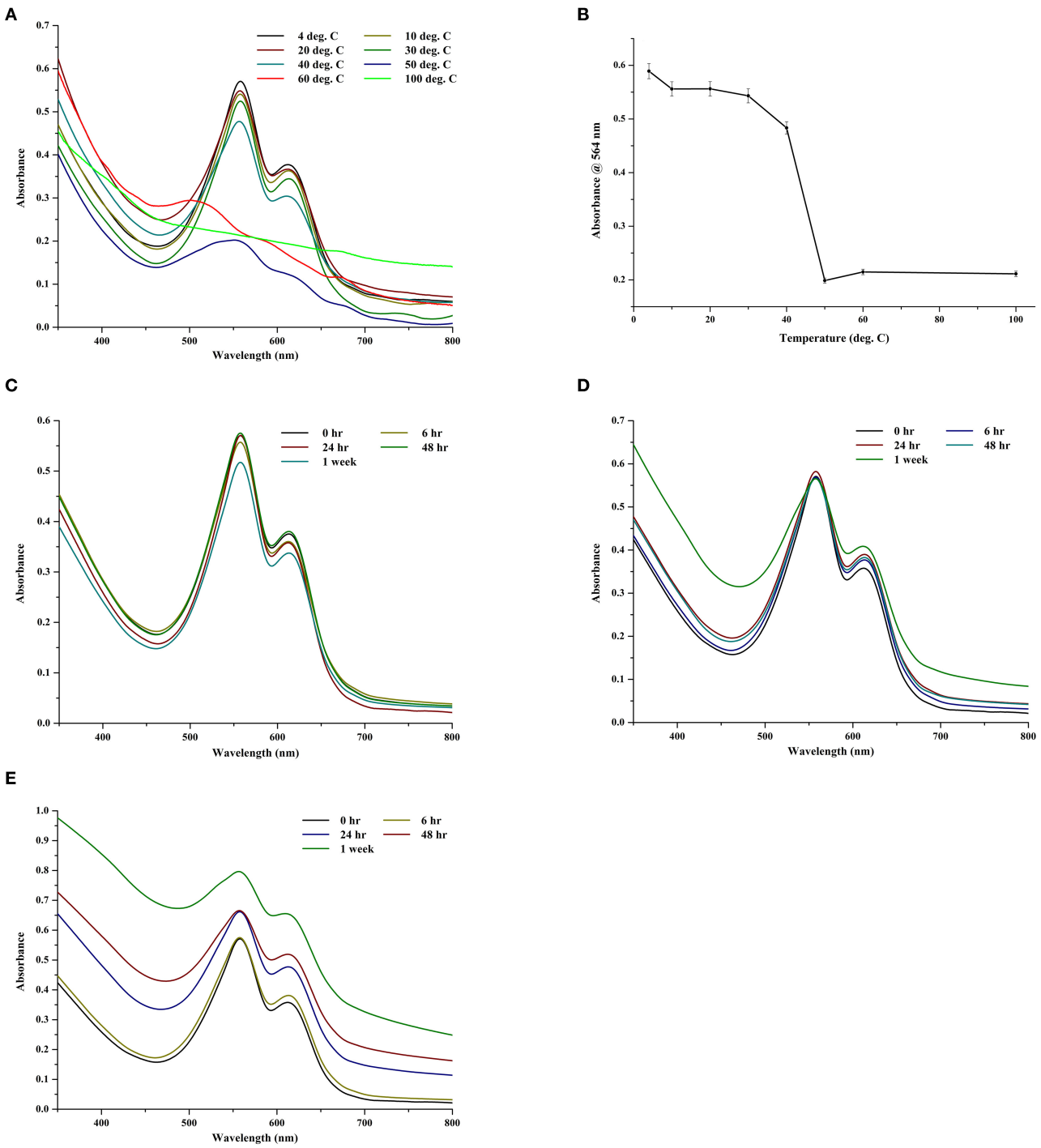

FIGURE 7 | (A) Effect of different temperatures on crude CPE extract. **(B)** Trace absorbance intensities at 564 nm for CPE extract kept at different temperatures for 2 h. **(C)** Effect of storage at −64°C on crude CPE extract. **(D)** Effect of storage at 4°C on crude CPE extract. **(E)** Effect of storage at 10°C on crude CPE extract.

at the trace intensities (**Figure 6B**), there is a sharp decrease in the absorbance profile after an exposure of 1 h, while there is a significant difference between the intensities after 1 and 8 h of exposure. The intensity stabilizes to an extent after 6 h of exposure, after which the color of the tube became visibly faint compared to the control (data not shown).

Stability of the Crude Extract Under Different Temperatures

The thermal and storage stability of the CPE extract was investigated in different temperatures in the dark (**Figures 7A,B**). For the 2-h data, the extract was comparatively stable till 30°C; thereafter, it began to show signs of degradation (**Figure 7A**). At 100°C, it was almost completely denatured with visible loss of color and negligible fluorescence (data not shown). If we look at the trace intensities of CPE during the thermal stability test (**Figure 7B**), there was a sharp decrease in the absorbance after 40°C, which suggests the denaturation of the pigment.

To study the effect of storage, the extract was stored in dark at −64, 4, and 10°C for 1 week and the spectra were collected at appropriate intervals. Predictably, the extracts at −64°C showed negligible loss of color (**Figure 7C**). At 4°C, the chromophore was stable after a week but the protein backbone of CPE began to show signs of deterioration (**Figure 7D**). However, the long term (1 week) storage at 10°C had the maximum deterioration on the extract (**Figure 7E**) wherein both the chromophore and the protein backbone began to degrade. There was a visible loss of color along with a smell of rotting, possible due to the decomposition of sulfur in the amino acids.

DISCUSSION

Choice of Buffer and Cell Disruption Methods Influence CPE Extraction

The extraction of CPE is a complex model with various factors playing a role. The downstream processing of the crude extract and the steps involved in it depends solely on the nature and amount of contamination. The presence of salts has an effect on the solubility of proteins (Arakawa and Timasheff, 1982); small concentrations of salts (<1 M) may actually increase the solubility of proteins as a result of the "salting in" mechanism (Timasheff and Arakawa, 1988). In other words, dilute buffers have better protein extraction abilities. As it is favorable to have as low levels of contaminating proteins as possible, phosphate buffer was the choice since it had the maximum CPE : total protein ratio (*w*/*w*).

Two main buffer types have been reported for the extraction of CPE: tris chloride and phosphate buffers. However, there are wide variations between the ionic strengths utilized for their preparation (Soni et al., 2010; Mishra et al., 2011; Parmar et al., 2011; Senthilkumar et al., 2013b; Munier et al., 2014; Sonani et al., 2014; Patel et al., 2018). Both these buffers have been used for seaweeds as well as cyanobacterial extraction procedures and have been effective. However, there is a lack of knowledge about other buffer and/or de-ionized water for the extraction of CPE. There are very few reports on Goods' buffers or de-ionized water as a solvent to extract phycobiliproteins. Peterson and colleagues have used a 0.03 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/PIPES combination buffer for the isolation of phycobiliproteins from heterocysts or intact cells of *Anabaena variabilis* (Peterson et al., 1981) while another study has used water for extracting CPE and CPC (Mubarak Ali, 2013). The choice of buffer is dictated mostly by the availability and economics of the chemicals and their ease of use. While de-ionized water is the simplest extractant available, it also solubilizes the non-targeted proteins, through osmolysis, which contaminate the extract. Phosphate salts of sodium and potassium are one of the most easily available and thus, are widely used for making the buffers.

Cell disruption system is another area which needs a comparative study for proper conclusions. Enzymatic hydrolysis, ultrasonic cavitation, repeated freeze–thaw and manual crushing are some of the methods utilized for extraction of phycobiliproteins. Enzymatic hydrolysis is especially useful for those organisms which have a tough cell covering and/or outer sheath to protect the cells. For instance, it specifically dissolves the cell wall components in seaweeds and has been reported in a few studies (Reis et al., 1998; Santiago-Santos et al., 2004; Dumay et al., 2013) with reported yields ranging from 0.5 to 150 mg g^{-1}. However, purified enzymes are not traditionally required for cyanobacterial cultures since their cells are devoid of any specialized protection.

Repeated freezing at sub-zero temperatures followed by instant thawing is intended to shock and rupture the fragile cell walls and is considered to be one of the gentler techniques from the point of view of the proteins, since there is no heat generation involved. As such, it is one of the most common methods used for phycobiliprotein extraction (Soni et al., 2010; Mishra et al., 2011; Parmar et al., 2011; Ghosh et al., 2016, 2018, 2020; Patel et al., 2018; Ghosh and Mishra, 2020) with recoveries ranging from ∼29 to 102 mg g^{-1} of cell dry weight in the crude extract.

Ultrasonication is a cell breakage method which uses ultrasonic sound waves to achieve acoustic cavitation—the rapid formation, growth and collapse of air bubbles in a liquid medium. It is a rapid method for cellular disruption. The rapid formation and collapse of bubbles can lead to a considerable release of heat energy, a factor that may contribute to denaturation. Johnson et al. (2014) have reported total PBP yields ranging from 19 to 42 mg g^{-1} dry mass basis using sonication as a cell disruption method while (Safi et al., 2014) have reported total water-soluble protein yields between 8.5 ± 0.0 and 67.0 ± 0.9% in 5 different micro and macroalgae when they utilized sonication as a method for cell breakage. Sun et al. (2009) have also utilized ultrasonic cell disruption for R-phycoerythrin extraction from *Heterosiphonia japonica* at a power of 350 W for 10 min keeping the temperature constant at 0°C.

Manual crushing of biomass with or without using an abrasive is also reported in some studies (Gómez-Lojero et al., 1997; Munier et al., 2014). Due to generation of heat from the crushing process, the CPE extracted runs the risk of being denatured even before it is completely extracted into the buffer. Although 33.76% of the extracted protein using this method comprised of CPE, the low recoveries and purity ratio was a major reason for not selecting this particular method.

Due to the lower levels of contamination, phosphate buffer [42.46% (*w*/*w*) CPE in total extracted proteins] and freeze–thaw [33.72% CPE (*w*/*w*) in total extracted proteins] method were chosen for further experiments.

Optimization of the Extraction Conditions Using Statistical Optimization

Statistical optimization of the extraction conditions involved optimization of three processes: pH (A), buffer molarity (B) and number of freeze thaw cycles (C). The solid:liquid ratio (SLR) was kept constant at 0.125 ml mg^{-1} and the buffer volume was adjusted according to the dry weight in each tube. To take advantage of the salting—in effect, low molarity buffers were prepared which were able to achieve more extraction compared to higher molarity ones. The number of cycles also played a role in the extraction process; however, increasing the number of cycles would also increase the total protein content. Keeping this in mind, the trick was to moderate the freeze thaw cycles with low molarity buffer (pH 8).

The amount of total proteins was also less in pH 8 buffers, compared to pH 6.8 and 5.5, which makes purification of the crude extract easier and also avoids unwanted contamination. Since this process is being optimized for a food ingredient, it is necessary to reduce unwanted molecules in the extraction process. The purity ratio (3.84) was also better in pH 8 buffer. If we could choose one condition, it would be to use pH 8 low molarity buffers for maximal CPE extraction, with minimal contaminating proteins.

During the verification of the model, the experimental and theoretical values agreed with each other, which indicates the model suitability and efficacy.

Stability of CPE Crude Extract

The stability of the crude extract was determined on the basis of absorbance readings for the CPE samples. The extract was stable under the experimental conditions, with no unwanted odors and colors. The color stability of CPE depends on the chromophore, while the structural stability is significantly dependent on the protein backbone.

The results from the pH experiments suggest that CPE extract is stable for short durations, within a pH range of 3–8 range (**Figure 5A**), which is beneficial for the beverage industry. Many of the beverages and drinks have an acidic pH, so a colorant stable under such conditions is highly desirable. The light exposure duration experiments suggest there is decrease in the stability of CPE extract after 1 h of exposure, which can be seen in the absorbance spectra (**Figure 6A**). The stability of the extract to temperatures as high as 40°C for 2 h is advantageous since it protects CPE from short duration fluctuations in temperature. Above 40°C, there was considerable degradation in CPE stability (**Figure 7B**). The storage of CPE extract under different temperatures showed stability at −64°C (**Figure 7C**) which was expected since it reduces the water activity of the protein to a minimum. Long duration storage at 10°C was found to be unfeasible since CPE showed signs of degradation within 24 h of storage with peak broadening and appearance of a shoulder peak (**Figure 7E**). The shoulder increased in prominence with time till it was clearly noticeable after a week. Sub-zero temperatures are, obviously, ideal for long-term storage; it is necessary to avoid repeated freezing and thawing of the samples and not to break the cold chain.

A comparative study performed by a research group has shown the stability of phycoerythrin from 2 species of seaweeds. It concluded that phycoerythrin from *Porphyridium cruentum* and *Grateloupia turuturu* was stable up to 40°C, in a pH range of 4–10 and could withstand up to 8 h exposure to a light intensity of ∼34 μmol m^{-2} s^{-1} (Munier et al., 2014). Moreover, the sensitivity of these pigments is said to increase in dilute solutions. A study has reported the stability of phycoerythrin from *Palmaria palmata* up to 60°C and also within a pH range of 3.5–9.5 (Galland-Irmouli et al., 2000). In comparison, our extract also gave similar results and could be a strong contender for the food color market in the near future.

CONCLUSION

This study establishes an optimized protocol for the extraction of CPE for the food industry and also studies the stability of the crude pigment under different physiological conditions, such as pH, temperature and light exposure. Preliminary screening involving different buffers and extraction method suggested phosphate buffered system with repeated freeze thaw to be the ideal approach for a high yield of CPE with lesser contaminants. Further in the optimization process, 0.1 M phosphate buffer (pH 8) with 7 freeze thaw cycles was observed to be ideal for wholesome extraction of CPE. However, the number of freeze thaw cycles had a significant impact on its extraction due to the varying extents of cell breakage. More the number of cycles, more was the amount of total proteins extracted in to the buffer along with CPE. The crude extract was also studied with regards to stability whereby it was found that CPE extract is stable up to 40°C for 2 h and could endure prolonged storage at 4°C. It was also found stable within a pH range of 3–8. Although still in the preliminary stages, such a colorant would be immensely beneficial for the food industry.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

TG was responsible for conducting the experiments, data collection and analysis and writing the manuscript. SM provided critical guidance during the study and manuscript preparation. All 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/fsufs.2020.00102/full#supplementary-material

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Bacterial Pigments: Sustainable Compounds With Market Potential for Pharma and Food Industry

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The continued universal application of synthetic colorants for decades have caused environmental pollutions and human health vulnerabilities. So, it was indispensable to discover novel natural colorants such as microbial colorants which were safer and better than synthetic colorants. The potential of bacterial pigments for mass production of diversified coloring properties was first prospective and is now getting the notable importance and attention of both the researchers and industries. Literature establishes that the natural colorants produced from microbes were applied in food and pharma products successfully. Apart from serving as food colorants, bacterial pigments have several pharmacological activities like anti-microbial, anti-cancer, anti-oxidant, anti-inflammatory and anti-allergic properties with large economic potential. And, there is vast scope for easy and cheap production of natural colorants in all seasons from bacterial sources, compared to plant sources. Tactics in strain improvement, fermentation conditions, metabolic engineering, and easy extraction techniques are needed to produce high end products. This review highlights the significance of bacterial colorants and summarizes its application in food and pharma industries. Further, the major challenge of lower stability of bacterial pigments and the solution to address it is also appraised.

Keywords: natural colorants, bacterial colorants, scale-up, genetic engineering, food grade pigments, pharmacological activities, challenges, instability

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INTRODUCTION

Industries of late, have resorted to use many important microbial metabolites like antibacterials, antifungals, vitamins, enzymes, pigments, etc., for varied applications as being alternative ones to synthetic products. Changing nature of such kind of the industrial behavior have enthused both academia and industries to discover renewable and biodegradable natural products from microbial resources. Nevertheless, the task of discovering potent novel microbial pigments for the optimal applications in food and pharma products, in spite of several research studies is still a challenging one requiring appropriate approaches (Sen et al., 2019) and this review suggests the ways and means to overcome such challenges in this field.

The production of microbial pigments has two basic approaches:

a) Finding new strains of pigment producing microbes from diverse environments and enhance their productivity.
b) Achieving enhanced yields from the already recognized species either by strain improvement and process development.

Currently, the use of natural colorants has been encouraged and protected by laws and this inclination will continue in the coming years too. Public opinion toward natural colorants is encouraging due to their safety and biodegradable capabilities besides their production economy. Natural stains are slowly replacing synthetic colorants and this change has encouraged the possibilities for the high production of natural dyes and pigments and their sustainable utilization in all prospective fields. Yet there are many challenges in front of us; as natural colorants are sometimes less stable and covering a limited range of shades only and are not extensively exploited like current artificial colorants. Moreover, they require substantial material to attain equivalent color strength (Rodriguez-Amaya, 2016). Amidst various hurdles, the most important hurdles in the production of natural colorants are getting regulatory approval for new products and developing natural colorants which can resist to heat, light and acidic environments. Focusing the investigations more on the prospects of genetic engineering and up-scaling the pigment production.

Growth drivers for natural colorants

- There is an increased awareness on the harmful influence of synthetic colorants and the resultant boosts in the demand for natural colorants.
- In some international markets, there are restrictions and limitations in manufacturing and trading of synthetic colorants which drives toward alternate natural colorants.
- There is an increase in demand to color unique products like toys, crayons, textile printing, hand-made paper etc. using natural colorants.

The prospective of bacterial pigments in health care has stimulated attention toward the search for alternate natural colorants. These colorants receive significant consideration because of strict rules and regulations applied to synthetic pigments. The advances in fermentation processes have enabled the bacterial pigment production to reach business scale. However, two existing fundamental issues are to be addressed: (1) economic and (2) marketing difficulties. So, alternative strategies such as smarter screening methods, apt fermentation processes, cost-effective down streaming, genetic engineering for strain improvement etc. should be involved more effectively in valued natural pigment production from bacteria (Ramesh et al., 2019). Such approaches would be a matter of great interest on the production of natural pigments and its economics from industrial perspectives (Majumdar et al., 2019). This review appraises the issues of bacterial colorants, fermentation strategies, genetic engineering for strain improvement, scale up of bacterial colorants from lab scale to industrial level, pharmacological applications, food colorants besides the stability issues of bacterial colorants.

INDUSTRIAL IMPORTANCE AND MARKET POTENTIAL

Owing to the hazardous nature of synthetic colorants, the current exploitations and the prospective of microbial pigments as natural colorants in the food industry are promising. Food and Drug Administration (FDA) already approved pigments existing in the market such as riboflavin (*Ashbya gossypii*), β-carotene and lycopene (*Blakeslea trispora*), Arpink Red (*Penicillium oxalicum*), astaxanthin (*Xanthophyllomyces dendrorhous*), and *Monascus* pigments (Venil et al., 2013). FDA and EFSA (European Food Safety Authority) assess the safety of food additives through international guidelines and codes of practice. During the assessment process, the need for color requirement must be described with strict toxicity testing prior to inclusion for assessment. Also, when there are changes in the manufacturing conditions for food applications, safety of additives have to be reexamined (Scotter, 2015).

The estimation of the universal food colorant market is anticipated to touch 3.75 billion USD by 2022. The marketplace is determined by the organizations' procedures to develop item claim through colorants, interest in clean name items, and headway in scientific novelties. The farming colorant market is anticipated to grasp 2.03 billion USD by 2022 (www.bccresearch.com). The global market polls estimated the market potential of carotenoids for the years 2018–2024 in foods, beverages, pharmaceuticals, cosmetics, animal feed and dietary supplements as 26.1, 9.2, 6.5, 34.8, and 23.5%, respectively. Presently, 80–90% of the carotenoid synthesis is fulfilled by chemical synthesis (Saini and Keum, 2019). The market value of natural carotenoids is less (24%) than synthetic carotenoids (76%) because of their high cost (Market Research Report, 2016). The low cost of synthetic carotenoids ($250–2,000 kg^{-1}) is the main factor behind its huge market, whereas natural carotenoids have higher market values ($350–7,500 kg^{-1}) according to the Deinove biotechnology company (Deinove, 2019). The plant-derived carotenoids are expensive and hence scientific interest in bacterial carotenoid production has escalated in recent years because of their sustainability and cost-effectiveness (Ram et al., 2020).

Carotenoids have many medicinal and health-improving properties and are also utilized in nutraceuticals, make-ups, pharmaceuticals and their usage is probably going to be expanded in the following years. Thus, carotenoids are considered as profitable business prospects for the food, healthcare and beautifying agent ventures sooner rather than later (Sathasivam and Ki, 2018). The worldwide carotenoid (astaxanthin, beta-carotene, canthaxanthin, lutein, lycopene, zeaxanthin) platform is evaluated to be at USD 1.53 billion by 2021. The important carotenoid-containing biomass from *Haematococcus* sp. and *Chlorella* sp. are sold at a cost of 40–50 USD per kg in the open market. ß-carotene acts as an antioxidant and destroys the impact of free radicals in the human assimilation up-to numerous folds

(Fiedor and Burda, 2014). Mounting interest for beta-carotene because of its high viability and therapeutic properties is setting off the market for beta-carotene. Astaxanthin market is foreseen to reach USD 814.1 million by 2022 at a CAGR of 8.02% from 2017. Europe has moved emphatically toward the utilization of natural colorants and leads the way and they utilize 85% of natural colorants (www.bccresearch.com). Expanding interest for processed and ready to eat food in India, China and Middle-East is expected to drive food colorant market in Asia Pacific.

DIFFERENT SHADES OF BACTERIAL PIGMENTS

Eco-friendly and non-toxic bacterial colorants are gradually replacing synthetic colorants in food, pharmaceuticals, textiles and cosmetics (Chiba et al., 2006). The current concern is on producing coloring materials using microbes (Usman et al., 2017). Pigment synthesis depends upon the microbes and fermentation conditions. Pigment production in a mini bioreactor should be carried out before proceeding to a pilot plant for commercial production (Banerjee et al., 2013).

Microbial pigments include astaxanthin, canthaxanthin, carotenoids, melanins, granadaene, indigoidine, flavins, quinones and more specifically monascins, prodigiosin, pyocyanin, rubrolene, scytonemin, violacein, phycocyanin possessing various activities like anti-oxidant, anti-carcinogenic, anti-inflammatory and anti-obesity properties (**Table 1**) (Fernandez-Orozco et al., 2011; Kim et al., 2011). Bacterial pigments can magnify the existing palette of colors used in numerous applications. Bacterial pigments are considered safe and can be used as natural colorants which will benefit the human health and save the ecosystem (Malik et al., 2012). Many promising carotenoid producing bacterial strains belonging to *Arthrobacter, Flavobacterium, Chryseobacterium* and *Zobellia* genera were isolated from King George Island, Antarctica. They produce 10 different carotenoids like zeaxanthin, β-cryptoxanthin and β-carotene (Vila et al., 2019).

Bacteria produce different shades of color like red (*Serratia marcescens, Gordonia jacobaea*), red-yellow (*Kocuria* sp., *Chryseoacterium artocarpi*), yellow (*Micrococcus*, *Hymenobacter* sp. and *Chryseobacterium* sp.), green (*Pseudomonas* sp.), blue (*Corynebacterium insidiosum, Erwinia chrysanthemi, Vogesella indigofera*), purple (*Chromobacterium* sp.) as shown in Munsell color system (**Figure 1**).

GENETIC ENGINEERING FOR STRAIN IMPROVEMENT

Through genetic engineering, bacteria could be modified to produce pigment of interest. The remarkable increase in fermentation efficiency and the subsequent decrease in cost have made it possible through mutagenesis to select prospective pigment producing strains (Venil et al., 2014; Rao et al., 2017). Protein engineering by random and rational approaches is possible through the developments in metabolic engineering. These approaches may support the improvement of pigment production biologically. The use of recombinant microorganisms provides pathway to modify the active components to develop standard pigments with attractive pharmaceutical properties. To produce valuable bacterial pigments in good qualities, genetic engineering has proven to be highly active in a value effective manner (Numan et al., 2018). Pigment producing bacterial strain was engineered to overproduce pigment and thereby changing the color and structure. The blue pigment, actinorhodin producing *Streptomyces coelicolor* has been genetically engineered to produce bright yellow polyketide, kalafungin and used to produce anthraquinone type pigment. The cell factories to effectively produce pigments has been developed by heterologous expression by biosynthetic pathway from known pigment producers (Sankari et al., 2018). The biosynthetic pathway for microbial pigments is fully understood by identifying and engineering the genes for overproduction. Cloning the pigment biosynthesis gene into microbial vectors like *E. coli, Bacillus subtilis, Pseudomonas putida, Corynebacterium glutamicum, Pichia pastoris* is the cost-effective and economic way for industrial production processes (Sen et al., 2019). The carotenogenic genes from *Xanthophyllomyces dendrohous* or *Agrobacterium aurantiacum* are genetically modified to produce carotenoids (lycopene, astaxanthin, β-carotene) (Heider et al., 2014).

The biosynthetic pathways of bacterial pigments have delivered chances for heterologous gene expression in recombinant bacteria. The advancement in genetic engineering would make bacterial pigments available in market, if the genomes of native pigment producing bacteria could be altered suitably. The genetic engineering is encouraged by the possible industrial uses wherever value-added strategies for developing strains are adopted to enhance the pigment production (Saini et al., 2020). The genetic engineering of bacteria to produce higher level of pigments is highly possible through biotechnology (Pham et al., 2019).

The targets for pigment production through genetic engineering include red (lycopene), orange (β-carotene) and the purple (violacein) pigment producing bacteria that contributes significantly for pigment production. Alper et al. (2005), Farmer and Liao (2001), and Yoon et al. (2006) studied the overproduction of lycopene and isoprenoids through genetic engineering and testified that they possess industrial significance (Ajikumar et al., 2010). Because of the various prospective applications of violacein as antimicrobial and antitumor agents, it has been the major topic of research in metabolic engineering.

Pseudomonas sp. produces blue pigment, pyocyanin comprised of N-methyl-1-hydroxyphenazine; MvfR gene, producing a transcription factor activates phnAB genes (Norman et al., 2004). Mavrodi et al. (2001) studied that these genes produce quinolone, which are important for pyocyanin synthesis. Jayaseelan et al. (2014) have analyzed the anti-microbial activity of pyocyanin and its use as bio-control agents. Melanin has various applications and widely used in cosmetics, eyeglasses, sunscreens etc. Melanin is produced by *Bacillus* sp., *Pseudomonas* sp., and is used to screen recombinant strains and in the treatment of metastatic melanoma (Surwase et al., 2013).

TABLE 1 | Bacterial pigments with high potential to be used as natural colorants.

Bacteria	Pigment	Color	Bioactivity	Status	References
Agrobacterium aurantiacum *Paracoccus carotinifaciens*	Astaxanthin	Pink-Red	Antioxidant, anticancer Anti-inflammatory, antioxidant Anticancer	RP/IP	Ambati et al., 2014
Bacillus cereus	Azaphenanthrene	Green	Anticancer, antibacterial, textile dyeing	RP	Banerjee et al., 2011
Rhodococcus maris *Kocuria* sp.	Beta-carotene Carotenoids	Bluish-Red Yellow	Used to treat various disorders such as erythropoietic protoporphyria. Reduces the risk of breast cancer Anti-cancer activity	DS	Kirti et al., 2014
Bradyrhizobium sp. *Lactobacillus pluvialis*	Canthaxanthin	Orange	Antioxidant, anticancer	RP	Dufossé, 2006; Ram et al., 2020
Chryseobacterium artocarpi	Flexirubin	Yellowish-Orange	Treatment for chronic skin disease, eczema, gastric ulcers	RP	Venil et al., 2015
Streptococcus agalactiae	Granadaene	Orange-Red	Antioxidant, detoxify ROS	DS	George and Nizet, 2009
Proteobacteria	Heptyl prodigiosin	Red	Antiplasmodial	DS	Lazaro et al., 2002
Corynebacterium insidiosum *Erwinia chrysanthemi*	Indigoidine	Blue	Protection from oxidative stress Antioxidant Antimicrobial	NK	Reverchon et al., 2002; Chu et al., 2010
Pseudomonas guinea	Melanin	Black	Antioxidant activity	NK	Tarangini and Mishra, 2013
Pseudomonas sp.	Phenazine	Yellow	Biological control	NK	Arseneault et al., 2013
Pseudomonas sp.	Phycocyanin	Blue, Green	Cytotoxicity, neutrophil apoptosis, proinflammatory	IP	Murugan, 2012
Serratia marcescens *Pseudoalteromonas rubra*	Prodigiosin	Red	Anticancer, DNA cleavage, immunosuppressant	IP	Melvin et al., 2000; Deorukhkar et al., 2007; Feher et al., 2008
Bacillus sp.	Riboflavin	Yellow	Nutritional supplement	RP	Lim et al., 2001
Bacillus subtilis	Riboflavin	Yellow	Food additives		Schwechheimer et al., 2016
Streptomyces echinoruber	Rubrolone	Red	Antimicrobial	DS	Dharmaraj et al., 2009
Staphylococcus aureus	Staphyloxanthin	Golden	Antioxidant, detoxify ROS	NK	Liu et al., 2005a,b; Clauditz et al., 2006
Cytophaga/Flexibacteria	Tryptanthrin	Light dark yellow	Antioxidant, anticancer	NK	Solieve et al., 2011
Streptomyces sp.	Undecyl prodigiosin	Red	Antibacterial, antioxidative, UV-protective, anticancer	NK	Liu et al., 2005a,b; Stankovic et al., 2012
Janthinobacterium lividium, *Pseudoalteromonas tunicate*, *Chromobacterium violaceum*	Violacein	Purple	Anti-bacterial, anti-viral, anti-leukemic, anti-fungal, anti-parasitic, anti-protozoal, anti-oxidant and anti-ulcerogenic.	NK	Venegas et al., 2019
Xanthomonas oryzae	Xanthomonadin	Yellow	chemotaxonomic and diagnostic markers	NK	Schoner et al., 2014
Flavobacterium sp., *Paracoccus* sp.	Zeaxanthin	Yellow	Photo protectant, antioxidant	DS	Berry et al., 2003

Industrial status adopted from Dufossé (2018).
DS, Development stage; IP, Industrial production; RP, Research project; NK, Not Known.

Sphingobacterium multivorum produces zeaxanthin and utilize deoxyxylulose-5-phosphate (DXP) pathway for its enhanced production (Ram et al., 2020). Numerous bacteria were explored for IPP biosynthesis, mevalonate and DXP pathways. Additionally, the identification of genes (IPP biosynthesis) in metabolic engineering of bacteria have increased the production of carotenoids (Lange et al., 2000; Calegari-Santos et al., 2016; Dufossé, 2018). Isorenieratene and the derivatives, the aryl carotenoids, are not attainable for industrial applications (Valla et al., 2003). Aryl carotenoids produced in culture medium by *Brevibacterium linens* is insufficient for commercialization and researchers have developed novel syntheses. Nevertheless, genetic modifications of pigment producing bacteria with potent antioxidant activity could be a substitute for carotenoids (polyenic-phenolic) (patent application WO 2008009675).

Violacein has been produced by *Chromobacterium violaceum* (Durán and Menck, 2001), *Janthinobacterium lividum* (Pantanella et al., 2007), *Pseudoalteromonas luteoviolacea* (Thogersen et al., 2016), *Duganella* sp. (Wang et al., 2009), *Collimonas* sp. (Choi et al., 2015a), and *Pseudoalteromonas* sp. 520P1 and 710P1 (Yada et al., 2008). Many researchers have cloned and expressed the genes for violacein production which occur within a single operon, vioABCDE (Kothari et al., 2017) and they have cloned and expressed these genes in other bacterial hosts, including *E. coli* (Rodrigues et al., 2013). A cosmid vector, pHC79, was cloned by violacein gene cluster

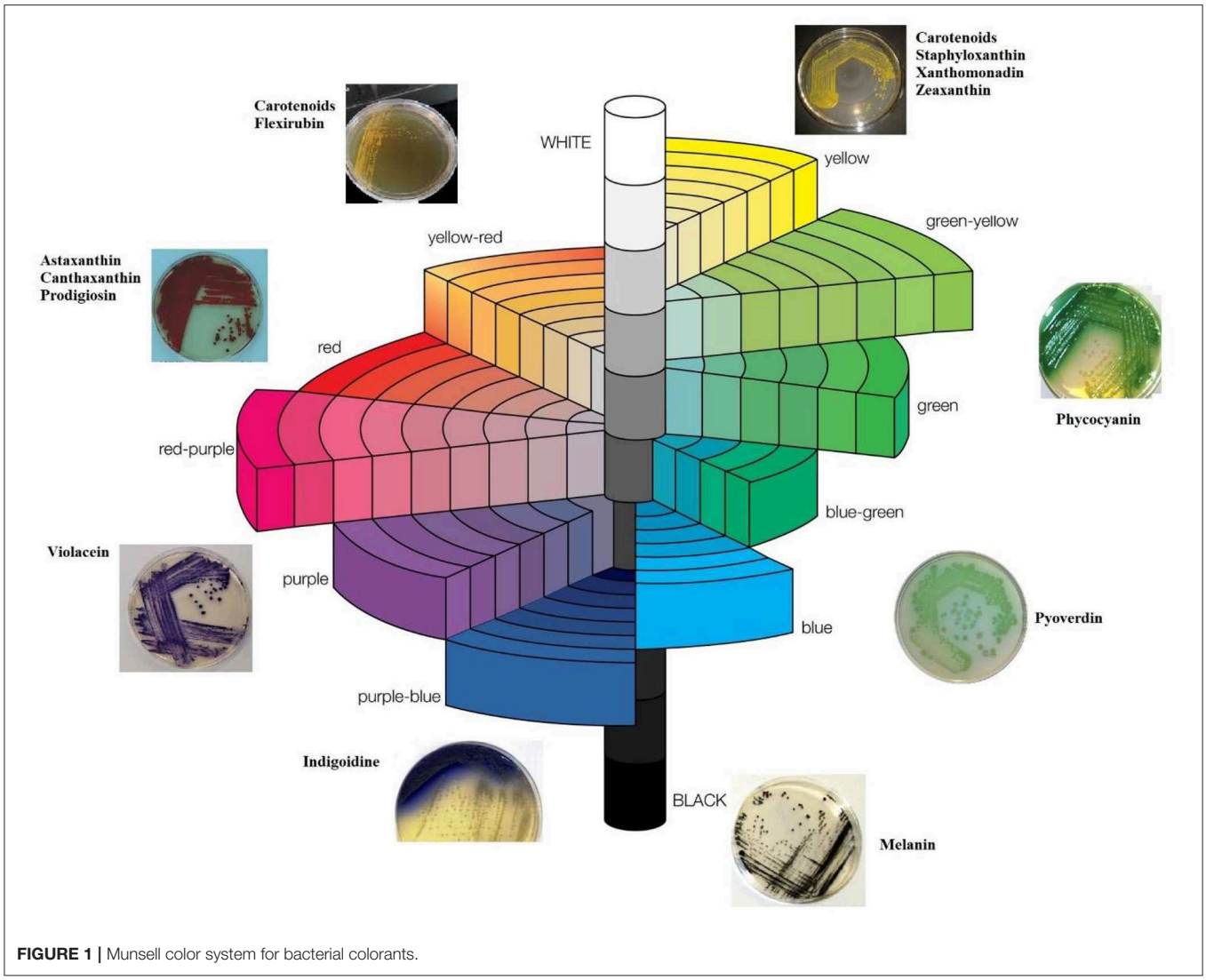

FIGURE 1 | Munsell color system for bacterial colorants.

and this was unstable and lost more than 50% of the bacterial growth at 15th generations (Choi et al., 2015b). The vioABCDE operon in pUC18 vector generates stable IncP plasmid and this plasmid is more stable for 100 generations without antibiotics, making it a valuable tool for violacein production (Ahmetagic and Pemberton, 2010).

For bacterial pigments, the biosynthetic pathway is a very crucial step and this is followed by identification and engineering the genes responsible for production of pigments (Sen et al., 2019). Cloning the pigment genes to microbial vectors (*E.coli, Bacillus subtilis, Corynebacterium glutamicum, Pichia pastoris*) is a cost-effective process for industrial fermentation. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-CAS9 technology in genetic engineering has lately been used for the large scale production of bioactive components. The colorant gene is injected into its genome via CRISPR-CAS9 system to engineer and produce natural colorants (Sen et al., 2019).

The carotenoid production, mainly β-carotene and torularhodin from *R.mucilaginosa* KC8 is enhanced using metabolic engineering and mutagenesis (Wang et al., 2017). The ultimate aim of metabolic engineering is higher production with downstream process and purification (Watstein et al., 2015). High carotenoid contents can be achieved by over expressing the isopentenyl-diphosphate delta-isomerase and 1-deoxy-D-xylulose-5-phosphate synthase gene in the DXP pathway (Choi et al., 2010). *E.coli* mutant was created by inserting carotenoid biosynthetic genes from *Deinococcus wulumuqiensis,* resulting in a 2.2-fold increase in lycopene production compared to wild strains (Xu et al., 2018). Metabolic engineering for strain improvement leads to the development of highly efficient strains producing pigments catering for industrial needs (Aruldass et al., 2018). The production of lysine and astaxanthin from *Corynebacterium glutamicum* was achieved when modified through chromosomal deletion and integration using suicide vector pK19mobsacB (Henke et al., 2018). *Rhodobacter sphaeroides* was genetically re-designed to obtain enhanced production of lycopene (Su et al., 2018). Park et al. (2018) have reported that with latest computational prediction tools, the use

of flux variability scanning based on the enforced objective flux algorithm to identify amplified gene targets, has resulted in to produce high content of astaxanthin in *E.coli*.

Advancement of the metabolic engineering in the industry sector will necessitate further large scale fermentation. Baseline methods for pigment production at laboratory level has been achieved in many studies and further researches centering on the increased production in terms of pathway design and structure are necessary. This trend will move metabolic engineering strategies beyond production of colorants to valuable natural products with prospective applications in pharmaceuticals, cosmetics, food industry etc. The expansion of engineering tools will also widen the scope for pigment production and this field is in the rise. It will be interesting to observe the future outcomes of metabolic engineering with new streams by altering the metabolome of pigmented bacteria (Yadav et al., 2012). The success of metabolic engineering is the beginning of sustainable biotechnological production. The strain development targets will be increased by genome, proteome and system biology. The breakthroughs in metabolic engineering will boost up microbial biotechnology on pigment production (Kumar and Prasad, 2011).

Genome Shuffling

Genome shuffling is the latest development for phenotypic enhancement that has established major attention toward industrially important strains (Magocha et al., 2018). The advantage of this technique is that genetic changes can be made in a microorganism without knowing its genetic background i.e., simplicity and does not require expensive genetic tools. Genome shuffling can induce mutations at diverse points for complex phenotypes without genomic data of the targeted strains (Biot-Pelletier and Martin, 2014). Violacein pathway is reported for the first time in *Saccharomyces cerevisiae* and expressed by Lee et al. (2013b). The high violacein producing strains can be screened by a combinatorial method thereby increasing the metabolic flux in this pathway. By chromosome rearrangement and modification by SCRaMbLE, the prodeoxyviolacein production is improved in haploid yeast (Wang et al., 2017). Genetic engineering will play a central role in the development of high pigment producing strains and counters high capital investment by warranting a successful commercialization.

FERMENTATION STRATEGIES

Fermentation strategies are widely used to meet the market demands for the enhanced production of bacterial pigments which have wide-ranging uses in pharmaceutical, chemical and food industries. Commercial production of bacterial pigment is possible when fermentation technology combined with genetic engineering technology is applied in achieving high yields of pigments. Bacterial pigments cannot be compared with synthetic pigments because of its low yield (Nasrabadi and Razavi, 2011). Different types of fermentation strategies are employed and they can be determined by the type of strains used and pigments extracted (Nigam and Luke, 2016).

Medium optimization is the most critical strategy for large scale production to meet the market demand. In earlier times, medium optimization was conducted using traditional methods which is time consuming, costly and have numerous trial runs. Medium optimization is becoming more effective, economical and robust with the advancement of current mathematical and statistical methods (Singh et al., 2016). Many researches claim that substantial improvements for medium optimization were achieved using experimental designs. Response surface methodology (RSM) was applied to improve fermentation parameters by avoiding unnecessary addition of components to the medium and to meet the nourishing demand for pigment producing bacteria. In addition to estimating the association of the medium components and product yield, the concentration of medium components also are evaluated to reach maximum production. By optimizing the carbon and nitrogen sources, high pigment production at higher concentration can be attained (Wang et al., 2011). However, RSM has some limitations in designing an objective function. The reliability of the technique will be confronted by the interaction between the factors and the response, which will increase the difficulty of the study (Pal et al., 2009; Kim et al., 2011). To overcome the drawbacks and to continually improve the efficiency of the RSM, various optimization algorithms have been designed over the past few decades.

In this perspective, Artificial Neural Networks (ANNs) are applied in modeling of biological systems and gained attractiveness in the intelligent decision making. Prior specification is not required in ANN and it can optimize non-linear functions. Pilkington et al. (2014) reported that ANN will have higher accurateness than RSM. The ANN comprises three layers of information known as input layer, hidden layer and output layer and is appropriate for medium optimization as it creates data containing hidden pattern.

Particle Swarm Optimization (PSO) algorithm, an easy to program one, offers more attractive choice over other methods to enhance non-linear hitches with multi-variables (Wang et al., 2008). Kennedy and Eberhart introduced Particle swarm optimization (PSO) (Du and Swamy, 2016), involving simulating behaviors to find the most suitable results. Literature exposes that several PSO optimization strategies in task scheduling (Jamali et al., 2016; Prathibha et al., 2017), medical (Jothi, 2016; Ryalat et al., 2016), oil and gas (Salehi and Goorkani, 2017; Siavashi and Doranehgard, 2017), batik production (Soesanti and Syahputra, 2016) have been positively applied in biochemical processes because of their controlled parameters to solve optimization problems (Liu et al., 2008).

Expensive synthetic medium demands an alternative cost effective fermentation process for pigment production from natural resources. Bacteria is one of the important source of pigment due to its breeding ease and abundance. Production of pigment from bacteria is subjective to various constraints like, pH, temperature, agitation, aeration, carbon source and nitrogen source (Tuli et al., 2015). However, pigments have to be produced in large quantities in a shorter time by integrating the bacterial pigments into preferred products. So, optimizing the bacterial growth is mandatory to find suitable physical factors.

Following further experiments, these factors on bacterial growth identify suitable conditions for the growth of bacteria to boost the pigment production. Musa and Yusof (2019) conducted a study which emphasized the key parameters to enhance the bioactivity and production of bacterial pigments for their commercial use in various industrial fields. The violet and red bacteria maximized the production in the presence of light with low shaking speed whereas the yellow bacteria enhanced the production in the absence of light with high shaking speed.

Bacterial pigments can be produced both by submerged and solid state fermentations. The production of β-carotene increased by 3.47-fold in submerged fermentation by the upregulation of carotenoid biosynthetic genes in *Exiguobacterium acetylicum* S01 under optimized conditions of 1.4 g/L, peptone 26.5 g/L, pH 8.5, and temperature 30°C (Jinendiran et al., 2019). In solid state fermentation, bacteria are cultivated on the surface of solid substrates/agro-industrial waste which ultimately saves the medium cost and thereby acting as a waste management tool too (Tuli et al., 2015). Solid state fermentation provides adequate environment for microbes to grow and simultaneously produce higher pigment with less cost of production (Ring et al., 2018). In SSF, substrates are utilized very slowly and steadily and hence the same substrates can be used for longer fermentation process (Panesar et al., 2015). Agro-industrial waste can be utilized to lower the production cost and to protect the environment. β-carotene from *Rhodotorula rubra* was produced in a fruit waste medium obtained from orange, pomegranate and pineapple waste (Korumilli and Mishra, 2014). Carotenoids are produced using whey filtrate and peat extract; whereas riboflavin and astaxanthin produced in grape must, beet molasses. These are interesting prospects for pigment production using agro-industrial wastes (Korumilli and Mishra, 2014). A wide range of bacterial strains such as *Serratia marcescens, Pseudomonas magneslorubra, Vibrio psychroerythrous, Serratia rubidaea, Vibrio gazogenes, Alteromonas rubra, Rugamonas rubra, Streptoverticillium rubrireticuli,* and *Streptomyces longisporus* have shown their potential in pigment production utilizing agro-industrial waste (Venil et al., 2020).

SCALE-UP FROM PETRI DISH TO LAB SCALE AND INDUSTRIAL FERMENTERS

Pigment production depends up on the bacteria and the physico-chemical parameters during fermentation and hence it is an essential requisite to analyse the pigments in a lab scale fermenter before scaling up in industrial fermenters. The product formation during scale-up from shake flasks to bench-scale bioreactors and commercial bioreactors is influenced by the physical, chemical and biological factors on bacterial growth. Mimicking the environment in different stages of scale up and the associated challenges arised needs to be addressed initially for different bacteria and their pigments (Wehrs et al., 2019). The main difference in physical stress between lab-scale and large-scale is operating pressure. As the volume in the bioreactor increases, height of the water column creates hydrostatic pressure gradient. This pressure directly influences the enzyme activity, cell membrane permeability that are vital to cell viability and metabolic flux (Lara et al., 2006). In commercial bioreactor, the hydrostatic pressure increases the concentration of dissolved gases, thereby affecting the medium pH. The chemical stress in large scale production is the imperfect mixing which results in gradients of parameters like pH, temperature, dissolved oxygen and also the concentration of the nutrients (Deparis et al., 2017).

The most important chemical stress in large scale production arise from raw material and microbial contaminants. To minimize the production cost, agro-industrial residues like molasses, sugarcane juice, sugar beet juice, agro residues etc. are utilized for pigment production. Microbial contamination of raw materials can further increase the deleterious impurities and reduce the production quality (Mukhopadhyay, 2015; Mohammed et al., 2017). These factors should be addressed at the early stage of strain cultivation i.e., in shake flasks where sterile and pure raw materials are commonly used for screening and optimization. The strain improvement via genetic engineering would address these issues and process scaling will be more predictable in developing novel bacteria as production forum for pigments. Regulatory approval, toxicity, stability and investment are the key bench marks for bacterial pigments to bring the colorants from Petri dish to market (Malik et al., 2012). Monascus pigment has been prohibited in Europe and United States due to the presence of mycotoxin but used in Asia for centuries as a food colorant (Dufossé et al., 2005). Therefore, researches are now focusing to develop strains that can withstand extreme pH and temperature to meet industrial standards and also to avoid toxin producing strains for its potential application.

PHARMACOLOGICAL ACTIVITIES

Bacterial pigments have shown their efficiency to treat various diseases and have antimicrobial, anticancer and immune-suppressive properties. Bacterial pigments have substantial clinical uses for diagnosing diseases like cancer, leukemia, diabetes mellitus etc. (Kumar et al., 2015). The bacterial pigment, melanin protect the human skin from UV radiation and is used as sun cream blocks (Rao et al., 2017).

Bioactive compounds of bacterial isolates like, prodigiosin (red), violacein (violet), flexirubin (yellowish-orange), carotenoids (yellow - orange) and pyocyanin (blue-green) serve as novel compounds for antimicrobial, antiviral, antitumor, antiprotozoa, antioxidant, anticancer and much more activities. In tune with these characteristics of the bioactive compounds of bacteria, Solieve et al. (2011) too have earlier reported that red pigment has highest antibacterial activity followed by orange, yellow and green pigments.

The xanthophylls (adonirubin and astaxanthin) act as nutraceuticals that prevents cancers, heart attacks and strokes (Long, 2004; Kim et al., 2012a). Astaxanthin (red pigment) is a valuable carotenoid that has great potential in pharmaceuticals and feed industry. Li et al. (2018) have also confirmed that prodigiosin has strong therapeutic potential and anticancer properties. Moreover, prodigiosin has valuable applications as it possesses anti-bacterial, anti-fungal, anti-protozoal,

cytotoxic, and anti-inflammatory properties (Panesar et al., 2015). Earlier, Kim et al. (2008) have reported that the pigment from Hahella chejuensis possesses immuno suppressant and antitumor properties.

Antimicrobial Activities

The foremost human health issue is the infection caused by the multidrug resistant (MDR) bacteria that are resistant to commercial antibiotics (Van-Duin and Paterson, 2017). The Center for Disease Control and Prevention (CDC) has categorized numerous disease causing bacteria such as the extended spectrum β-lactamase (ESBL) Enterobacteriaceae, methicillin-resistant *Staphylococcus aureus* (MRSA), *Mycobacterium tuberculosis* and *Streptococcus pneumoniae* as serious threats (Center for Diseases Control and Prevention, 2018). The investigation of new antibiotic candidates is instantly required to overcome this issue. Venil et al. (2013) have reported that bacterial pigments like melanins, carotenoids, flavins, monascins, quinones, and violacein possesses anti-microbial properties.

The multi-drug resistant bacteria like *Acinetobacter baumannii, Enterobacter aerogenes, E. coli*, MRSA and *Staphylococcus hemolyticus* were inhibited by orange-pigmented bacteria *Pseudoalteromonas flavipulchra* isolated from an Indonesian stony coral (Ayuningrum et al., 2017). The marine pigmented bacteria are reported to produce potent antimicrobial compounds such as marinoazepinone B; marinoquinoline I; marinopyrazinone B; marino-quinolines A, C and D (Choi et al., 2015; Romanenko et al., 2015; Kalinovskaya et al., 2017). Sibero et al. (2019) have isolated and identified yellow pigment (β-carotene) producing bacterium from an unidentified coral as *Vibrio owensii* TNKJ.CR.24-7 (MH488980.1) and the crude pigment extract inhibited ESBL E.*coli, Klebsiella pneumoniae* and MRSA strains. Selvameenal et al. (2009) have reported that *Streptomyces hygroscopicus* producing yellow pigment showed activity against MRSA, VRSA, and ESBL cultures.

Suresh et al. (2015) have characterized the proteinaceous red pigment from *Halolactibacillus alkaliphilus* MSRD1 isolated from seaweed of marine environment and reported that the pigment inhibited *Staphylococcus aureus* and *Salmonella typhi*. The pigments extracted from *Rhodotorula glutinis* with mutagens (UV irradiation and sodium azide) have more antibacterial activity (120-fold higher) compared to the wild strains. According to Bhosale and Gadre (2001), the pigments had more antibacterial effect on Gram positive bacteria (sensitive to the pigment) than Gram negative bacteria. Srilekha et al. (2017) have isolated a novel marine strain *Salinicoccus* sp. producing pinkish-orange pigment and reported that the pigment exhibited maximum antimicrobial activity against *Staphylococcus aureus* and minimum activity against *Klebsiella pneumonia* and *Pseudomonas aeruginosa*.

Duran et al. (2012) isolated a bacterial strain RT102 containing the mixture of violacein and deoxyviolacein and reported its antibacterial activity against Gram positive and Gram negative bacteria at a high concentration of the pigment. Ealier, Agematu et al. (2008) have reported that the violet pigment (deoxyviolacein and violacein) from *Massilia* sp. showed robust activity against *B. subtilis, E. coli* and *S. aureus*. Lapenda et al. (2015) have reported that prodigiosin exhibited antimicrobial activities against different pathogens like *E. coli, E. faecalis, S. pyogenes, Acinetobacter sp. P. aeruginosa* and *S. aureus*. Mumtaz et al. (2019) have reported that prodigiosin from *Chromobacterium prodgiosum* showed antagonistic activity against *Bacillus subtilis, Corynebacterium diphtheriae* and *Staphylococcus aureus*. Prodigiosin from *Neisseria* spp., showed strong activity against fungal strains like *Aspergillus* sp., *Candida* sp., *Trichoderma* sp. (Wagh and Mane, 2017). Fariq et al. (2019) isolated three pigment producing colonies namely *Aquisalibacillus elongates* MB592, *Salinococcus sesuvii* MB597 and *Halomonas aquamarina* MB598 and have reported that the pigments showed excellent antimicrobial activity. Bisht et al. (2020) have testified that violacein and deoxyviolacein showed antimicrobial activity against pathogenic fungi, *Rosellinia necatrix*.

The carotenoids produced by *Halomonas* sp. exhibited antimicrobial activities against *Klebsiella* sp., *S. aureus, P. aeruginosa*, *Escherichia coli* and *Streptococcus pyogenes* (Ravikumar et al., 2016). Zerrad et al. (2014) showed that melanin from *Pseudomonas balearica* strain U7 possessed antimicrobial activity against phytopathogenic strains. Pyocyanin showed activity against urinary tract pathogens like *S. aureus, S. saprophyticus, S. epidermidis, E. coli* and *C. freundii* (Mohammed and Almahde, 2017). El-Shouny et al. (2011) have reported that pyocyanin showed activity against all Gram-positive bacteria and *C. albicans*. These bioactive compounds will provide promising future in biomedical research. However, when monitoring the antimicrobial activities of microbial pigments, sometimes researchers use non-pure pigments. Then the observed antimicrobial effect is not related to the pigment itself but to another compound and this factor has to be reckoned while designing such studies.

Anticancer Activities

The chemotherapy is still the customary treatment method for the most dreadful disease of cancer and this treatment is cytotoxic to normal cells which affects not only tumor development, but also worsens patient's recovery (Felisa et al., 2015; Ravin et al., 2017). Hence, there is an endless demand to develop cheaper, safer natural products in the treatment of cancer to challenge the dreadful human disease. Bacterial pigments seem to have huge potential as a source of anticancer compounds and deserve a comprehensive investigation (Srilekha et al., 2018). Prodigiosin, from *Serratia marcescens* induces apoptosis in human cancer cell lines, hematopoietic cancer cell lines (Jurkat, NSO and HL60) and human oral cancer HSC-2 cells. Campas et al. (2003) have reported that the cytotoxic effect of prodigiosin showed significant activity against B-cell chronic lymphocytic of leukemia patients. The pigment from *Salinicoccus* sp. was found to exhibit inhibitory action against the growth of human cancer cell lines for the development of anticancer drugs (Srilekha et al., 2018). Prodigiosin from *Pseudoalteromonas* sp. 1,020 exhibited cytotoxicity against U937 leukemia cells (Wang et al., 2012).

The risk of breast cancer was shown to be reduced by the novel compound from yellowish pigment produced by *Rhodococcus maris* (Elsayed et al., 2017). The red pigment from

Athrobacter sp. G20 isolated from the Caspian Sea exhibited remarkable anticancer activity against esophageal cancer cell lines (Afra et al., 2017) and carotenoids from *Kocuria* sp. QWT-12 showed anticancer activity against breast cancer cell lines MCF-7 (Rezaeeyan et al., 2017) (IC50 of 1 mg/ml) whereas carotenoids from *Haloferax volcanii* showed activity against human liver carcinoma cell lines HepG2 (53.52%) (Sikkandar et al., 2013). Derivatives of anthraquinone from *Alternaria* sp. ZJ9-6B showed activity against human breast cancer cell lines (Huang et al., 2015).

Violacein has shown its anticancer efficiency in numerous cell lines and Melo et al. (2000) found that the violacein (IC50 5-12 μM) was highly cytotoxic to V79 fibroblasts. Uveal melanoma cell lines, 92.1 and OCM-1 are found to be sensitive to violacein (GI50 ∼1.69–2.21 μM) and these results demonstrate that the violacein induces apoptosis in cancer cells (Saraiva et al., 2004) which is significant for defense against the diseases and cancers (Walsh et al., 2008). Alshatwi et al. (2016) have reported that violacein induced apoptosis in human breast cancer cells through upregulation of TNF-α expression and the p53 dependent mitochondrial pathway. Venegas et al. (2019) have now reported that violacein induced activation of inflammatory response, signaling through MAPK pathway, cytokine-cytokine receptor interaction and toll-like receptor signaling in raw 264.7 cells. Violacein could bind to hTLR8 to imidazoquinoline compounds by *in silico* analysis.

The phycocyanin interacts with non-specific targets from membrane to nucleus and kills cancer cells that are resistant to chemotherapy (Silva et al., 2018). The pyocyanin inhibited HepG2 human hepatoma cell proliferation and apoptosis (Zhao et al., 2014). The mutant strain *P. aeruginosa* S300-8 produces potent pyocyanin and this compound inhibited the growth and apoptosis of pelvic rhabdomyosarcoma cells (Hall et al., 2016). The melanin pigment from *Streptomyces glaucescens* NEAE-H showed cytotoxic activity against HFB4 skin cancer cell line (El-Naggar and El-Ewasy, 2017). There is a constant demand to develop effective anticancer drugs and these bioactive pigments from bacteria appear to be the most promising sources for new drug discoveries.

Antioxidant Activities

Antioxidants play a significant role to humans in protecting against various infections and degenerative diseases by inhibiting and scavenging free radicals. Many synthetic antioxidants are used to retain oxidation process which have potential health hazards and researchers are focusing to screen alternative antioxidants from natural sources (Lee et al., 2014). Carotenoids extracted from *Kocuria marina* DAGII, thermophilic bacteria *Meiothermus* and *Thermus* strains exhibits potent antioxidant properties (Rezaeeyan et al., 2017). Correa-Llanten et al. (2012) isolated carotenoid producing *Pedobacter* from an Antarctic bacterium which showed excellent antioxidant capacity. The carotenoid (flexirubin) from *Fontibacter flavus* YUAB-SR-25 showed significant antioxidant activity and inhibited lipid peroxidation (Prabhu et al., 2013).

Antonisamy and Ignacimuthu (2010) have testified that violacein from *C. violaceum* protects against oxidative damage by defense mechanism. Pyomelanin from *Burkholderia cenocepacia* C5424 was capable of protecting cells from oxidative stress (Keith et al., 2007). Lycopene has applications in various food products like nutritional food, soups, cereals for breakfast, beverages, surimi, dairy products, chips, spreads, pastas, snacks, and sauces (Chandi and Gill, 2011). Lycopene suppresses tumor cell proliferation in MSF-7 tumor cells (Numan et al., 2018). Fiedor and Burda (2014) reported that β-carotene suppresses the adverse effect of free radicals in humans. Majumdar et al. (2019) explored the bacterial isolate, *Planococcus* sp. TRC1 as carotenoid pigment producer which showed appreciable antioxidant activity leading to industrial applicability and additionally its potential to bio remediate the paper mill pulp effluent. These results show that the pigments are prospective ones for eventual use in pharmaceutical and food applications.

Anti-inflammatory and Anti-allergic Activities

Inflammation is an immunological defense mechanism induced in response to mechanical injuries (Menichini et al., 2009; Mueller et al., 2010). Oxidative stress plays an important role in endothelial dysfunction (Schramm et al., 2012), lung disease (Paola-Rosanna and Salvatore, 2012), gastrointestinal dysfunction (Kim et al., 2012b) and atherosclerosis (Hulsmans et al., 2012). Lee et al. (2013a) reported that ankaflavin possessed anti-allergic activity in both lung cell line (A549) and lungs ovalbumin (OVA)-challenged mice. Radhakrishnan et al. (2016) studied the soil isolate from desert producing yellowish-orange pigment from *Streptomyces* sp. D25 which showed strong antioxidant activity. Srilekha et al. (2018) isolated a marine pigmented bacteria, *Micrococcus* sp. which has strong potential as anti-inflammatory and wound healing agent and this is influenced by the antibacterial property of the pigment. Egeland (2016) reported another carotenoid, fucoxanthin from cyanobacteria possessing its anti-cancer, anti-inflammatory and anti-obesity properties. Thus, the bioactive pigment showed strong wound healing property and could be a good source for anti-inflammatory compounds.

POSSIBLE APPLICATIONS APART FROM COLORANTS AND PHARMACY

Food industries are looking for substitutions to synthetic food colors such as sunset yellow, tartrazine, and quinoline yellow (**Table 2**). Zeaxanthin from *Flavobacterium* sp. is used as an additive in poultry feeds (Pasarin and Rovinaru, 2018). Sajilata et al. (2008) reported that fish and crustaceans convert zeaxanthin to red-colored pigment, astaxanthin, which enhanced desirable red coloration of the shells. These pigments are considered safe and approved by FDA (Neeraj et al., 2011). Riboflavin (vitamin B2) are used in beverages, instant desserts and ice creams.

Carotenoids play an important role as sunscreen by maintaining the quality of foods by protecting from intense sunlight (Tendulkar et al., 2018). The carotenoids like torulene and torularhodin are being investigated in detail by many researchers and Matz et al. (2004) reported that torularhodin

TABLE 2 | Rainbow (VIBGYOR) colorants possible with bacterial pigments.

VIBGYOR (Violet-Indigo-Blue-Green-Yellow-Orange-Red) is a popular mnemonic device used for memorizing the traditional optical spectrum.

protects from oxidative stress. The concentration of aryl carotenoids from *B. linens* possessing antioxidant activity is not sufficient for commercialization at a competitive price and genetic modifications could be an alternative. Kurdziel and Solymosi (2017) reported that phycobiliproteins have great market value and they can be used as biochemical tags and food colorants due to their high color tone.

STABILITY AND SOLUBILITY OF BACTERIAL PIGMENTS

Though having great potential in terms of applications, natural colorants have only limited markets due to their poor stability. Sen et al. (2019) have now reported that various techniques are available to produce stable natural pigment with increased shelf life and market potential in a cost-effective manner. The most important distress among natural colorants is anthocyanin and because of its low stability, it has only restricted its use as natural colorants (Babaloo and Jamei, 2018).

Encapsulation plays an important role in maintaining the stability of pigments, thereby rising their shelf life. Physical and chemical attributes of food, stability during processing and storage and regulatory issues are the important criteria in selecting natural colorants for food applications (Sen et al., 2019). The value-added anthocyanin's stability would possibly give a chance in a multiplicity of food colorants. Beta-carotene possessing nutraceutical activity can be used as food colorants and is limited by solubility, stability, melting point and low bioavailability (Gutiérrez et al., 2013; Liang et al., 2013). β-carotene is quite unstable and degrades during food processing and storage (Mao et al., 2009). Therefore, to address the distresses of stability, encapsulation of β-carotene is carried out to enhance the stability for its application in food industry.

Encapsulation

Bacterial pigments are quite unstable at high temperatures, light, oxygen and hard to retain their characteristics when they are exposed to certain inherent conditions (Rao et al., 2017). Microencapsulation method is an alternative one to increase the stability and solubility which entraps the active components into the microparticles. The microencapsulation is packing any solid, liquid or gas in microparticles of size ranging from mm to nm. The active compound, bacterial pigment becomes the core material and the packaging material is the wall material. The wall material should have low viscosity, emulsifying properties, biodegradable, low hygroscopicity as well as low cost (Barros and Stringheta, 2006). The wall materials used to encapsulate bacterial pigments include maltodextrins, modified starch, inulin, furcellaran etc. (Ozkan and Ersus, 2014). Encapsulated colorants have better solubility with improved stability to ambient conditions, leading to an increased shelf life. The wall material plays important roles by protecting the active material from light, temperature, humidity, oxygen as well as matrix interactions. The objective of encapsulating bacterial pigments for food industry applications include protecting core material, increasing shelf life and most importantly, controlling the release of pigment and suppressing any type of aroma. There are various reports available for encapsulating bacterial pigments viz., encapsulation of anthocyanin by spray drying in which maltodextrin has been used as the wall material (Silva et al., 2013), encapsulation of β-carotene by freeze drying using modified starch as wall material (Spada et al., 2012). These encapsulated powders have been applied in cakes, yogurt, soft drinks and found to be stable and effective (Rocha et al., 2012).

Under specific conditions, the encapsulated material can be protected and released in a controlled way to widen its scope in food applications (Rosas et al., 2017). Bacterial pigments because of their bioactive components can be used as food colorants and in the pharma industry (Venil et al., 2015) and the stability of the pigment may be affected by several factors, such as temperature, light, pH, oxygen, etc. The above said factors directed researchers to search for inexpensive processes aimed at increasing the shelf life and stability of bacterial pigments (Venil et al., 2016).

The stability of these bioactive bacterial pigments can be improved using the encapsulation technique which entraps a sensitive ingredient inside the coating material. The flexirubin from *Chryseobacterium artocarpi* CECT8497 showed that microencapsulation increased the stability by giving great protection to flexirubin compared to its free form. Moreover, the antioxidant activity is higher for microcapsules and the boosted properties indicate that this colorants from *C. artocarpi* CECT8497 can be used as natural colorants in food industry (Venil et al., 2016). Namazkar et al. (2013) reported that non-encapsulated prodigiosin from *Serratia marcescen* has poor stability on exposure to light, pH and temperature whereas the encapsulated prodigosin increases the stability and solubility and it can be used as an alternative to existing synthetic colorants.

Nano-Emulsion

Nano-emulsions are droplet size of 100 nm or even less and can be prepared to encapsulate bacterial pigments. It contains water, oil and emulsifier and the addition of emulsifier is the most crucial step in the formation of nano-emulsions which decrease the tension between water and oil phases. Most commonly used emulsifiers are surfactants but proteins and lipids can also be used. Compared to micro or macro emulsions, nano-emulsions have improved applications due to its large surface area, kinetic stability and resistance to physical or chemical change (Gupta et al., 2016). These nano-emulsions for food colorants possess various advantages like non-irritant and non-toxic, making them suitable for applications in food industry. Nano-emulsions stabilize the colorants within the emulsion and has no undesired taste (Jaiswal et al., 2015). Nano-emulsion can also decrease the amount of colorant required to obtain the desired color and hence it is a cost-effective method. The formation and stability of nano-emulsion with β-carotene were reported and stabilized using β-lactoglobulin, a biocompatible emulsifier (Yi et al., 2014). Several methods for encapsulation of β-carotene in suitable delivery system, such as nano-emulsion, micro-emulsion, liposome, solid lipid nanoparticles and complex assemblies with macromolecules are reported with increased bioavailability and so there is a scope for further research in this

field (Cornacchia and Roos, 2011; Qian et al., 2012; Liang et al., 2013; Donhowe et al., 2014).

FUTURE PERSPECTIVES AND CONCLUSIONS

Natural colorants are being extracted from microorganisms and are used for various applications. Of them, pigments extracted from bacterial sources are noteworthy and have better prospective applications due to their easy and cost-effective production processes. Many bacteria produce different shades of coloring pigments prospective to meet the emerging market needs and industrial applications. Pigments extracted from the trustworthy strains of bacteria have exhibited their diversified activities like anti-microbial, anti-cancer, anti-oxidant, anti-inflammatory and anti-allergic activities and thus exposing their potential for medicinal applications. Further, the bacterial pigments have exhibited their potential applications in food, agriculture and other industries too. To enhance the stability and shelf life of the pigments, strategies of encapsulation and nano-emulsions have been tried and their effectiveness testified.

Yet comparing to the emerging global market needs, the volume of bacterial pigment production is less. There is a better scope to upgrade their overall production volume so as to meet the market demands by applying various biotechnological tools. In this aspect, genetic engineering to strain improvement, genome shuffling, fermentation strategies to scale-up production to industry level etc. play vital roles for maximum production of bacterial pigments at cheap cost with high stability. As the market for bacterial pigment products is globally large for eventual applications, ecofriendly and economic bacterial pigment production has a better prospective.

AUTHOR CONTRIBUTIONS

CV: conceptualization, original draft preparation, and writing. LD: writing, review, and editing. PR: review. All authors contributed to the article and approved the submitted version.

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Antioxidant Content, Capacity and Retention in Fresh and Processed Cactus Pear (*Opuntia ficus-indica* and *O. robusta*) Fruit Peels From Different Fruit-Colored Cultivars

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During processing, the peels and seeds from cactus pear fruits are usually discarded. These "waste" products contain valuable bioactive compounds. This study investigated the antioxidant content and antioxidant potential of fresh and processed (juiced, dried, preserved, and chutney) cactus pear fruit peels from different fruit-colored cactus pear cultivars. Cactus pear peels contained high levels of antioxidants and demonstrated high antioxidant activity. The highest contents were found in dried peels, while the preserves had the lowest contents. Products on the positive side of Factor 1 of the PCA plot are mostly associated with the antioxidants PCA analysis showed that products, rather than cultivars, seem to cluster together, e.g., juices and fresh peels, chutneys, preserves and dried peels. Robusta and its products cluster together, as well as with betalains. The % DPPH, carotenoids and phenolics are grouped together, with % chelating activity closely correlated with ascorbic acid. Dried products from all cultivars correlated closely with % DPPH, carotenoids and phenolics, especially dried peel from Gymno-Carpo (orange), Ofer (orange), Meyers (red), and Nepgen (green). Red and orange preserves formed a cluster, while green preserves and chutney from all cultivars clustered together, as did fresh peels and juices. Purple fruit peel products had the highest % DPPH, % chelating activity, betalains, phenolic compounds and carotenes. Ascorbic acid dominated in orange and red fruit peels. Purple and orange were the colors of cactus pear fruit cultivars that might be the best choice in terms of antioxidant content. The cultivar that presented the best fruit peel from an antioxidant point of view for preservation was Robusta. Cactus pear fruit peels should be included in processed products such as juice, dried fruit and chutneys. These processed products are multi-component food ingredients and are therefore multi-component nutraceuticals which retained their antioxidant properties.

Keywords: ascorbic acid, betalains, carotenoids, phenolics, prickly pear peels

INTRODUCTION

The cactus pear (*Opuntia ficus-indica*) originated from Mexico (Inglese et al., 2002) and is a member of the *Cactaceae* family. It is found in Latin America, South Africa and the Mediterranean. The cactus pear plant is a fleshy bush or small tree and the fruit is an elongated oval-shaped berry with a thick peel that accounts for 33–55% of the fruit, while the pulp makes up about 45–67% (Piga, 2004; Arrizon et al., 2006). The pulp contains 2–10% seeds, which are good sources of unsaturated edible oils (López-González et al., 1997; De Wit et al., 2016, 2017, 2018). The fruit is usually consumed fresh, however interest to process it into different products such as juice, jam, marmalade and jelly is increasing (Cardador-Martínez et al., 2011). During processing, the peels and seeds are usually discarded. These agro-industrial "waste" products contain valuable bioactive compounds and dietary fiber (Pimienta-Barrios, 1994; Amaya-Cruz et al., 2018). In general, cactus pear fruit peels contain total soluble solids (TSS) (8.03–15.4 °Brix), organic acids (titratable acidity, TA, of 0.61–3.4 g.L^{-1}), protein (1.53% DW), lipids (0.32% DW), total fibers (5.83% DW), ash (3.4% DW), fructose (27–81.8 g.L^{-1}) and glucose (57–128 g.L^{-1}) (De Wit et al., 2010; Hernández García et al., 2020). Sucrose is also present. Dietary fibers include both soluble (mucilage and pectin) and insoluble fibers (lignocellulosic material). Processing of common fruits generates large amounts of peels which are known to contain high dietary fiber contents. Furthermore, Pérez-Jiménez and Saura-Calixto (2018) reported fruit peels as sources of non-extractable polyphenols or macromolecular antioxidants. The most commonly cultivated *Opuntia* spp. include *O. ficus-indica*, *O. megacantha, O. streptacantha and O. amyloclaea* (Galati, 2001). Fruits and young cladodes of Opuntia have traditionally been used to treat diabetes, indigestion, hypertension, asthma, oedema and burns in folk medicine (Pimienta-Barrios, 1994; Galati et al., 2003; Stintzing et al., 2005).

The health-promoting and health- improving capacity of cactus pear fruit pulp is of interest (Kuti, 2000; Stintzing et al., 2005; Arrizon et al., 2006) especially for the development of nutraceutical and functional foods (El-Said et al., 2011) and is attributed to bioactive compounds such as phenolic and polyphenolic compounds, pectin, carotenoids, betalain pigments, vitamins, and enzymes with antioxidant properties (Kuti, 2004; Abou-Elella and Ali, 2014; Patil and Dagadkhair, 2019). Polysaccharides contributing to antioxidant values in *O. macrorhiza* peels were reported by Amamou et al. (2020). Antioxidant properties of betalains could be related to and have synergistic effects with other bioactive molecules such as tocopherols, organic acids, reducing sugars and polyunsaturated fatty acids (Pérez-Jiménez and Saura-Calixto, 2018). The presence of antioxidants in plants makes it beneficial for health to consume plant products. Antioxidants are compounds that protect cells against reactive oxygen molecules' oxidative effects. Oxidative stress is caused by an imbalance between these reactive oxygen species and antioxidant content. This oxidative stress causes cellular damage and leads to conditions and ailments such as diabetes, cancer, CVD, aging and neurodegenerative disorders (Panche et al., 2016). Fruit products are important for the prevention of degenerative diseases such as cancer, hyperglycaemia, hypercholesterolemia, arteriosclerosis, diabetes and gastric diseases because of the presence of antioxidant phenolic compounds in cactus pear plants (Butera et al., 2002; Stintzing et al., 2005). The antioxidant activity of cactus pear fruit is double than that of apples, pears, bananas, tomatoes and white grapes and is comparable to that found in red grapes, grapefruit and oranges (Cerezal and Duarte, 2005). El-Said et al. (2011) pointed out that it is known that fruit contains vitamin C but studies on other antioxidants in peels are lacking. These authors also pointed out that one of the major needs within the prickly pear industry is the development of new processed products, especially fruit by-products. These "new" functional components will open up new possibilities to add value. Fermentation could be used to develop new food products, such as wine, vinegar and balsamic vinegar (acetic acid fermentation) (Sáenz, 2015). Innovative extraction techniques such as ultrasound-assisted extraction of phytocompounds could be employed (as was done in dragon fruit peels) (Bhagya Raj and Dash, 2020). On a larger and industrial scale, biofuels, i.e., bioethanol could be produced from organic materials containing carbohydrates and have high sugar contents as well as from the lignocellosic material (consisting of lignin, hemicellulose and cellulose) using saccharification and fermentation by enzymes simultaneously (Casabar et al., 2019) as was done for pineapple peels.

Currently, limited literature on the antioxidant content of cactus pear fruit peels is available. Cardador-Martínez et al. (2011) reported on the revalorization of cactus pear by-products as a source of antioxidants. These authors suggested that some of these waste product constituents should be obtained and used as additives in the food, pharmaceutical and cosmetic industries. Cactus pear peels have been reported useful as a marmalade (El Kossori et al., 1998), while the seeds were a source of dietary fiber (Ramadan and Mörsel, 2003a), oil (López-González et al., 1997; Habibi et al., 2002; Ramadan and Mörsel, 2003b; De Wit et al., 2016, 2017, 2018), and D-xylan (Habibi et al., 2002, 2005). Cactus pear peels have been used for the extraction of phenolic compounds and polysaccharides (Melgar et al., 2017; Pérez-Jiménez and Saura-Calixto, 2018; Amamou et al., 2020) and are a rich source of dietary fiber, vitamin C and betalains (Jiménez-Aguilar et al., 2015). These compounds have been reported to have a positive affect against coronary heart disease, high blood pressure, diabetes and cancer (to name a few).

Cactus pear fruit is susceptible to microbial spoilage. Preservation techniques are applied to extend the storage life and also to diversify the processed products (Joubert, 1993; Piga et al., 2003; Sáenz et al., 2004). It was previously assumed that processing would damage the antioxidant content and -potential of cactus pear fruits. According to Sacchetti et al. (2008), the antioxidant potential of processed fruit products, after processing and during storage, is mostly dependent on the quality of the fresh fruit, processing procedures and storage conditions. Tesoriere et al. (2005) reported that the vitamin E and betalains appeared to be unaffected by fruit processing. Piga (2004) described the ascorbate-sparing effect of polyphenols. Ryan and Prescott (2010) uncovered a whole new research question on whether heat treatments affect

antioxidant capacity in processed fruit products. Some juices studied had higher antioxidant capacity in long life (heat-treated) versions. This contradicts traditional beliefs that the scavenging ability of individual antioxidants is destroyed by heat. Ryan and Prescott (2010) provided three explanations for the protection of the antioxidants: firstly, antioxidant potential increased after processing because more antioxidant components are released due to the heat that disrupts the cell walls. Secondly, heat treatments destroy oxidative enzymes that would normally destroy antioxidants. Thirdly, during heat treatments new structural groups are formed which enhance antioxidant potential. The third reason was proposed as being the most probable. It was also found that antioxidant potential of gallic acid increased after heat treatments because new hydroxyl groups are formed due to structural changes of the polyphenols. These structural changes cause the antioxidant to be more stable to pH, which allow it to continue its activity throughout the digestive tract. In a study done by Lee et al. (2008) the effect of home processing and light exposure on flavonoid content was evaluated. It was found that "cooking" methods had varying effects on the content and that exposure to fluorescent light increased the content.

Currently very little information is available on processing effects on the antioxidant content of cactus pear fruit peels. In a recent study of Barba et al. (2017) it was speculated whether phytochemicals can loose their properties, or be transformed into anti-nutrients, depending on the processing conditions. The authors concluded that processing and preservation techniques strongly influence the stability of phytochemicals present in *Opuntia* fruits. Conventional and novel non-thermal technologies are efficient to recover high contents of value-adding compounds of *Opuntia* fruit by-products and wastes (such as ultrasonic extraction as was mentioned earlier for dragon fruit peels). This current study was done to firstly obtain a basic profile of the main antioxidants present (contents) and its antioxidant potential and to investigate if different fruit peel colors (tonalities) influence this profile. Secondly, it was attempted to determine if these identified antioxidants would be retained in the products after the peels were processed into different products to prolong shelf-life. The data is presented on an "as is" basis, implicating the antioxidant contents of the final products as the consumer would use them. Therefore, in the current study, the antioxidant content (betalain-, ascorbic acid-, carotenoid-, and total phenolic contents) and antioxidant potential (% DPPH scavenging and % Fe chelating activity) of fresh and processed (juiced, preserved, dried and chutney) cactus pear fruit peels from different fruit-colored cactus pear cultivars was investigated. Different preservation methods were applied to the peel of each of the five cultivars, in order to obtain results for antioxidant content and potential in the peels of the fruit, after preservation methods were applied. It was important to produce marketable products for the South African public. The products had to be well-known, everyday food that South Africans are accustomed to. In South-Africa, the *Opuntia ficus-indica* plant is mainly cultivated for the fruit destined for the local and European markets. There is currently a small but well developed commercial sector in South-Africa, but the plant as a whole is mainly under-utilized and under-valued. It is only through research such as this study that the true value of this easily cultivated plant may be realized as a healthy food resource. These processed products could be regarded as multi-component nutraceuticals. Published results on a previous study by De Wit et al. (2019) on the antioxidants of the different tissue types (vegetal parts) are included as a starting point to this study for comparison purposes of fresh peel. It was therefore important to compare the results of the products manufactured from the peels of different cultivars with fresh unprocessed control peels.

Materials and Methods

Fruit Collection

Fruit was collected at an experimental orchard to the West of Bloemfontein (29° 10′ 53′ S, 25° 58′ 38″ E), Free State Province, South Africa (De Wit et al., 2010). The complete randomized block designed orchard consisted of 42 spineless cactus pear cultivars. The plants were 3 m apart in rows spaced at 5 m, resulting in 666 plants ha^{-1}. Treatments were replicated twice with five plants per treatment (Coetzer and Fouché, 2015). Fruits were picked at the 50% color-break stage to ensure an even degree of ripeness and also because it is the point of optimum sugar content (°Brix) and firmness in this non-climacteric fruit (does not ripen or change after harvesting) (Felker et al., 2008). This is also considered the commercial maturity (50% external color) stage, when both the parenchyma and chlorenchyma of the peel portions have similar colouration as the edible pulp portion (Felker et al., 2008). Fruit size ranged from 67 to 216 g. Fruit from four cultivars of the *O. ficus-indica* species, namely Meyers (red), Gymno-Carpo (orange), Ofer (orange), and Nepgen (green), and one cultivar from the *Opuntia robusta* specie, namely Robusta (purple) were picked, refrigerated at 4 ± 1°C and processed within 3 days after harvesting (De Wit et al., 2010; Du Toit et al., 2015, 2018b). The different colored phenotypes (cultivars) of cactus pear fruit are related to betalin- and carotenoid content (Cano et al., 2007; Amaya-Cruz et al., 2018) and green, orange, yellow, red and violet (purple) colors are available due to the large genetic variability. It is important to note that the same methodology was used as reported in the study of De Wit et al. (2018) on cactus pear fruit pulp. The current study however, makes use of the fruit peels and although the peel is part of the fruit, the physiological composition differs with regard to the cellular structure. The cell wall construction is different, resulting in higher fiber content in the peel and more juice in the pulp (fruit). It is therefore accepted that the chemical content of the pulp and peel will be different (Salisbury and Ross, 1991).

Sample Preparation

Working with cactus pear fruit is problematic as it is covered with hair-like thorns (glochids) that may cause severe discomfort when penetrating the skin. The glochids were consequently removed by brushing under cold running water while held with tongs. All fruit were then blanched in boiling water for 30 s, followed by cooling in cold water (4 ± 1°C). This allowed bare handed handling and easy peeling of the outer skin from the peel, similar as is practiced for tomatoes (Brown, 2008). After skin removal, the peel was separated from the fruit by the cut

and tear method. Depending on the cultivar, the amount of peel may vary between 40 and 50% of the whole fruit. Juice, dried peel, preserves and chutney were prepared for each cultivar replication in triplicate. Juice was the first choice as it would be the most obvious product to market since the fruit has such vibrant colors. Drying is the oldest and one of the easiest preservation methods and therefore had to be included in the study. Chutney was included as this method includes sugar, acid as well as spices in the preservation technique. Preserved peel in syrup was included due to its visual appeal and being a very tasty product (Food Preservation, 1986). Since the formulation of products such as peel juice, chutney and preserved peels differ drastically it is not possible to compare products on the same peel content. Since formulations contain different ingredients, the logical and possible way to evaluate results is to compare the manufactured products on an "as is" basis (as the consumer consume it) with the fresh peels as was done in this study.

Peel Juice Preparation

For juice preparation, the peels of 20 fruits of each cultivar were liquidized in a Milex 4-in-1 multi-purpose Mean Juice Machine (Model MMJ004) (low speed setting for soft fruit) for 60 s. The juice was pasteurized in a water-bath until the internal temperature reached 72°C and kept for 5 min at 72°C, shocked in cold water and then frozen at −20 ± 1°C.

Dried Peels

For preparation of dried peels, ten fruits of each cultivar were washed and peeled and the peels were blanched at 80°C for 5 min. The fruit peels were cut into slices (7.5 ± 0.5 mm), dried in a convection oven at 70 ± 1°C for 18 h (Kuti, 2000) and then vacuum packed and frozen (– 20 ± 1°C) until further analysis. The peels were dried from an initial moisture content of ≈ 80–89% until a final moisture content of between ≈ 9–18%.

Preserved Peels

Preserved peels (a sugar-based product) were prepared as follows: 20 peels of each cultivar were weighed and jarred according to the open-kettle method (Food Preservation, 1986). The peels were cooked in boiling water for 5 min until just tender. It was transferred into prepared boiling syrup [250 ml sugar (sucrose) dissolved in 500 ml water] for 10 min to allow the syrup to permeate through the peels. The preserved peels were transferred to hot, sterilized jars. The jars were filled with boiling syrup to the brim in order to exclude air from the jar. Metal lids were used to cover the jars and screwed down tightly. The steam from the fruit condensed and formed a vacuum that completed the seal (Food Preservation, 1986).

Chutney Preparation

Chutney is a low pH (acid) preserved relish-type condiment using sugar, acid and heating. Chutney was prepared from peels as follows: the peels of 20 cactus fruits of each cultivar were washed, weighed and liquidized as explained above. The liquidized peel was weighed and the amounts of added ingredients were calculated according to the following formula: 69% fruit peels, 13.8% sugar, 12.95% vinegar, 4.2% seasoning and flavoring, which consisted of cayenne pepper (0.01%), minced onion (3.45%), salt (0.22%), powdered ginger (0.22%), powdered mustard (0.06%) and powdered garlic (0.24%) (Food Preservation, 1986). All the ingredients were added to a stainless steel saucepan and allowed to boil slowly until it was thick and dropped off the spoon in flakes and had the consistency of jam. It was stirred occasionally using a wooden spoon. The prepared chutney was poured into hot sterilized jars and sealed immediately, labeled and stored at room temperature. Antioxidants from spices reportedly include flavonoids, phenolic acids, lignins, essential oils, and alkaloids (Yashin et al., 2017). Herbs and spices have therapeutic effects such as anti-carcinogenic, anti-diabetic, anti-ulcer, anti-inflammatory properties and protect against endocrine disease, oxidative damage to red blood cells and renal disease. In a study done by Hossain et al. (2008), the antioxidant capacity of 30 spices and herbs were evaluated, with garlic ranking the lowest. According to this ranking, the antioxidant capacity of the spices used in chutney preparation, ranks as follows: onion > ginger > cayenne pepper > mustard > garlic. The contribution of each individual spice used was not determined, but it contributed to the antioxidant capacity of the chutney product as a whole.

Antioxidant Measurements

For determination of iron chelating activity, DPPH radical scavenging, betalain content, ascorbic acid content and total phenolic compound content, aqueous extracts were prepared for all the processed products. The products were liquidized with 50% distilled H_2O, strained and the volume of the filtrate determined and aliquots frozen at – 20 ± 1°C (Du Toit et al., 2018a,b). A hexane/acetone/ethanol extract was prepared from all the processed products for carotenoid determination. Two grams of product (tissue) was homogenized with 10 ml hexane/acetone/ethanol (50:25:25) mixture, centrifuged and the hexane layer then recovered (Du Toit et al., 2018a,b).

Chelating Activity

Chelating activity of the antioxidant was determined using an amended method (Sumaya-Martínez et al., 2011) of the method of Gülçin et al. (2007). One hundred microliters of aqueous extract, containing 50 µl ferric (II) chloride solution (2 mM) and 4.5 ml methanol was vortexed for 10 s. Two-hundred µl ferrozine (5 mM) was added and the solution was centrifuged. Blank solutions containing both aqueous extract and methanol were also prepared and were included to make the absorbance measurement of the purple fruit possible. Absorbance of the supernatant was determined at 562 nm using a Genesys 10 Vis Thermospectronic spectrophotometer and the chelating activity (as % FW) determined.

DPPH Radical Scavenging Activity

DPPH (2,2′-diphenyl-1-picrylhydrazyl) was determined according to the methods of Morales and Jiménez-Pérez (2001) and Sumaya-Martínez et al. (2011). Five-hundred µl of the DPPH solution (7.4 mg/100 ml^{-1} ethanolic solution) was added to 100 µl of the aqueous extracts, vortexed for 10 s, left to stand for 1 h and then centrifuged at 13416 xg for 5 min at 4°C. Absorbance was measured at 517 nm with a Genesys 10 Vis

Thermospectronic spectrophotometer (Gülçin et al., 2007) using a blank solution containing aqueous extract and ethanol. Results were expressed as % FW.

Betalains

Betalains were determined according to the methods reported by Castellanos-Santiago and Yahia (2008) and Stintzing et al. (2005). The aqueous extract was centrifuged at 13416 xg for 5 min in a 12 Hettich centrifuge. The photometric quantification of the supernatant was done on a Genesys 10 Vis Thermo Spectronic spectrophotometer (Stintzing et al., 2005). Measurements were done in triplicate and the betalain content [which comprises of the red-violet betacyanins (Bc) and the yellow betaxanthins (Bx)] was calculated according to the following equation. Thereafter all values were converted to mg/kg fresh weight (FW):

Bc / Bx (mg/g) = (A x DF x MW x 1,000) / (ϵ x l) (De Wit et al., 2019).

Ascorbic Acid

Ascorbic acid was determined by means of titration according to James (1995). The aqueous cactus pear product extracts were titrated with 0.04% 2,6 dichlorophenolindophenol solution. Results were expressed as mg/100 g FW.

Carotenoids

Carotenoid content was determined after the samples were homogenized with 10 ml hexane:acetone:ethanol (50:25:25,v/v) and centrifuged at 5668 xg at 4°C for 5 min, after which the top-layer of hexane was recovered and the volume adjusted to 25 ml with hexane. The absorbance was measured at 450 nm according to the methods described by Kuti (2004) and Fernández-López et al. (2010) using an extinction coefficient of β-carotene, E1% = 2590. Results were expressed as μg/g FW.

Total Phenolics

Total phenolic content was determined using 2 g of the aqueous extract, which was centrifuged, and 0.2 ml of the extract was combined with 1 ml Folin-Ciocalteu reagent and 0.8 ml sodium carbonate solution. Absorbance was read at 765 nm in a Genesys 10 Vis Thermospectronic spectrophotometer after 30 min. The polyphenol content was expressed as milligrams of gallic acid equivalents per liter (mg l^{-1} GAE), following a calibration curve made with pure gallic acid at 0,50, 100, 150, 200, 250, 300, and 350 mg l^{-1} (Kuti, 2000). The units were expressed as mg/kg FW.

Statistical Analysis

Analyses were done on the two replications per cultivar. Three replications of each type of product were manufactured. Results were expressed on an as is basis of the end products, as it would be consumed by the consumer. The NCSS Statistical Software package (version 11.0.20) was used for statistical analysis. Results were expressed as mean ± standard deviation. The effect of cultivar and processing method on antioxidant properties of cactus pear fruit peels was analyzed with one-way analysis of variance and the means compared with the Tukey-Kramer multiple comparison test (NCCS 11, 2018). The multivariate statistical procedure, principal component analysis (PCA) was used to investigate and simplify the relationship between products manufactured from different colored fruit peel samples with regard to their % DPPH, % chelating activity, ascorbic acid (mg/100 g), betacyanins (mg/kg), betaxanthins (mg/kg), betacyanins + betaxanthins (mg/kg), carotenoids (μg/g) and phenolics (mg/kg) (variables) (NCCS 11, 2018).

RESULTS AND DISCUSSION

Chelating Activity

Percentage chelating activity determines the reducing power of an antioxidant, i.e., the ability to reduce Fe^{3+} and the ability to donate an electron. Antioxidants cause reduction of Fe^{3+} to Fe^{2+}, therefore a change of color of the solution is indicative of the reducing power of the compounds (Butera et al., 2002). Furthermore, metal ion chelating capacity plays an important role in the antioxidant mechanism, since it reduces the concentration of the catalyzing metal in lipid peroxidation (Divya et al., 2016). Chelating agents that form œ bonds with metals are effective secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal. Although not significantly different, the general trend observed in the % chelating activity for peels, was that the highest chelating % was observed in dried products (average 86%) (the removal of moisture caused a more concentrated product), followed by fresh (average 78%), juice (average 77%), chutney (average 75%) and the lowest in preserves (average 67%) (**Table 1**). Robusta (*O. robusta*; purple) peel was the cultivar with the highest levels (91.4% for preserves to 97.3% for fresh peels) throughout all the products (**Table 1**). In a study done by Divya et al. (2016) on bitter orange, it was found that peels provided a higher chelating activity than the pulp.

DPPH Radical Scavenging

The % DPPH indicates the free-radical scavenging activity of the antioxidants. DPPH is a protonated radical (that has absorption maxima at 517 nm) that decreases with scavenging of the proton radical (Divya et al., 2016). Antioxidants react with DPPH (the nitrogen-centered free radical) that convert to 1,1 diphenyl-2-picryl hydrazine due to their hydrogen donating ability. This will intercept the propagation of the free radical chain of oxidation and will form thereby stable end products. The DPPH assay for antioxidant activity is measured by a decrease in the absorbance when the DPPH radical receives a hydrogen radical or an electron from an antioxidant compound to become a stable diamagnetic molecule (Butera et al., 2002). According to the % DPPH data, in general, the average values for chutney, dried and fresh peels were the highest and almost similar (96%, 95%, 93%, respectively) (**Table 1**). Chutney had higher values (94.6 for Gymno-Carpo to 97.3% for Robusta) than dried (93.3 for Nepgen to 95.6% for Meyers) and fresh (91.2 for Gymno-Carpo to 96.3% for Meyers) cactus pear peels. The values for fresh, dried and chutney did not differ significantly, but juice and preserves were significantly lower, with preserves having the lowest values (average in juice 89% vs. average in preserves 77%). Robusta had the highest values for preserves and juice, while Meyers had the highest values for fresh and dried products, although by very slight margins. Percentage DPPH levels for peels were higher (89.9% average)

TABLE 1 | The effect of cultivar and product type on the antioxidant properties of fresh and processed cactus pear peels.

Product	Cultivar	Chelating activity (%)	DPPH (%)	Ascorbic acid (mg/100 g)	Betacyanins (mg/kg)	Betaxanthins (mg/kg)	Betacyanins + Betaxanthins (mg/kg)	Carotene (μg/g)	Phenolics (mg/kg)
Fresh	Gymno C	72.50 ± 9.01^{abcde}	91.18 ± 2.11^{efgh}	68.04 ± 38.66^{bcde}	2.21 ± 0.21^{ab}	1.55 ± 0.15^{ab}	3.75 ± 0.37^{ab}	3.99 ± 1.32^{ab}	14.04 ± 2.48^{ab}
	Meyers	70.00 ± 10.90^{abcd}	96.25 ± 2.91^{gh}	86.28 ± 21.90^{de}	6.87 ± 0.75^{ab}	4.81 ± 0.53^{ab}	11.69 ± 1.28^{ab}	1.79 ± 0.35^{ab}	58.88 ± 25.60^{abcdef}
	Nepgen	81.67 ± 5.20^{cdefgh}	91.67 ± 1.04^{efgh}	55.88 ± 7.71^{abcde}	0.89 ± 0.37^{a}	0.62 ± 0.26^{a}	1.52 ± 0.63^{a}	3.46 ± 0.40^{ab}	15.96 ± 8.06^{ab}
	Ofer	69.17 ± 3.82^{abcd}	93.85 ± 0.75^{fgh}	64.24 ± 16.73^{abcde}	1.11 ± 0.22^{a}	0.78 ± 0.15^{a}	1.89 ± 0.37^{a}	4.80 ± 0.59^{ab}	21.31 ± 14.73^{abc}
	Robusta	97.32 ± 0.38^{h}	91.65 ± 0.44^{efgh}	61.16 ± 25.48^{abcde}	42.62 ± 8.79^{c}	29.84 ± 6.15^{c}	72.46 ± 14.94^{c}	6.06 ± 2.83^{b}	7.44 ± 4.48^{a}
Chutney	Gymno C	68.33 ± 3.82^{abc}	94.55 ± 0.62^{fgh}	41.99 ± 10.16^{abcd}	0.86 ± 0.06^{a}	0.60 ± 0.04^{a}	1.46 ± 0.10^{a}	1.27 ± 0.16^{a}	95.41 ± 2.80^{fg}
	Meyers	66.67 ± 5.20^{abc}	95.30 ± 0.58^{fgh}	35.75 ± 7.54^{abcd}	3.66 ± 1.72^{ab}	2.56 ± 1.20^{ab}	6.22 ± 2.92^{ab}	1.36 ± 0.32^{a}	87.53 ± 0.72^{defg}
	Nepgen	$75.83 \pm 8.04^{abcdefgh}$	98.25 ± 1.00^{h}	18.39 ± 1.16^{ab}	1.52 ± 0.50^{a}	1.07 ± 0.35^{a}	2.59 ± 0.85^{a}	0.51 ± 0.29^{a}	69.11 ± 17.46^{bcdef}
	Ofer	72.50 ± 2.50^{abcde}	94.57 ± 0.56^{fgh}	27.22 ± 5.43^{abc}	0.65 ± 0.10^{a}	0.45 ± 0.07^{a}	1.10 ± 0.17^{a}	0.66 ± 0.26^{a}	93.26 ± 1.95^{fg}
	Robusta	94.29 ± 0.66^{efgh}	97.34 ± 0.15^{h}	37.98 ± 6.98^{abcd}	6.38 ± 0.75^{ab}	4.47 ± 0.53^{ab}	10.85 ± 1.28^{ab}	1.89 ± 0.65^{ab}	47.91 ± 7.93^{abcdef}
Dried	Gymno C	79.17 ± 3.82^{bcdefgh}	94.61 ± 1.12^{fgh}	78.65 ± 28.3^{cde}	2.51 ± 0.75^{ab}	1.76 ± 0.52^{ab}	4.27 ± 1.27^{ab}	81.92 ± 2.83^{de}	87.78 ± 9.52^{defg}
	Meyers	95.20 ± 0.13^{efgh}	95.63 ± 1.21^{fgh}	66.87 ± 31.53^{bcde}	4.57 ± 2.75^{ab}	3.20 ± 1.93^{ab}	7.77 ± 4.68^{ab}	81.27 ± 0.56^{de}	100.96 ± 59.49^{fg}
	Nepgen	85.00 ± 9.01^{cdefgh}	93.33 ± 3.63^{efgh}	63.23 ± 15.88^{abcde}	0.33 ± 0.23^{a}	0.23 ± 0.16^{a}	0.56 ± 0.38^{a}	78.72 ± 2.76^{d}	78.52 ± 19.92^{cdefg}
	Ofer	74.17 ± 7.22^{abcdefg}	94.66 ± 1.48^{fgh}	47.58 ± 18.25^{abcd}	1.65 ± 0.72^{ab}	1.16 ± 0.50^{ab}	2.81 ± 1.22^{ab}	83.31 ± 3.50^{e}	89.23 ± 8.81^{efg}
	Robusta	96.46 ± 1.21^{gh}	94.71 ± 0.17^{fgh}	109.46 ± 27.80^{e}	42.47 ± 12.81^{c}	29.73 ± 8.97^{c}	72.20 ± 21.78^{c}	72.78 ± 3.20^{c}	126.82 ± 39.66^{g}
Juice	Gymno C	73.33 ± 14.65^{abcdef}	88.37 ± 5.38^{defg}	33.07 ± 17.67^{abcd}	0.59 ± 0.11^{a}	0.42 ± 0.08^{a}	1.01 ± 0.19^{a}	1.07 ± 0.01^{a}	59.56 ± 11.97^{abcdef}
	Meyers	69.17 ± 15.07^{abcd}	87.67 ± 8.58^{def}	39.12 ± 6.07^{abcd}	0.91 ± 0.48^{a}	0.64 ± 0.34^{a}	1.56 ± 0.82^{a}	1.38 ± 0.13^{a}	47.74 ± 9.00^{abcdef}
	Nepgen	77.50 ± 8.66^{bcdefgh}	85.41 ± 1.08^{de}	35.08 ± 6.41^{abcd}	0.27 ± 0.08^{a}	0.19 ± 0.05^{a}	0.46 ± 0.13^{a}	0.68 ± 0.26^{a}	53.55 ± 4.98^{abcdef}
	Ofer	69.17 ± 2.89^{abcd}	88.65 ± 3.10^{defg}	29.65 ± 2.34^{abc}	0.90 ± 0.22^{a}	0.63 ± 0.15^{a}	1.52 ± 0.37^{a}	0.85 ± 0.11^{a}	52.06 ± 7.89^{abcdef}
	Robusta	95.64 ± 0.90^{fgh}	96.12 ± 0.14^{gh}	37.00 ± 2.78^{abcd}	5.62 ± 1.46^{ab}	3.93 ± 1.02^{ab}	9.55 ± 2.48^{ab}	3.03 ± 0.15^{ab}	54.74 ± 8.64^{abcdef}
Preserves	Gymno C	53.33 ± 6.29^{a}	68.23 ± 2.26^{ab}	19.77 ± 1.13^{ab}	2.65 ± 3.58^{ab}	1.86 ± 2.50^{ab}	4.51 ± 6.08^{ab}	1.62 ± 0.05^{ab}	34.73 ± 1.82^{abcde}
	Meyers	53.33 ± 3.82^{a}	82.37 ± 1.76^{cd}	23.19 ± 12.14^{ab}	0.88 ± 0.11^{a}	0.62 ± 0.08^{a}	1.49 ± 0.19^{a}	1.60 ± 0.04^{a}	31.58 ± 17.56^{abcd}
	Nepgen	79.17 ± 1.44^{bcdefgh}	75.32 ± 2.58^{bc}	33.12 ± 11.91^{abcd}	1.22 ± 0.36^{a}	0.86 ± 0.25^{a}	2.08 ± 0.62^{a}	1.06 ± 0.05^{a}	78.52 ± 13.32^{cdefg}
	Ofer	57.50 ± 15.21^{ab}	63.19 ± 2.51^{a}	43.29 ± 16.66^{abcd}	0.59 ± 0.14^{a}	0.41 ± 0.10^{a}	1.00 ± 0.23^{a}	0.79 ± 0.02^{a}	15.94 ± 9.56^{ab}
	Robusta	91.40 ± 1.68^{defgh}	93.41 ± 0.11^{efgh}	11.67 ± 1.30^{a}	12.31 ± 4.17^{b}	8.62 ± 2.92^{b}	20.93 ± 7.08^{b}	2.69 ± 0.04^{ab}	74.73 ± 14.95^{cdefg}
AVG		76.71	89.85	46.71	5.77	4.04	9.81	17.54	59.89
STD		14.14	9.05	27.02	11.60	8.12	19.71	31.33	34.57
N		6	6	6	6	6	6	6	6
Significance level		$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

Means with different superscripts in the same column differ significantly. Values for fresh peels were reported in De Wit et al. (2019).

than that reported for cladodes (84.1%) [44] and fruit pulp (87.9% average) (Du Toit et al., 2018b). Abou-Elella and Ali (2014) reported % DPPH values of up to 97.7% in fresh peels, however, the color of the cultivar was not indicated. In a study by Abdel-Hameedl et al. (2014) it was found that the juice of the peels had the highest % DPPH in red fruits (compared to yellow fruit peels) and that DPPH radical scavenging was higher in the peels than in the pulp. For oranges, it was found that the % DPPH found for the peel was higher than that of the pulp (Divya et al., 2016).

Ascorbic Acid

Main vitamins in *Opuntia* spp. include vitamin E, vitamin C, vitamin K, and tocopherols and their contents depend on the cultivar types (Diaz Medina et al., 2007). Being an important nutritional antioxidant, the ascorbic acid content in cactus pear fruits is notably higher than the average ascorbic acid content in regularly consumed fruits such as plums (7 mg/100 g fresh fruit), nectarines (10 mg/100 g fresh fruit) and peaches (9 mg/100 g fresh fruit) (Gil et al., 2000). The average ascorbic acid content was 46.7 mg/100 g. Ascorbic acid levels in peels ranged from 11.7 mg/100 g in Robusta peel preserves to 109.5 mg/100 g for dried Robusta peels (**Table 1**). The lowest values were observed in preserves (26.2 mg/100 g average), chutney (32.3 mg/100 g average) and juice (35 mg/100 g average), with much higher values in fresh peels (67.1 mg/100 g average) and the highest in dry peels (73.2 mg/100 g average). Most products and cultivars showed statistically similar results. Interestingly, the two products with the highest ascorbic acid content were fresh and dried peels, while chutney and juice had lower, and very similar, results. Preserves had the lowest ascorbic acid content. This could possibly be ascribed to the heat-sensitivity of ascorbic acid to the high temperatures applied during chutney and preserves production. The levels for peel juice were approximately 50% that of the values for fresh peel, the same as was found by Gurrieri et al. (2001). Fernández-López et al. (2010) reported ascorbic acid values for fresh peels of 14.5–23.3 mg/100 g, while Diaz Medina et al. (2007) reported the highest ascorbic acid values in the red-skinned fruit (815 mg/g). El-Said et al. (2011) found 590 mg/kg ascorbic acid in peels, based on FW, although no color was specified. Abdel-Hameedl et al. (2014) found values of 70.17 mg/100 ml ascorbic acid in the juice from red cactus pear fruit peels. In a study by Barba et al. (2017) it was found that high pressure processing (HPP) and pulsed-electric field (PEF) treatment on cactus pear juice had higher vitamin C values than the juice receiving a mild pasteurization treatment. In a study done by Divya et al. (2016) it was found that the higher ascorbic acid content in the bitter orange pulp caused the reducing capacity to be higher in the pulp than in the peels.

Betalains

The cactus pear fruits are characterized by different colors due to the combination of the purple-red betanin and the yellow-orange indicaxanthin pigments (Cerezal and Duarte, 2005). Betalains also contribute most to cactus pear classification (Moussa-Ayoub et al., 2014). Betalains are excellent radical scavengers with an antioxidant activity 3–4 × higher than ascorbic acid, rutin, and catechin (Cai et al., 2005). Betanin extracts were reported to have anticancer activity (Butera et al., 2002). Fruits of cactus pear contain different betalains whose concentration depends on species, cultivar and geographic region (Del Socorro Santos Díaz et al., 2017). Betalains occur in vacuoles as zwitterions, which implicates that peel and pulp amounts will differ. Heat degradation was also described, although different conditions to that applied in the current study (Damodaran and Parkin, 2017). A value of 39.3 mg/100 g FW was reported for *O. ficus-indica* fruits (no color mentioned) and 80 mg/100 g betacyanin for *O. stricta* fruits (Patil and Dagadkhair, 2019). Beetroot contains ≈ 50 mg/100 g betanin, while purple cactus pear may contain up to 100 mg/100 g betanin FW. In general, the betacyanin (Bc) values were higher than the betaxanthin (Bx) values (5.77 vs. 4.04 mg/kg). Robusta (purple cultivar) had the highest Bc values in all the products, followed by Meyers (red fruit peel). Although not significantly different, Nepgen (green peel) had the lowest Bc values. Dried and fresh products (averages of 10.31 and 10.74 mg/kg, respectively) had the highest values, followed by preserves (3.53 mg/kg) and chutney (2.61 mg/kg), and lastly juice (1.66 mg/kg). Interestingly, Bx contents did not follow the same pattern as observed for Bc, with the highest average values observed in fresh peels (7.52 mg/kg), followed by dried (7.22 mg/kg) and preserves (2.47 mg/kg), with the lowest values in chutney (1.83 mg/kg), and juice (1.16 mg/kg). Bx values were the highest in Robusta (purple peel) followed by the red Meyers and orange Gymno-Carpo fruit peels. The values for betalains (betacyanins + betaxanthins) were generally lower in peel (average of 13.57 mg/kg) than in fruit pulp (18.52 mg/kg average) and cladodes (16.17 mg/kg average) (Du Toit et al., 2018a,b). The lowest Bc and Bx values were found in Nepgen (as was expected), which is a green fruit/peel. Abou-Elella and Ali (2014) reported betacyanin values of 2.94 and betaxanthin values of 2.06 μg/ml in cactus pear fruit peels. Yellow cultivars contained, according to Butera et al. (2002), the highest amounts of betalains, followed by red and white cultivars, while in the current study, purple and red cultivars contained the highest concentrations of betalains, followed by orange and lastly green cultivars. Interestingly, it was reported by Mena et al. (2018) and Amaya-Cruz et al. (2018) that Bc was only found in red cactus pear peel and that red peels showed more Bx contents than yellow and green fruit peels. These authors also reported a greater variety in Bx compounds in the red peels than that found in green and yellow-orange peels. According to Moussa-Ayoub et al. (2014) red fruit peels contains mainly Bc, while yellow-orange fruit peels contain mainly Bx. According to these authors, green fruit cultivar peels contained no betalains. In a study by Barba et al. (2017) it was found that high pressure processing (HPP) and pulsed-electric field (PEF) treatment on juice had a similar effect on Bc than a mild pasteurization treatment, i.e., no difference between HPP and PEF and pasteurization on Bc.

Carotenoids Measured as β-Carotenes

Carotenoids represent the major water-insoluble pigment in the peels (El-Said et al., 2011). β-carotene is pro-vitamin A, which play an important role in cell integrity. Yellow fruits, in general, have higher carotenoid concentrations than other colored fruit (Fernández-López et al., 2010). Carotenoids contribute to the

appearance and antioxidant content, and combat human chronic diseases (Jaramillo-Flores et al., 2003; Abou-Elella and Ali, 2014). Although not very high in cactus pear fruits, they contribute to antioxidant properties. The highest carotenoid contents were found in yellow-skinned cactus pear fruit (23.7 mg/g) (Diaz Medina et al., 2007). It has been reported that processed products often contain similar amounts of carotenoids than the fresh counterparts (Rickman et al., 2007). Carotenoid values were higher in marmalade than in the fresh fruit, especially the β-carotene and lutein (Leopoldo et al., 2012). Values of 2.97 mg/100 g were found in Egyptian prickly pear peels, although no color was mentioned (El-Said et al., 2011).

Carotenoid levels in the cactus pear peel products under study varied from 0.51μg/g (Nepgen, green, chutney) to 83.3 μg/g (Ofer, orange, dried). In general, very high contents were found in dried products (80 μg/g), while much lower values were found in fresh (4.04 μg/g), preserves (1.6 μg/g), juice (1.4 μg/g), and chutney (1.2 μg/g). The high levels could be as a result of the concentrated nature of the dried peel. Robusta was the cultivar with the highest values for all products, followed by Meyers and Gymno-Carpo with very little difference between the remaining two cultivars. Fernández-López et al. (2010) reported total carotenoid values of 2.58–6.68 μg/g for cactus fruit (unpeeled). The average carotenoid value (17.5 μg/g) reported for the peels and its products were higher than that reported for the pulp carotenoid value (4.26 μg/g) and noticeably lower than that reported for the cladodes (36.9 μg/g) (Du Toit et al., 2018a,b) (**Table 1**). Interestingly, it seems as if the contributing effect of the spices added to the chutney was negligible when compared to preserves and juice. Cano et al. (2007) investigated the carotenoids in the peels of red and yellow-orange cactus pear fruit and reported nine xanthophylls and four hydrocarbon carotenes. The main carotenoid in the peel was (all-E)-lutein and (all-E)-β-carotene. Higher values (16.48–19.2 μg/100 FW) were found in the peel than in the pulp. Amaya-Cruz et al. (2018) found the greatest variety of carotenoids in the green peel than the other peel colors.

Total Phenolics

Flavonoids are secondary metabolites and are categorized in different classes such as alkaloids, terpenoids, and phenolics. Its antioxidant properties are ascribed to their ability to reduce free radical formation and to scavenge free radicals (Panche et al., 2016). They are very effective against lipid peroxidation that causes diseases such as atherosclerosis, diabetes, hepatotoxicity, inflammation and aging. Phenolic compounds, including their functional derivatives, can be defined as substances possessing an aromatic ring, carrying one or more hydroxyl groups. Their chemical structures and concentrations are variable and depend on variety, ripeness stage and kind of plant tissue (Wallace, 1986). Flowers and peels could exhibit higher phenolic contents than fruit and cladodes, with about 45.7 g/100 g FW. It is therefore recommended to exploit these materials to obtain bio-compounds with antioxidant characteristics (El-Mostafa et al., 2014). Antioxidant properties of phenols are attributed to their redox properties—they act as reducing agents, H_2 donators, singlet oxygen quenchers and metal chelators (Abdel-Hameedl et al., 2014). Phenolics are reckoned to be the main antioxidants in cactus pears (Abdel-Hameedl et al., 2014; Moussa-Ayoub et al., 2014). Polyphenolics are more stable against radicals than vitamins (Patil and Dagadkhair, 2019). Flavonoids (glycosilated flavols, dihydroflavonols, flavones, and flavonols) are more efficient antioxidants than vitamins because phenolic compounds are able to delay pro-oxidative effects in proteins, lipids and DNA because of the generation of stable radicals (Shahidi and Wanasundara, 1992). Flavonoids present in *Opuntia ficus-indica* include kaempferol, quercitin, narcissin, dihydro-kaempferol, dihydroquercitin and eriodictyol and total phenolic values of 218 mg GAE/100 g FW and total flavonoid values of 19.4 mmol quercitin/ g sample FW were reported (Patil and Dagadkhair, 2019). Phenolics were higher in processed peel products than fresh peels (**Table 1**). In general, dried peels had the highest content of total phenolics (96.7 mg/kg), followed by chutney (78.6 mg/kg), while preserves (47.1 mg/kg) and juice (53.5 mg/kg) had values in a similar range with fresh peels having the lowest values (23.5 mg/kg). Chutney and dried products had the highest contents of phenolics (dried Meyers peel 100.96 mg/kg and Robusta 126.82 mg/kg). Juice had very similar results in all cultivars (47.74–59.56 mg/kg), while the values in preserves were not consistent (**Table 1**). Overall though, there were very high individual results but no one cultivar could be singled out as having the highest phenolic content across the different peel products. The average total phenol value found for all products (59.9 mg/kg) were lower than that reported for the pulp and its products (97.79 mg/kg) (Du Toit et al., 2015, 2018b) as well as for fresh cladodes and its preserved products (130.55 mg/kg) (Du Toit et al., 2018b). In products such as chutney, preserves and juice, the possible effect of the added ingredients and spices, as well as the higher temperature, on the total phenolic content could be speculated on. The presence of phenol compounds such as alicin in garlic and shogao in ginger was reported to contribute to antioxidant activity (Hossain et al., 2008). Total phenolic values of 164.6–218.8 mg GAE/100 g was found by Fernández-López et al. (2010), which were higher in the red skin varieties than in peaches, plums and nectarines. Peels contain higher amounts of phenolics, since phenolics have a tendency to accumulate in the dermal tissues of plant bodies because of their potential role in protection against UV radiation. They also act as attractants in fruit dispersals and as defensive chemicals against pathogens and predators. Cardador-Martínez et al. (2011) reported higher values (376 mg/kg) in unripe green-yellow cultivars than in red-purple (44 mg/kg) cultivars, while Abou-Elella and Ali (2014) reported values between 221.3 μg gallic acid/100 g and 1507μg gallic acid/100 g dry weight. It was found by Divya et al. (2016) that the peel extracts of bitter orange had higher antioxidant activity than the pulp extracts. This was attributed to the higher concentrations of phenolic acids and their derivatives in the peels. Phenolic compounds content vary in different plant parts. An interesting observation was made in strawberries, where achenes (real dry fruits on strawberries) contained 10x more phenolics that the fruit flesh (Ariza et al., 2017). As already mentioned, boiling of onions resulted in lower losses of flavonoids than for example frying. Dehydrated onions contained low amounts of flavonoids. The size, as well as the

distribution in the onion bulb affected the flavonoid content (Lee et al., 2008). Different results were reported by various authors. Amaya-Cruz et al. (2018) reported the presence of 68 extractable polyphenols and 15 hydrolysable polyphenols in the peel of green, yellow-orange and red cactus pear fruit. Green peels had the highest content of extractable polyphenols, while the peel of green and yellow-orange fruit contained the most hydrolysable polyphenols. Betalains, carotenoids and phytochemical contents were the highest in the red peels. Total phenolics found by these authors were 30% higher in the green peel (12.28 GAE mg/g) than in the yellow-orange (8.62 GAE mg/g) and red peel (9.64 GAE mg/g) and were similar to values reported by Jiménez-Aguilar et al. (2015) (9.94–12.75 GAE mg/g). These authors also stated that total phenolics were higher in green and yellow-orange peels than in red peels. According to them, green peels would have a greater beneficial effect on health than the red and yellow-orange peels since it contains a greater diversity, variety and abundance of flavonols and phenolic acids than red and yellow-orange peels. Jiménez-Aguilar et al. (2015) mentioned that peels contained higher TP than pulp, seeds or juice, while Moussa-Ayoub et al. (2014) reported the lowest TP values found in green colored cactus pear peels compared to the red and yellow-orange peels. These authors also reported that cultivars from South Africa had higher phenolic contents than the Egyptian and Sicilian cultivars studied. Abdel-Hameedl et al. (2014) reported highest values in red fruit peels (1152.97 mg GAE/100 ml), higher than in yellow fruit peels (786.01 mg GAE/100 ml).

Regarding flavonols, it was found by Moussa-Ayoub et al. (2014), that fruit color did not influence the flavonol profile and that green and yellow-orange cultivars produced similar profiles. Red fruits however, contained higher contents of flavonol glycosides compared to yellow-orange and green cultivars. The two cultivars from South Africa showed the highest flavonol contents and thus the effect of location on antioxidant content. Surprisingly, the pulp of *O. ficus-indica* contains no flavonols, however, flavonols such as isorhamnetin occurs exclusively in the fruit's peels (2.2–4.1 mg/g DW) and might be higher than that found in some other common fruits (Moussa-Ayoub et al., 2014). Conflicting results were reported by Amaya-Cruz et al. (2018) who found higher flavonoid contents in green and yellow-orange cultivars than in the red cultivar, while Abdel-Hameedl et al. (2014) found that flavonoids and flavonols are higher in red peels than in yellow peels, but the total flavonoids and flavonols in pulp of red and yellow peels are higher than in the peel of red and yellow peels. Furthermore, flavonols were higher in the peels than in the pulp of cactus pear fruit and higher in peels from thornless cultivars than peels from spiny cultivars (Barba et al., 2017).

PCA of Cultivar and the Antioxidants Properties of Cactus Pear Peel

In **Figure 1**, F1 and F2 explained 72.80% of the variation (F1: 50.62%; F2: 22.18%). It can be seen that products, rather than cultivars, seem to cluster together, e.g., juices and fresh peels (close to 0), chutneys (top left quadrant), preserves (left bottom quadrant) and dried peels (top right quadrant). Products on the positive side of Factor 1 are mostly associated with the antioxidants. It is evident that Robusta and its products cluster together (bottom right quadrant) as well as with betalains (Bc, Bx and Bc+Bx). Robusta is also the only cultivar where its products cluster together. It is also clear that % DPPH, carotenoids and phenolics are grouped together in the upper right quadrant, with % chelating activity closely correlated with ascorbic acid. Dried products from all cultivars correlated closely with % DPPH, carotenoids and phenolics, especially dried peel from Gymno-Carpo (orange), Ofer (orange), Meyers (red) and Nepgen (green). Furthermore, it is also visible that red and orange preserves form a cluster, while green preserves and chutney from all cultivars cluster together. Mostly fresh peels and juices cluster together. The position of the vectors in Factor 1 indicated a high correlation between all antioxidants. It was reported that DPPH correlated highly with phenolics and flavonoids (Butera et al., 2002), while ascorbic acid showed a good correlation with antioxidant activity. According to Patil and Dagadkhair (2019) ascorbic acid is responsible for 30–40% of antioxidant activity in cactus pear fruit. This correlation is also evident in the current study with ascorbic acid clustering with % chelating activity. Total phenolics correlated linearly with reducing power, free radical scavenging and total antioxidant activity (Abdel-Hameedl et al., 2014) and red peel and pulp had a higher DPPH radical scavenging ability than yellow peel and pulp, implicating a higher antioxidant activity in red peels than in yellow peels. Divya et al. (2016) reported higher retention of total phenolics in pickles than in sweet preserves of bitter orange peels and attributed this trend to heat processing causing more destruction than mechanical processing (destruction). The antioxidant activity of sweet preserves was higher than that of pickles. This was attributed to the liberation of low molecular weight antioxidants on thermal treatment. In the present study, chutney had higher total phenolics than juices and preserves, all products undergoing a heat treatment. It is also known that phenolic compounds may bind to food proteins covalently and non-covalently. These proteins include mostly milk caseins and soy glycinin and β-conglycinin (Zhang et al., 2014). It also binds to enzymes such as maltase, lipase, protease (trypsin), usually proteins with regions which are proline-rich (such as found in cereals) and hydrophobic (Zhang et al., 2014). These types of interactions are therefore not expected in cactus pear fruit peels, since only sucrose and stachyose are reported to be present in peels and no starch (El-Said et al., 2011). Different heat treatments have different effects on flavonoid contents as was reported for home-processed onions, e.g., while frying resulted in a 23% loss of flavonoids, baking caused no losses (Lee et al., 2008).

According to Fernández-López et al. (2010) ascorbic acid contributed 68% to antioxidant activity, but that % DPPH correlated weakly with betalains (Kugler et al., 2007) and carotenes. However, in the present study, % DPPH correlated strongly with carotenes and phenolics, but also showed no correlation with betalains. Melgar et al. (2017) found a correlation between DPPH and Bc as well as between DPPH and total phenolics. Antioxidant activity usually correlated strongly with the amount of phenolics. Phenolics in peels are good electron donors and could therefore terminate the radical chain reaction by changing free radicals to more stable products (Butera

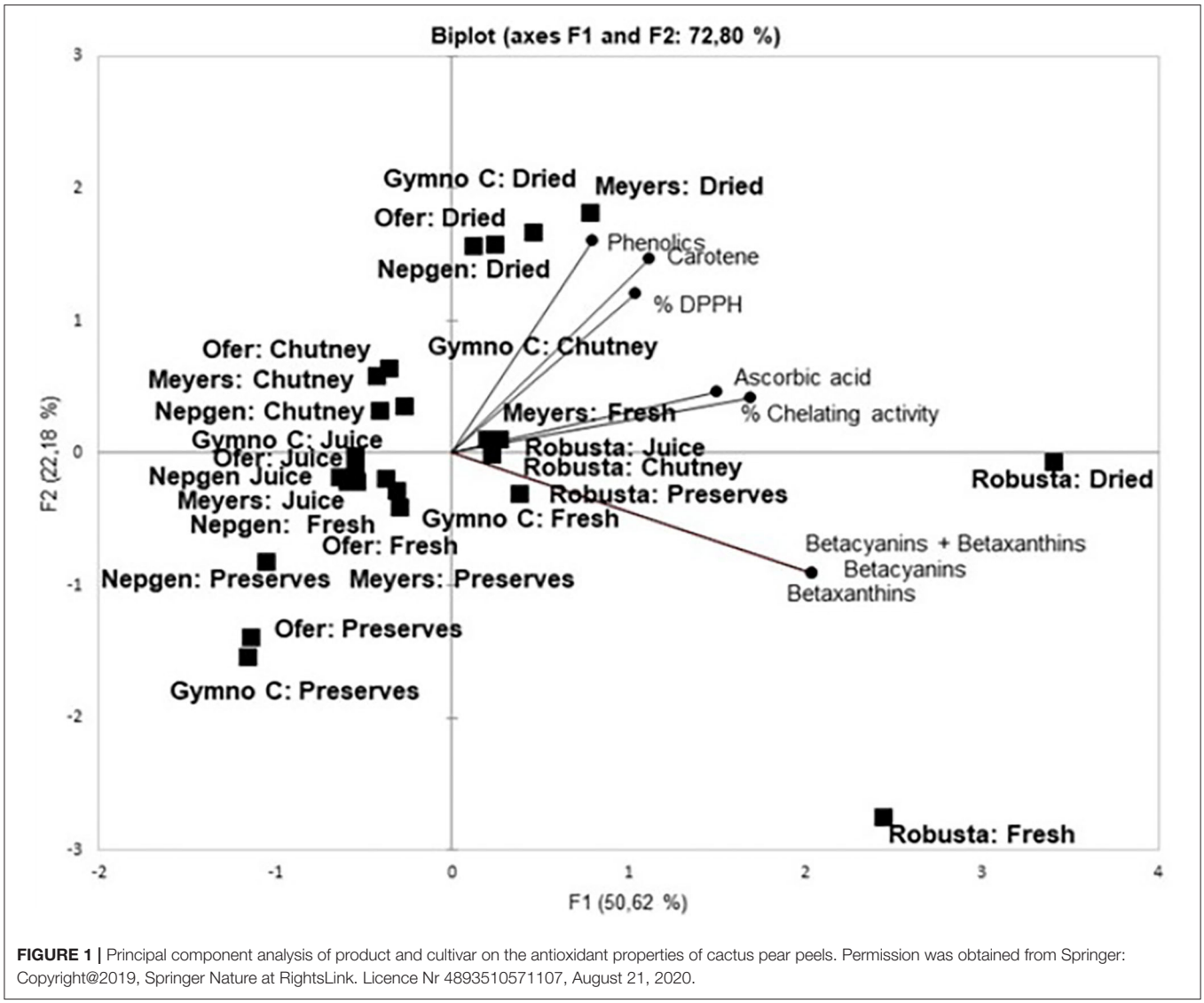

FIGURE 1 | Principal component analysis of product and cultivar on the antioxidant properties of cactus pear peels. Permission was obtained from Springer: Copyright@2019, Springer Nature at RightsLink. Licence Nr 4893510571107, August 21, 2020.

et al., 2002). Divya et al. (2016) also described a high association between total phenols and free radical scavenging and metal ion chelation. The extent of association depended on the polyphenol and the transition metal concentration.

Chain reaction by changing free radicals to more stable products (Butera et al., 2002). Divya et al. (2016) also described a high association between total phenols and free radical scavenging and metal ion chelation. The extent of association depended on the polyphenol and the transition metal concentration.

In the lower left quadrant, it seems that preserved products are clustered together and are not closely related to any of the antioxidants (lowest values in **Table 1**) while in the upper left quadrant the chutneys seemed to bundle (high values in **Table 1**). Robusta products were scattered around the betalain content marker (lower right quadrant). Ofer (fresh and juice) (on the left) are not associated with any of the antioxidants.

The PCA figure correlates with data from **Table 1**, since the products with the highest contents and –capacities were situated nearby the associated markers, whereas products with similar results (chutney and preserves) were bundled together. Peel showed antioxidant content similar to that of fruit (Du Toit et al., 2018b), implying that both fruit and peel would contribute to excellent antioxidant capacity; therefore the peel should be included in processed products such as juice, dried fruit and chutneys, where possible. In the study done by Divya et al. (2016) it was found that the higher antioxidant activity in thermally processed sweet preserves were attributed to the disruption of the peel matrix and caused leaching of phytonutrients. Heat treatment, infrared radiation, fermentation and proteases liberate and activate low molecular weight antioxidants. It was found by these authors that total phenols increased in sweet preserves and chili pickles. An increase in flavonoid content of onions subjected to fluorescent light was also observed by Lee et al. (2008).

The cultivar that could be identified as the best fruit (pulp and peel) from an antioxidant point of view was Robusta. Robusta is not regarded as a popular fresh fruit product, because it lacks sweetness as well as acidity, resulting in an unpleasant

taste. However, after processing it was transformed into highly acceptable products when sugar and acids were added during processing. It is regarded as the only cactus pear cultivar in South Africa that is unacceptable as fresh fruit but is ideal to be used for processed products. It had high % DPPH and % chelating activity readings, very high betalain content and fair amounts of ascorbic acid, carotenes and phenolics.

Drying is the product/processing method of choice, regardless of cultivar. According to Vinson et al. (2005), dried fruit had denser nutrient contents and significantly higher phenolic antioxidant contents than fresh fruit. Dried fruit contains complex carbohydrates, is higher in fiber and has a longer shelf-life and should be recommended to be added to daily diets. Convenience foods are becoming more popular due to the modern lifestyles and consequently, dried products are popular because of the light weight, shelf-stability and small size (Sharma et al., 2011). Opposite results were however reported by Lee et al. (2008), for commercially dehydrated onion products. These were shown to contain very little or no flavonoids. The stability that betalains showed in the current study, with regards to heat and pH, was reported before (Mosshammer et al., 2006). Ascorbic acid may be retained or protected in certain circumstances during processing (Sáenz et al., 2004). Increased carotene contents after processing was reported by Jaramillo-Flores et al. (2003) while the phenolics displayed high levels in the current study. Consequently, processed cactus pear products may provide more antioxidants to the consumer than fresh fruit as was found by Divya et al. (2016) who reported that sweet preserves, chili pickles and salted pickles all had higher antioxidant activities than the fresh products. Onion by-products added to processed meat products, i.e., pre-cooked pork patties as antioxidants, increased the shelf-life (Cao et al., 2013). Color extracts (anthocyanins) from e.g., blueberries could be used in bakery products to add value and functionality as was reported by Primo da Silva et al. (2019).

CONCLUSIONS

Cactus pear fruit peels contain high quantities of antioxidants which contribute to considerable antioxidants capacities. It could be concluded that the processed cactus pear peel contains high levels of antioxidant as well as demonstrates high antioxidant capacity. Peels that are normally discarded as waste when only fruit is used, should be utilized for preservation purposes.

Cactus pear fruit peels should be included in processed products such as juice, dried fruit and chutneys, where possible. The best process for peels was drying while the preserves had the lowest values in terms of antioxidants.

Purple fruit peel products contained the highest % DPPH, % chelating activity, betalains, carotenoids and phenolics, while in orange and pink fruit peels, ascorbic acid dominated. Purple (first) and orange (second) colored cultivars could be the best choice in terms of antioxidant content. The cultivar that could be pinpointed as the best fruit peel from an antioxidant point of view for preservation was Robusta. It had high % DPPH and % chelating activity readings, very high betalain content and fair amounts of ascorbic acid, carotenoids and phenolics. Nepgen (green) was the cultivar with the lowest antioxidant content (not significantly), but demonstrated equally good antioxidant capacity levels. The current study may serve as a benchmark for antioxidant content, activity and retention in processed products, while future studies should focus on the analytical chemistry analysis of antioxidant compounds. Further research must include bioavailability and bioaccessibility studies on these "new" nutraceutical food products. These are defined by Ariza et al. (2017) as: "bioavailability is the ease with which compounds are assimilated by the body and have a positive effect on health, thus the fraction of the given compound or its metabolites that reaches systematic circulation without considering bioactivity", while bioaccessibility is defined as "the fraction of a food constituent that is released from a food matrix in the GIT and becomes available for absorption". Other new innovative products should be developed using processing and preservation methods such as fermentation.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

MD: conceptualization, project administration, supervision, and writing—review and editing. AD: data curation, investigation, and writing—original draft. AD and AH: formal analysis. MD and GO: methodology. GO and AH: validation. AH: visualization. All authors: contributed to the article and approved the submitted version.

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Bactericidal Effects of *Exiguobacterium* sp GM010 Pigment Against Food-Borne Pathogens

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Bacterium producing yellowish-orange pigment was identified (morphological, biochemical, and 16S rRNA) as *Exiguobacterium* sp GM010. The UV-visible spectrum of *Exiguobacterium* sp GM010 extract showing λ max at 465 nm revealed orange pigment characteristic. Pigment showed broad spectrum antibacterial action against gram positive and gram negative food-borne pathogens. The SYTO9 and propidium iodide (PI) staining revealed the cell membrane damage of food-borne pathogens under confocal laser scanning microscope (CLSM) indicating the bactericidal effect. This was evidenced by the fourier transform infrared (FTIR) spectrum, showing characteristic functional groups that mainly included hydroxyl, carbonyl, and carboxylic groups causing a system of delocalized electrons leading to destabilization of membrane and decrease in membrane potential that resulted in bactericidal effect. The pigment of *Exiguobacterium* sp GM010 were non-toxic against *Artemia franciscana* and can be a promising source to control the food-borne pathogens in food industries.

Keywords: *Exiguobacterium* sp GM010, pigment, marine bacteria, antimicrobial, food-borne pathogens

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INTRODUCTION

Food-borne diseases have become one of the most widespread public health problems. About two-thirds of all the outbreaks were due to consumption of microorganisms contaminated food and water. World Health Organization (WHO) estimates, unsafe food causes 600 million cases of food-borne diseases and 4,20,000 deaths annually, including children (Bajpai et al., 2013; Fukuda, 2015; World Health Organization, 2020). Hence, food safety is a major concern not only for developing countries but also for the developed countries. A study was conducted to characterize the prevalence and diversity of food-borne pathogens, recommended to minimize the risk of contamination in fields (Strawn et al., 2013). Recent reports have identified the prevalence of bacterial pathogens in quality export seafood (Bandekar, 2015) causes massive economic losses to the food industry.

To reduce the health risks and economic losses, the foods are treated with antimicrobial agents as preservative. There has been a major focus on the development of safe and efficient natural broad spectrum antimicrobials that can replace synthetic alternatives (Kim et al., 2013; Al-zoreky and Al–Taher, 2015). The marine microorganisms are excellent source of bioactive compounds, where antimicrobial compounds stand in the majority (Burgess et al., 1999; Nithyanand et al., 2011; Wiese and Imhoff, 2019). Many marine microorganisms have been isolated from different marine habitats to discover new bioactive compounds (Blunt et al., 2012, 2014). These bioactive compounds have a considerable importance (Newman and Cragg, 2007) for drug discovery industry due to their

TABLE 1 | Antimicrobial activity of pigment of *Exiguobacterium* sp GM010 against food-borne pathogens.

Strain/antibiotics	Inhibition zone (mm)*							
	Gram-positive					Gram-negative		
	***B. cereus* ATCC 11778**	***B. subtilis* ATCC 06633**	***S. aureus* FRI722**	***M. luteus* ATCC 9341**	***L. monocytogenes* Scott A**	***E. coli* EFR02**	***P. aeruginosa* ATCC 15442**	***Klebsiella* sp**
Exiguobacterium sp GM010	7.16 ± 0.28	7.66 ± 0.57	8.5 ± 0.5	10.83 ± 0.36	14.33 ± 0.57	6.66 ± 0.28	8.16 ± 0.28	8.1 ± 0.0.28
AMP	10	16	12	16	18	10	8	11
TET	18	18	16	21	21	12	16	16
CIP	16	19	14	23	18	14	15	14

**Zone of inhibition including the disc.*
AMP, Ampicillin; TET, Tetracycline; CIP, Ciprofloxacin are standard broad spectrum antibiotics.

structural diversity and biological activity. More than 22,000 bioactive compounds from marine organisms have been reported (Demain and Sanchez, 2009) that includes both prokaryotes and eukaryotes. The *Streptomyces* sp was widely studied microbial species from Indian coastal waters as a source of antibiotics (Chandramohan, 1997; Valliappan et al., 2014). There are report on the algicidal (Li et al., 2016), antifungal (Selvakumar et al., 2009) and antibacterial (Shanthakumar et al., 2015) effect of *Exiguobacterium* species, however mechanism of antibacterial effect of *Exiguobacterium* pigment on food-borne pathogens was not reported. In this study pigment producing *Exiguobacterium* sp GM010 was isolated, and characterized. The antibacterial properties of pigment against the food-borne pathogens, mode of action, and non-toxic effect suggested the potential applications in food industries.

MATERIALS AND METHODS

Food-Borne Pathogens

Food-borne pathogens *Escherichia coli* EFR02, *Staphylococcus aureus* FRI722, *Pseudomonas aeruginosa* ATCC 15442, *Bacillus subtilis subsp spizizenii* ATCC 06633, *B. cereus* ATCC 11778, *Klebsiella sp, Listeria monocytogenes* Scott A, and *Micrococcus luteus* ATCC 9341 were obtained from Food Safety and Analytical Quality Control Laboratory, CSIR-Central Food Technological Research Institute, Mysore, Karnataka, India. Stock cultures of pathogens were maintained on nutrient agar at 4°C and sub-cultured in nutrient broth at 37°C, before activity assay.

Isolation and Identification

Sediment samples collected from Tamil Nadu coastal regions were used for isolation of bacteria. The isolated chromosomal DNA (Marmur, 1961) was amplified using universal primers 27F (50-AGA GTT TGA TCC TGG CTC AG-30) and 1492R (50-GGT TAC CTT GTT ACG ACT T-30) (Lane, 1991). The 16S rRNA gene sequence was compared using the NCBI BLAST for similarity with the reference bacterial species in GenBank database. Multiple alignments of the sequences using the Clustal W program and phylogenetic tree construction using treeing algorithms were performed in MEGA X software package.

Antibacterial Activity and Mode of Action

The strain GM010 was inoculated into 100 ml of zobell marine broth (ZMB) in 250 ml conical flask and incubated for 3 days at 30°C, 150 rpm. After incubation, the pigment was extracted by solvent-solvent partition by adding equal volume of ethyl acetate to the culture broth. Ethyl acetate fraction was separated and concentrated. The stock of pigment was prepared by dissolving in DMSO (50 mg/ml) for antibacterial assay (Dhale et al., 2007). The overnight grown food-borne pathogen (**Table 1**) bacterial cultures (200 μl) were spread on nutrient agar. The 6 mm disks impregnated with the 200 μg of GM010 pigment were placed and incubated at 37°C for 24–48 h. The activity was determined by measuring the inhibition zones. The disks with DMSO and standard antibiotics were used as negative and positive control, respectively.

Confocal Laser Scanning Microscopy (CLSM)

The mode of inhibition of food-borne pathogens, was determined using LIVE/DEAD BacLight™ Bacterial Viability Kit (Invitrogen, Molecular probes Inc) according to manufacture's protocols. One ml of food-borne pathogen cells were treated with 200 μg of GM010 pigment in centrifuge tubes. After removing growth medium, the cells were washed with 0.85% saline and mixture of SYTO9 and propidium iodide (PI) was added to the treated cell. This was incubated in dark at room temperature for 20–25 min in dark. After incubation, the images were acquired in Zeiss LSM 700 CSLM to analyze samples. Images were acquired with 512 × 512 resolutions in at least three different fields. Zeiss ZEN software was used to acquire images. The laser was used at 488 nm for excitation and the emission was observed at 528 nm (SYTO9) and 645 nm (PI).

Scanning Electron Microscopic (SEM)

The morphological changes of food-borne pathogen cells were analyzed after treatment with GM010 pigment under SEM (Asensioa et al., 2005). Briefly, 1 ml of bacterial culture was added to centrifuge tube containing 200 μg of pigment. This was centrifuged after incubation at 37°C for 6 h, the pellets were rinsed with phosphate buffer and fixed with 3% glutaraldehyde

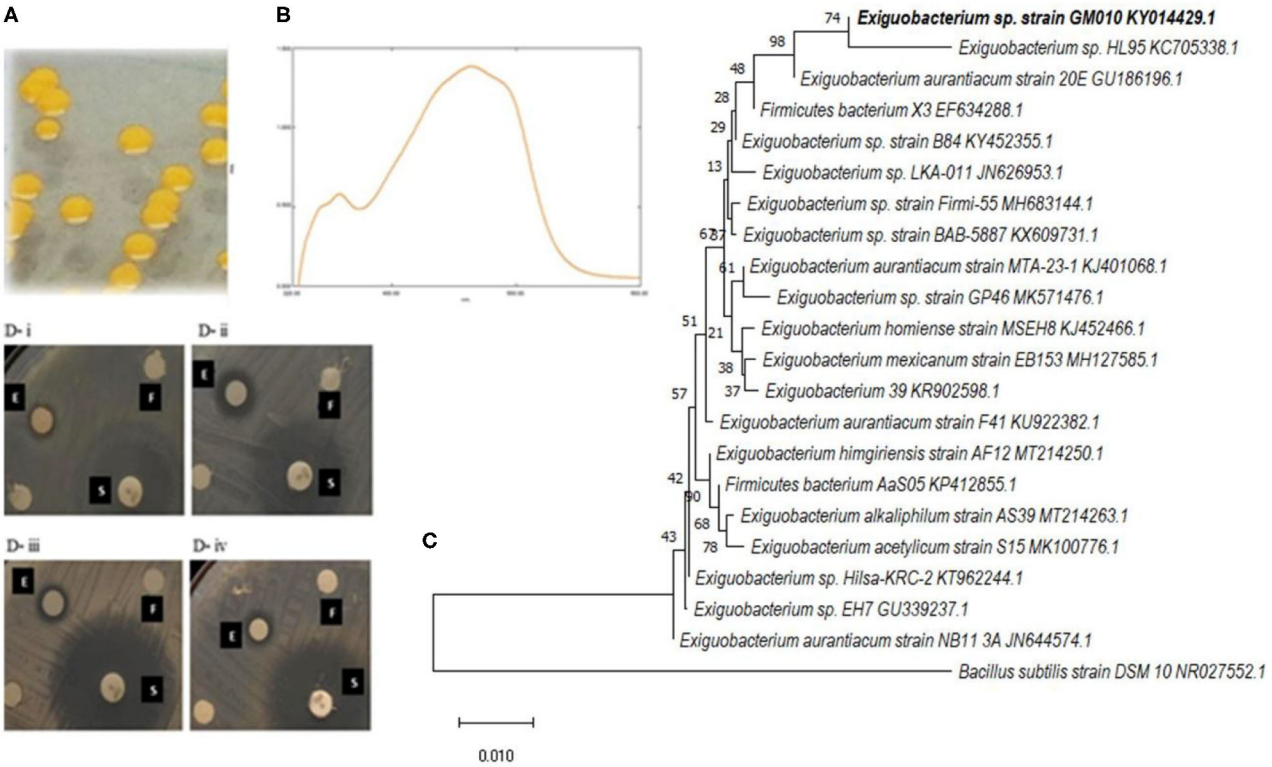

FIGURE 1 | The pigmentation was observed on zobell marine agar by strain GM010 **(A)** and the extract of strain GM010 showing absorption peak at 465 nm confirmed the pigment production **(B)**. Based on 16S rRNA gene sequence, neighbor-joining phylogenetic tree showing the relationship of strain GM010 with related species of *Exiguobacterium* **(C)**. Numbers at the nodes indicate percentage bootstrap values (500 replicates). Disc diffusion assay **(D)** showing inhibitory action of *Exiguobacterium* sp GM010 (E) against food-borne pathogens *B. cereus* ATCC 11778 **(i)**, *S. aureus* FRI722 **(ii)**, *Klebsiella* sp **(iii)** and *E. coli* EFR02 **(iv)**. The DMSO (F) and standard antibiotic (S) were used as negative control and positive control, respectively.

TABLE 2 | Minimal inhibitory concentration and minimal bactericidal concentration of pigment of *Exiguobacterium* GM010 against food-borne pathogens.

Strain/ Antibiotics (μg/ml)	**Gram +ve**										**Gram –ve**					
	***B. cereus* ATCC 11778**		***B. subtilis* ATCC 06633**		***S. aureus* FRI722**		***M. luteus* ATCC 9341**		***L. monocytogenes* Scott A**		***E. coli* EFR02**		***P. aeruginosa* ATCC 15442**		***Klebsiella* sp**	
	MIC	***MBC***	***MIC***	***MBC***	***MIC***	***MBC***	***MIC***	***MBC***	***MIC***	***MBC***	***MIC***	***MBC***	***MIC***	***MBC***	***MIC***	***MBC***
Exiguobacterium sp GM010	125	250	125	250	125	250	62.5	125	62.5	125	250	500	125	250	125	250
AMP	10	20	10	20	10	20	5	10	5	10	10	20	5	10	10	20
TET	5	10	5	10	5	10	10	20	10	20	5	10	10	20	5	10

AMP, Ampicillin; TET, Tetracycline are standard broad-spectrum antibiotics.

for overnight in refrigerator (4°C). After dehydration with series of ethanol, sample were examined under SEM (LEO 435VP, Japan). Tetracycline was used as positive control.

UV-Visible and FT-IR Spectroscopy

The UV-visible spectrum was recorded (UV–Visible 2450, Shimadzu Spectrophotometer, Japan) in the range of 400–700 nm to determine the pigment characteristic. The FT-IR spectrum was recorded using a fourier transform infrared (FTIR) spectrophotometer (Bruker IFS 25 model, Bruker, Germany) in the 4,000–400 cm^{-1} range in transmission mode. The characteristic functional groups of GM010 pigment were identified.

Determination of MIC and MBC

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of pigment was determined by broth microdilution method (Pandit et al., 2018). All tests were performed in nutrient broth. Overnight bacterial culture broth of each strain were prepared and the final concentration in each

well was adjusted to 2 $\times 10^4$ cfu/ml. A serial doubling dilution of the pigment was prepared in a 96-well microtiter plate over the range 15.625–2000 μg/ml. The plates were incubated at 37°C for 24 h. To determine MBC, the pigment treated broth from each well was inoculated on nutrient agar and incubated for 24 h at 37°C. Ampicillin and Tetracycline were used as positive control over the range of 1.25–160 μg/ml. The controls were maintained without pigment to demonstrate growth of bacterial pathogen.

Toxicity Assay

Toxicity assay was performed using *Artemia franciscana* nauplii (Pandit et al., 2019). About 100 μl of growth medium containing ∼26 nauplii were used for bioassay in microtiter plates. The GM010 pigment (100 and 1,000 μg ml^{-1}) was added to microtiter plates and incubatted at 27 ± 2°C for 36 h. Artificial sea water was used as negative control and potassium cyanide was used as positive control. The viability of cysts was monitored at different time intervals of 12, 24, 36 h independently (Babu et al., 2015). Under 10x magnification, percentage mortality was calculated using the formula.

$$\text{Mortality rate (\%)} = \frac{\text{Death nauplii} \times 100}{\text{Total nauplii}}$$

Statistical Analysis

Data were expressed as the mean ± standard deviation of triplicate measurements. Results were processed by 1-way analysis of variance (ANOVA). A Duncan multiple range test was used to determine significant differences. Differences at $P < 0.05$ were considered as significant.

RESULTS

Isolation of Active Strain

The heterotrophic marine bacteria were isolated on five different culture media from the samples collected from different ecological niche. Based on morphological characteristics 43 strains were selected to isolate antagonistic strains. Since the selection of antagonistic strains was usually affected by the species and number of indicator microorganisms (Shnit-Orland and Kushmaro, 2009), all 43 strains were screened against both Gram positive and Gram negative food-borne pathogens (Data not shown). Among these, pigment of strain GM010 inhibited both Gram-positive and Gram-negative bacteria compared to broad spectrum antibiotics. The antimicrobial activities showing the zone of inhibitions were summarized in **Table 1**.

Identification and Characterization

The isolate GM010 produced yellow to orange pigmentation on ZMA (**Figure 1A**). The physiological and biochemical characteristics of the strain GM010 were presented in **Table S1**. UV-vis spectrum of GM010 pigment showed absorption peak at 460–470 nm indicating orange pigment characteristic (**Figure 1B**). The 16S rRNA sequence analysis revealed that, the bacterium belongs to the phylum Firmicutes and *Bacillaceae* family. The strain GM010 was closely related to *Exiguobacterium* sp HL95 (**Figure 1C**). Therefore, the strain GM010 was a species of genus *Exiguobacterium* for which *Exiguobacterium* sp GM010 was proposed. The sequences were submitted in GenBank with accession number KY014429 (https://www.ncbi.nlm.nih.gov/genbank/).

Further to consider the applications of the pigment, the antibacterial functional properties were evaluated (**Figure 1D**). The zone of inhibition were observed in the range of 6.6–14.3 mm. The *Exiguobacterium* sp GM010 pigment has strongly inhibited *L. monocytogenes* Scott A (14.33 mm) followed by *M. luteus* ATCC 9341 (10.83 mm) compared to other food borne pathogens. Whereas, zone of inhibition for *E. coli* EFR02 observed was 6.66 mm. Further, the MIC values of *Exiguobacterium* sp GM010 pigment against the food-borne pathogens were found to be in the range of 62.5–500 μg/ml. The pigment was tested against both Gram positive and negative bacteria for antibacterial activity with a broth dilution microtiter system. The pigment inhibited *L. monocytogenes* Scott A and *M. luteus* ATCC 9341 at minimum concentration of 62.5 μg/ml and *Klebsiella* sp followed by *P. aeruginosa* ATCC 15442 was inhibited at minimum concentration of 250 μg/ml. Whereas, *E. coli* EFR02 was inhibited at 500 μg/ml concentration of pigment (**Table 2**). Even though these results revealed that *Exiguobacterium* sp GM010 pigment can inhibit both gram-positive and gram-negative food-borne pathogens, the MIC values were greatly varied. MIC determines antimicrobial susceptibility and sensitivity by measuring inhibition of bacterial growth. Whereas, MBC is the lowest concentration of test sample that results in a 99.9% reduction in the initial microbial density. MBCs values were generally within a two-fold dilution of the MIC value.

Mode of Antibacterial Action

The CLSM studies revealed mode of antibacterial action of *Exiguobacterium* sp GM010 pigment on food-borne pathogens. The SYTO9 and PI staining indicated loss of membrane integrity of food-borne pathogens treated with *Exiguobacterium* sp GM010 pigment and compared with tetracycline treatment. The appearance of red and yellow fluorescence indicated dead cells. Whereas, the live cells appeared green (control) did not receive any treatment (**Figure 2**). These results confirmed that, *Exiguobacterium* sp GM010 pigment disrupted the cell membrane of food-borne pathogens leading to bactericidal action. Further SEM observations revealed morphological deformation of food-borne pathogen cells, while untreated (control) cells were uniform in shape (**Figure 3**). The distorted structure as indicated by low electron density regions, ruptured cell wall, and cavity formation in the bacterial cells. Similar effects were also observed in tetracycline treatment (**Figure 3**).

The results of FT-IR spectrum of *Exiguobacterium* sp GM010 pigment revealed absorption bands characteristic for the functional groups of the components (**Figure 4**). Infrared spectrum showed characteristic functional groups that mainly included hydroxyl, carbonyl, and carboxylic groups. The strong broad band at 3,459 cm^{-1} was assigned to the presence of OH stretching in hydrogen bonds and N-H vibration. Absorption peaks between 2999 and 2915 cm^{-1} correlated to stretching frequencies of aliphatic C-H groups. This spectrum showed an

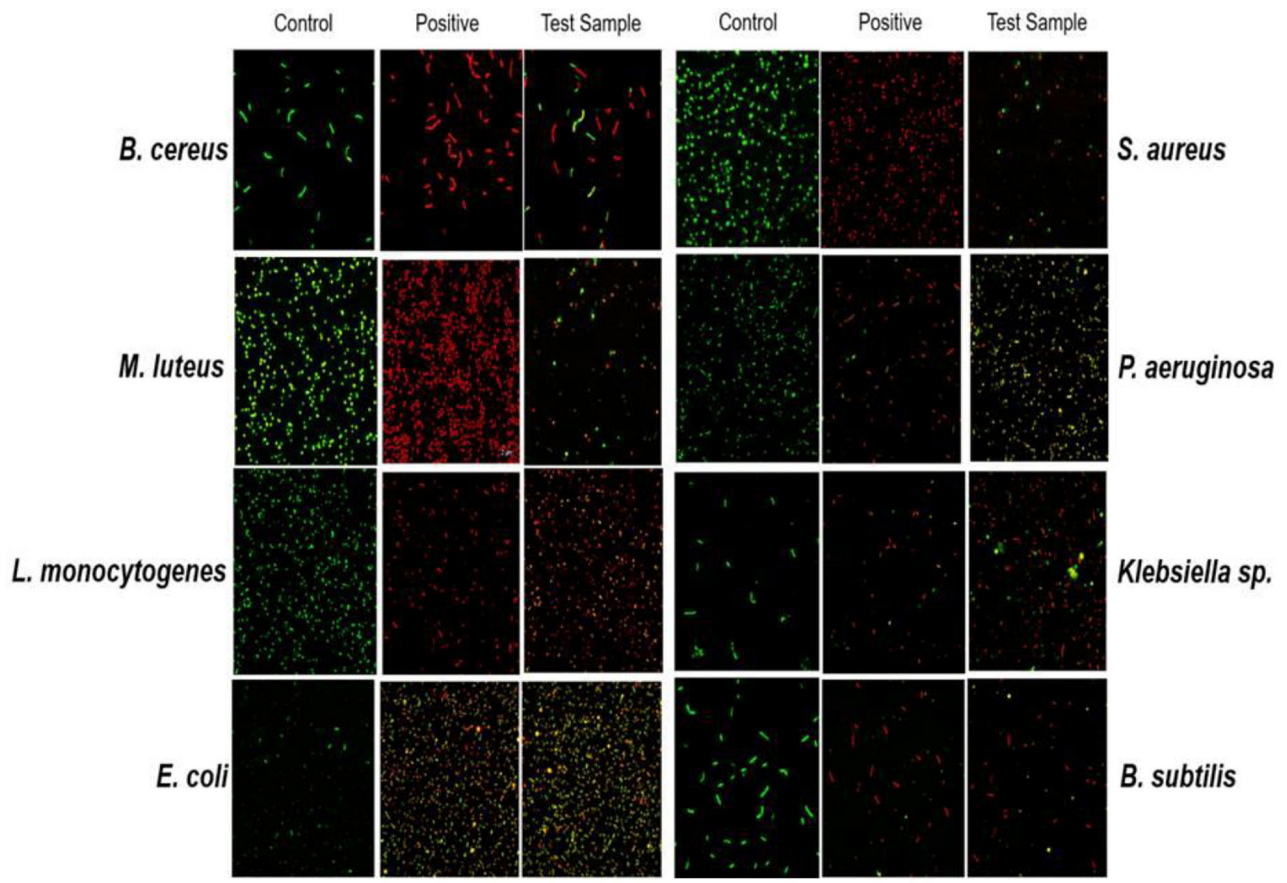

FIGURE 2 | Loss of membrane integrity of food-borne pathogens treated with *Exiguobacterium* sp GM010 pigment (test sample) were analyzed with CLSM using SYTO9/PI staining and compared with tetracycline (positive) and untreated cell (control). All the pictures were taken at 60X.

absorption band at 1,666 cm^{-1} that was indicative for stretching frequencies of an C=O group. The band at about 1,437 cm^{-1} was indicative for asymmetric vibrations of the carboxylic group. Peaks at 1,000–1,200 cm^{-1} correlated to C-O-C linkages of sugar components remaining in the extract.

Toxicity Effect

Toxicity of *Exiguobacterium* sp GM010 pigment was analyzed by estimating the mortality rate of *A. franciscana,* a relatively rapid way to detect toxic compounds (Meyer et al., 1982). The mortality rates at 100 $\mu g\ ml^{-1}$ of pigment were not significantly different at 12 h intervals of time. However, at 1,000 $\mu g\ ml^{-1}$ the *Exiguobacterium* sp GM010 has shown 38.33 ± 1.44% mortality at 36 h of treatment (**Figure 5**). The mortality rate (%) was compared with the Clarkson's toxicity assessment (Clarkson et al., 2004) and it was confirmed the non-toxic effect of *Exiguobacterium* sp GM010 pigment.

DISCUSSION

Marine bacteria *Streptomyces, Pseudomonas, Pseudoalteromonas, Bacillus, Vibrio*, and *Cytophaga* isolated marine environment have shown various biological activities (Azamjon et al., 2011). The majority of marine microorganisms are not easily culturable in the laboratory (Valliappan et al., 2014), due to dynamics of nutrient conditions. To isolate maximum number of marine bacteria, five different culture media were used for isolation of bacteria showing broad spectrum antibacterial activity. Further the NaCl concentration of the isolation media was increased to ensure the isolated bacteria were truly associated with marine niche.

Generally the Gram-positive bacteria are more sensitive to antibiotics compared to Gram-negative bacteria (Nikaido, 1996) and using more Gram positive indicator bacteria may lead to false positive results of more antibiotic-producing bacteria. Hence, the isolated marine bacteria were screened equally against both Gram positive and Gram negative food-borne pathogens (**Table 1**), to isolate potential marine bacterium (**Figure 1D**). Among the marine bacteria screened, a bacterium inhibited both Gram positive and Gram negative food-borne pathogens was identified based on morphological, biochemical, and molecular analysis (**Figure 1**). These results revealed, the isolated bacterium is belongs to the genus *Exiguobacterium.* Even though there are reports on *Exiguobacterium* species showing the algicidal (Li et al., 2016), antifungal (Selvakumar et al., 2009) and antibacterial (Shanthakumar et al., 2015) effect, there are no report on the

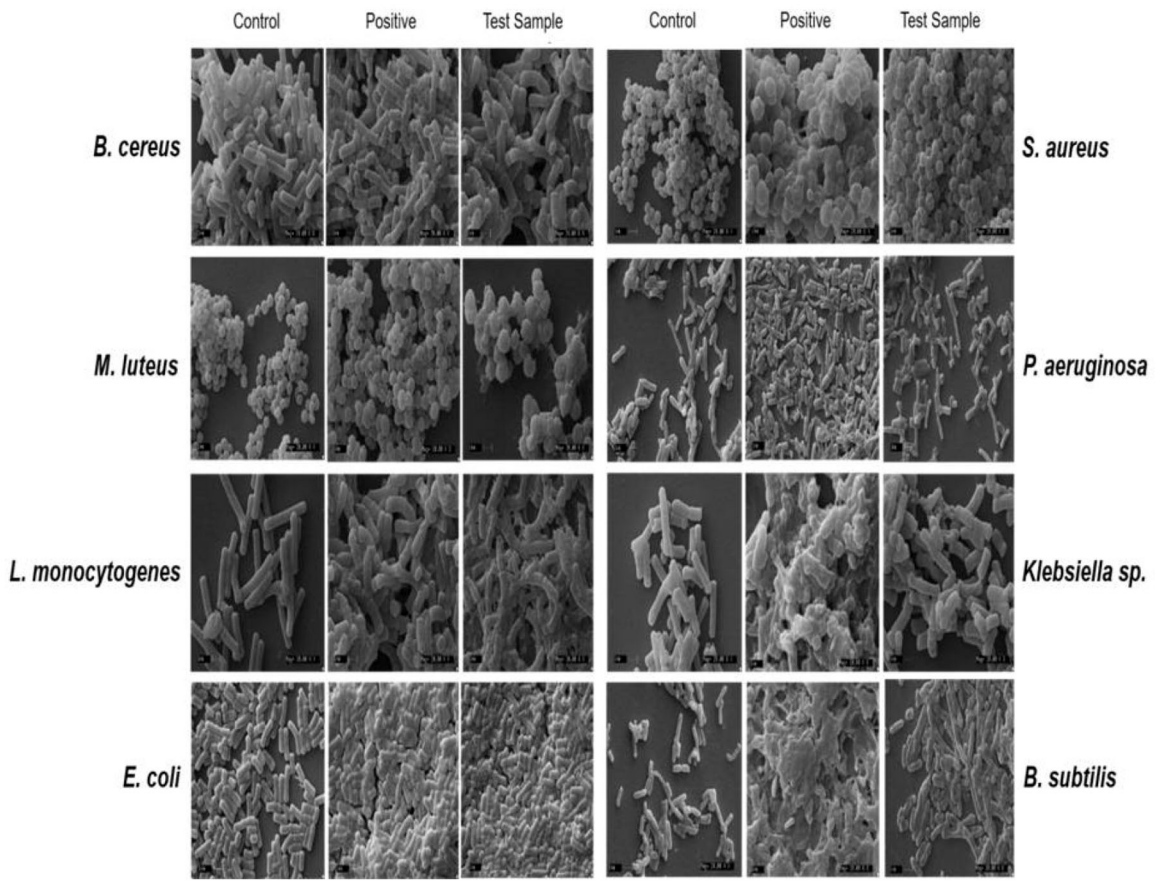

FIGURE 3 | Lysis of food-borne pathogens cells treated with pigment of *Exiguobacterium* sp GM010 (test sample) and tetracycline (positive) and untreated (control) were observed under SEM. Scale bar-1 μm.

antibacterial action of *Exiguobacterium* sp GM010 pigment and the mechanism of antibacterial action.

The *Exiguobacterium* sp GM010 pigment has shown antibacterial action against both Gram positive and negative food-borne pathogens. The cellular and membrane integrity is considered important to distinguish between viable and dead bacterial cells for physiological activities (Stiefel et al., 2015). Viable cells have intact membranes and cannot be penetrated by some staining compounds, whereas dead cells are considered to have disrupted or damaged membranes (Stiefel et al., 2015). The CLSM studies suggested that pigment of *Exiguobacterium* sp GM010 damaged the cell membrane (**Figure 2**) of food-borne pathogens. The SEM image analysis of the food-borne pathogens treated with pigment have shown shrunken shape compared to the untreated. This is due to the cell membrane damage caused the release of cytoplasmic content and lysis of cell as observed in the **figure 3**.

Several studies have reported that, functional group of the bioactive compounds can damage the membrane integrity of food-borne pathogens. The hydroxyl group present in the substances of *Vibrio* sp (Horta et al., 2014) and *Pseudoalteromonas phenolica* (Isnansetyo and Kamei, 2003) exhibited antibacterial action against *B. subtilis* and *S. aureus*. The characteristic functional groups of the algicidal substance produced by the *Exiguobacterium* sp. h10 mainly included carbonyl, amino, and hydroxyl groups (Li et al., 2016). Similarly 3,6,18-trione, 9,10-dihydro-12-hydroxyl-2methyl-5-(phenyl methyl) (5-alpha, 10-alpha)-dihydroergotamine and dipropyl-S-propyl ester molecules exhibit antibacterial action against clinical pathogens (Shanthakumar et al., 2015). The hydroxyl group of compounds and the presence of a system of delocalized electrons are important for the antimicrobial activity (Ultee et al., 2002). The hydroxyl groups destabilizes the cytoplasmic membrane and acts as a proton exchanger, thereby reducing the pH gradient across the cytoplasmic membrane. The resulting collapse of the proton motive force and depletion of the ATP pool (Ultee et al., 2002) eventually lead to cell death. This was evidenced by the FTIR spectrum, showing characteristic functional groups that mainly included hydroxyl, carbonyl, and carboxylic groups in *Exiguobacterium* sp GM010 pigment (**Figure 4**). These functional group cause a system of delocalized electrons leading to destabilization of membrane and decrease in the membrane potential that resulted in bactericidal action of *Exiguobacterium* sp GM010 pigment.

Toxicity of GM010 was evaluated by estimating the mortality rate of *A. franciscana*. This assay has been considered as efficient

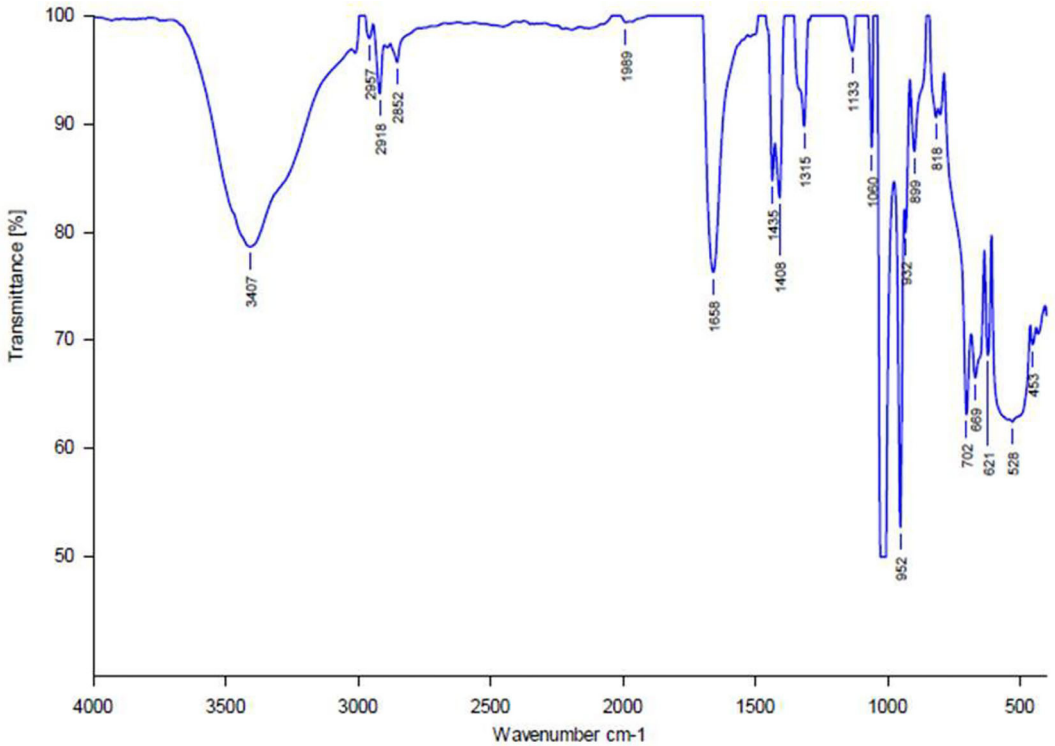

FIGURE 4 | Fourier transform infrared spectrum of *Exiguobacterium* sp GM010 pigment in the range 400–4,000 cm^{-1}.

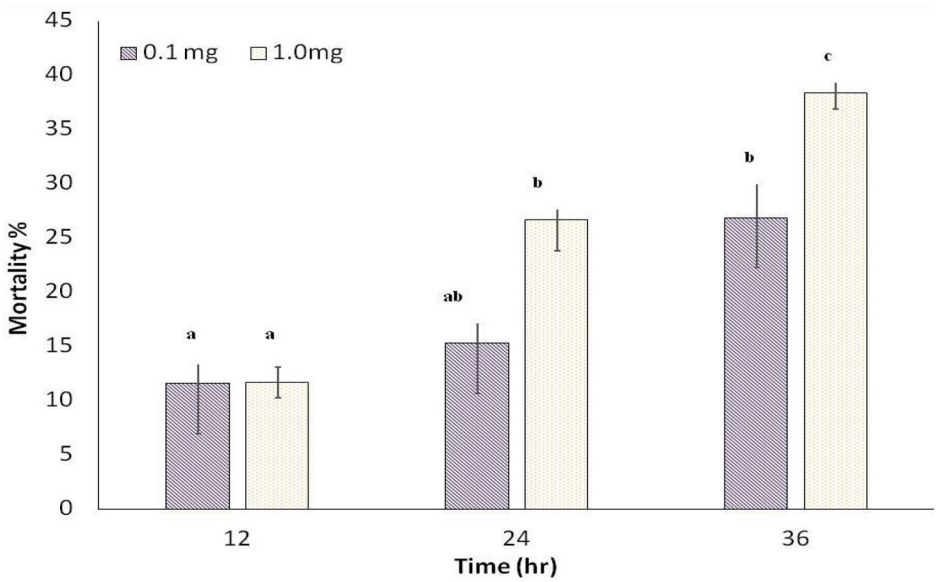

FIGURE 5 | Mortality responses of *A. franciscana* nauplii exposed to 100 and 1,000 μg ml^{-1} *Exiguobacterium* sp GM010 pigment. Data represent mean value of three replicate experiments of each concentration. Bars with different letters (a, b, c) are significantly different ($P < 0.05$).

inexpensive and a relatively rapid way to detect toxic compounds tool for toxicity assay and requiring only less quantity of sample (<20 mg) (Meyer et al., 1982). At 1,000 μg ml^{-1} the GM010 has shown 38.33 ± 1.44% mortality at 36 h of treatment (**Figure 5**). Accordingly, extracts with LC_{50} above 1,000 μg ml^{-1} are non-toxic, LC_{50} of 500–1,000 μg ml^{-1} are low toxic, extracts with LC_{50} of 100–500 μg ml^{-1} are medium toxic, while extracts with LC_{50} of 0–100 μg ml^{-1} are highly toxic (Clarkson et al., 2004).

The pigment of *Exiguobacterium* sp GM010 was considered as non-toxic, since the 50% mortality of *A. franciscana* napuli was not observed at 1,000 $\mu g\ ml^{-1}$ concentration.

CONCLUSIONS

The results of *Exiguobacterium* sp GM010 pigment showing bactericidal activity against food-borne pathogens and non-toxicity toward *A. franciscana* suggested the application in food preservation and safety efficacy, respectively. Even though the pigment is non-toxic to *A. franciscana* napuli, additional data on preclinical studies are necessary in order to confirm that pigment is free of cytotoxic effects at different doses of treatment.

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 in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

MD conceptualized, designed the experiment, and reviewed the paper. K-PM executed the experiments, data analysis, and wrote the paper. SP executed the toxicity assay. All the authors read 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/fsufs.2020.00142/full#supplementary-material

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Natural Pigments of Microbial Origin

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The world demands new solutions and products to be used as dyes for industrial applications. Microbial pigments represent an eco-friendly alternative as they can be produced in large amounts through biotechnological processes and do not present environmental risks, as they are easily decomposable. Moreover, some of these metabolites are recognized for their biological activities, which qualify them for potential uses as food colorants and nutraceuticals, protecting against degenerative diseases related with oxidative stress. Because of their genetic simplicity as compared with plants, microorganisms may be a better source to understand biosynthetic mechanisms and to be engineered for producing high pigment yields. Despite the origin of the pigmented microorganism, it seems very important to develop protocols using organic industrial residues and agricultural byproducts as substrates for pigment production and find novel green strategies for rapid pigment extraction. This review looks for the most recent studies that describe microbial pigments from microalgae, fungi, and bacteria. In particular, the underexploited tools of omics science such as proteomics and metabolomics are addressed. The use of techniques involving mass spectrometry, allows to identify different protein and metabolite profiles that may be associated with a variety of biotechnologically-relevant pathways of pigment synthesis.

Keywords: bioactive metabolites, microbial pigments, extraction, proteomics, metabolomics

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INTRODUCTION

Pigments have become an essential part of our daily lives and have extensive applications in many areas, such as agriculture, textiles, cosmetics, pharmaceuticals, foods, among others (Yusuf et al., 2017; Venil et al., 2020a). Synthetic dyes have been produced on a large scale to respond the high demand for improving the color appearance of diverse products. They still have an advantage in terms of large-scale production at economical price with consistent color quality and numerous color variation outweigh the benefits of natural dyes. However, several studies show that synthetic dyes can cause adverse effects in terms of consumer and occupational health and have a negative impact on the environment (Fuck et al., 2018; Zerin et al., 2020). Synthetic dyes are non-renewable, non-biodegradable, sometimes carcinogenic, and cause toxic waste pollution, presenting a huge challenge in disposing the byproduct waste in a cost effective way (Azman et al., 2018; Ramesh et al., 2019).

Many natural pigments besides fulfilling their function of giving color are known as interesting bioactive compounds with potential health benefits. These compounds have a wide range of applications in medicine, food, pharmacology, agrochemicals, cosmetics, among others (Venil et al., 2020b). Numerous microbial bioactive pigments have been discovered and many of them show antioxidant, anti-inflammatory, and/or antimicrobial properties (Ramesh et al., 2019).

The high demand for natural products is impelling an exponentially growing market, and the annual increase rate of the colorant market is estimated at ∼7% and is expected to reach $7.79 billion by the year 2020 (Dikshit and Tallapragada, 2018). Thus, the organics market and pigment industries represent vast commercial sectors that would be shortly dominated by microbial pigments (Novoveská et al., 2019). In comparison to plant and animal sources, microbial pigment production by fermentation technology is more dynamic and economic, resulting in biodegradable compounds that may have wide industrial applications as colorants (Silva et al., 2019; Venil et al., 2020a). Although microbial pigments are not widespread in colorant formulations, they represent an important alternative that has the long-term ability to compete with synthetic dyes (Zerin et al., 2020). The successful application of microbial pigments relies on high production yields, reasonable production costs, regulatory approval, pigment characterization, and stability to environmental factors such as temperature and light (Morales-Oyervides et al., 2017).

In this mini-review, recent studies describing pigments extracted from microalgae, fungi and bacteria are discussed, including potential industrial applications. This article focuses on the importance of finding natural microbial bioactive pigments and the need of understanding the metabolic pathways involved in their synthesis. The requirement for green methodologies for rapid pigment extraction and purification is also addressed.

MICROBIAL BIOACTIVE PIGMENTS

Pigments are extensively produced among microorganisms, including microalgae, fungi and bacteria. Although there is still no precise classification of all pigments that can be naturally synthesized by microorganisms, *in vitro* and *in vivo* studies indicate that some of these molecules can be helpful in the prevention or treatment of degenerative diseases (Shen et al., 2018; Sajjad et al., 2020). Some examples of microbial pigments showing bioactivities are presented in **Supplementary Table 1**.

Bioactive Pigments From Microalgae

Microalgae genera as *Nostoc, Dunaliella, Scenedesmus, Nannochloropsis, Haematococcus, Muriellopsis, Chlorella, Phaeodactylum, Spirulina, Artrhospira, Porphyridium, Agardhiella, Polysiphonia* produce different groups of pigments, such as carotenoids, chlorophylls and phycobiliproteins (PBPs), known as non-toxic water-soluble proteins mostly found in Rhodophyta (red algae), Cyanobacteria, and Cryptophyta (Yusuf et al., 2017; Noreña-Caro and Benton, 2018; Arashiro et al., 2020). Due to their strong absorbance and fluorescence properties as well as antioxidant and free radical scavenging activities, PBPs have been widely employed in food, cosmetics, pharmaceutical, and biomedical industries (Sonani et al., 2016). Some antioxidant pigments from microalgae are shown in **Supplementary Figure 1**. Microalgae pigments have additional biological functions, such as anti-inflammatory, antiangiogenic, neuro- and hepatic-protective, antiviral, anti-obesity, antidiabetic, anticancer, and anti-osteoporotic. They also may help to regulate cardiovascular diseases, cognitive function, protect from UV rays, enhance immune functions, present antiaging property, and prevent some blood-related disorders (Ambati et al., 2018; Saini et al., 2018).

Microalgae pigments have high commercial value as natural colorants in the nutraceutical, cosmetic, and pharmaceutical industries, as well as applications in clinical research and molecular biology, and as natural dyes in the textile industries, painting and color industries, food colorant and feed additive for poultry (de Morais et al., 2018; Okolie et al., 2019). Bottlenecks for effective pigment production should be overcome, and potential genetic and metabolic approaches could be used to achieve cost-intensive productivity (Saini et al., 2020).

Bioactive Pigments From Yeast and Filamentous Fungi

Fungi belonging to the *Monascaceae, Trichocomaceae, Nectriaceae, Hypocreaceae, Pleosporaceae, Cordycipitaceae, Xylariaceae, Chaetomiaceae, Sordariaceae, Chlorociboriaceae* families have been described as potent pigment producers (Ramesh et al., 2019). Certain genera of yeast like *Rhodotorula, Sporidiobolus, Sporobolomyces, Xanthophyllomyces,* and *Pichia* have also been recognized as pigment producers. Some of them have been reported to be prolific producers of torulene and torularhodin, β-carotene, poly-hydroxy carotenoids among others (Cipolatti et al., 2019; Kot et al., 2019).

Fungal pigments are mostly carotenoids, melanins and polyketides, namely flavins, phenazines, quinones, monascins, violacein and indigo, presenting a wide spectrum of colors (**Supplementary Figure 2**). Valuable bioactive properties like anticancer, antioxidant, antimicrobial, anti-inflammatory and immune-suppressor have been associated with fungal pigments (Mapari et al., 2009; Lopes et al., 2013). Thus, they present applications in the food and healthcare industries, as dyeing agents in the textile industry, and as cosmetic additives due to the capacity of absorb harmful UV rays (Chen et al., 2019; Lagashetti et al., 2019; Sajjad et al., 2020).

Bioactive Pigments From Bacteria and Actinobacteria

Most common bacterial pigments are carotenoids, aryl polyenes that in some cases are esterified with a dialkylresorcinol system, melanins, phenazines, quinones, tambjamines, prodigiosines, violacein (**Supplementary Figure 3**). These pigments are reported for their antioxidant and UV protection properties, and many bacterial pigments demonstrated potential biomedical applications such as antimicrobial, antimalarial, and anticancer properties (Sajjad et al., 2020).

The most common pigments from actinobacteria are melanins with colors ranging from black through brown to olive, carotenoids with colors ranging from red, yellow, and pink through to violet and thirdly, actinorhodin-related blue pigments (Rao et al., 2017). Pigments from the carotenoids group are also described, showing antioxidant activities and being harmless for safe use as a natural colorant in cosmetic, food, pharmaceutical and textile industries (Parmar and Singh, 2018).

TABLE 1 | Agro-industrial by-products used to produce orange and red pigments from *Monascus* strains.

Strain	Substrate	Process[a]	References
M. ruber LEB A4-5	Corn steep liquor	SmF, 30°C, 300 rpm	Hamano and Kilikian, 2006
M. purpureus NRRL 1992	Grape waste	SmF, 25°C, 100 rpm	Silveira et al., 2008
M. ruber MTCC2326	Rice brokens	SST	Rajagopal et al., 2009
M. purpureus CMU001	Meal from corn, peanut, soybean, coconut residue	SST, 30°C, 14 days	Nimnoi and Lumyong, 2009
M. purpureus NRRL 1992	Sugarcane bagasse	SmF, 27°C, 125 rpm	Silveira et al., 2013
M. purpureus ATCC 16436	Cob corn + glycerol	SST, 30°C, 150 rpm	Embaby et al., 2018
M. purpureus ATCC 16365	Orange peel	SSF	Kantifedaki et al., 2018
M. purpureus LQ-6	Rice straw hydrolysate	SmF 30°C, 150 rpm	Liu et al., 2019
M. purpureus CMU001	Brewer's spent grain	SmF, pH 6.5, 350 rpm	Silbir and Goksungur, 2019
M. purpureus FTC5357	Oil palm frond	SST, 30°C	Daud et al., 2020

[a]*SST, solid-state fermentation; SmF, submerged fermentation.*

MICROBIAL PIGMENT PRODUCTION USING AGRO-INDUSTRIAL BYPRODUCTS

A relevant aspect for the sustainable production of microbial pigments is the definition of an appropriate growth media, which should be cost-effective and result in high pigment yields. In this regard, the importance of recycling agro-industrial byproducts as growth substrates for microbial pigment production has been reported (Korumilli et al., 2020; Venil et al., 2020a). Bioconversion of agri-food waste to value-added products is very important toward zero waste and circular economy concepts. To reduce the environmental burden, food researchers are seeking strategies to utilize agro-industrial residues for microbial pigments production and further biotechnological exploitation in functional foods or value-added products (Usmani et al., 2020). Diverse agro-industrial wastes have been investigated for production of the well-known *Monascus* pigments (**Table 1**), and some processes using such inexpensive substrates presented high pigment yields (Embaby et al., 2018). In addition, industrial wastewaters are successfully used for production of phycocyanins and carotenoid pigments by microalgae (Singh et al., 2019; Arashiro et al., 2020).

Intracellular carotenoids (β-carotene, γ-carotene, torulene, and torularhodin) from *Rhodotorula* species have been produced by using different agro-industrial byproducts as sugarcane bagasse, wheat bran, rice bran, silage, whey, raw glycerol, corn steep liquor, sugarcane molasses, waste chicken feathers, fruit waste extract, and many others (Sharma and Ghoshal, 2019; Korumilli et al., 2020). These pigments could have applications in food and feed as well as in health, pharmaceutical products and cosmetics, generating a market value expected to reach over $2.0 billion by 2022 (Elfeky et al., 2019; Tang et al., 2019).

The economic viability of industrial-scale facilities that produce powdered astaxanthin and astaxanthin oil mixture from wheat bran and olive pomace was evaluated in a simulated solid-state fermentation study. An economic analysis was conducted for different fermentation conditions to identify the plant capacity that optimizes the process economics for a cost-effective bioprocess. The techno-economic analysis demonstrated that producing astaxanthin from agro-industrial waste is a feasible and promising technology (Dursun et al., 2020).

GREEN PROTOCOLS ON MICROBIAL PIGMENTS

Extraction Protocols

Microorganisms offer a tremendous diversity of pigmented molecules, but the methodologies and protocols applied for their extraction and purification are tedious, involving multiple steps, the use of diverse organic solvents, and still giving incongruent results. The variety of extraction protocols employed for microbial pigment extraction can be seen in **Supplementary Table 1**. The choice of extraction protocol is crucial, as the extraction solvents and conditions can drastically influence the final composition, quality, and efficiency of the process (Soares et al., 2016).

In order to minimize the use of organic solvents and preserve as much as possible the qualitative and quantitative compositions of the pigmented molecules, ecofriendly methodologies have been investigated. Although these techniques have been employed for extraction of many bioactive substances, their effective application for extraction microbial pigments should be further exploited (Kalra et al., 2020; Martínez et al., 2020). Some characteristics, advantages and disadvantages of these green methodologies are detailed in **Table 2**.

Ultrasound-assisted extraction (UAE) has been recognized as an efficient and environmentally safe extraction method. Enzymatic UAE was used for obtaining the natural food colorant C-phycocyanin from dry biomass of *Arthrospira platensis* and this method resulted in the highest yield (92.73 mg/g dry biomass) and extraction efficiency (78%) among the methods studied (Tavanandi and Raghavarao, 2019). Microwave assisted extraction (MEA) has been considered an excellent technique for the isolation of microalgae pigments due to its reproducibility, rapidity, uniform heating, and high extraction yields (Pasquet et al., 2011). As the most safe, non-toxic, non-flammable, non-corrosive solvent, water can be used as a green solvent in MAE and UAE for efficient extraction of several metabolites from

TABLE 2 | Green methodologies for microbial pigment extraction[a].

Method	Driving force	Principle	Advantages	Disadvantages
Ultrasound assisted extraction (UAE)	Acoustic cavitation	High-intensity ultrasound pressure waves accelerate the tissue rupture and releasing intracellular substances into a small amount of solvent	Fast, improved extraction yields, apparatus simple and easy to handle, safe, reduced solvent amount	Filtration step required, possible deterioration of compounds at high frequencies
Microwave assisted extraction (MAE)	Microwave power	Microwave radiation frequencies ranging from 300 MHz to 300 GHz as source of energy	Fast, reproducible, uniform heating, high extraction yields, easy to handle, reduced solvent amount	Filtration step required, expensive, risk of explosion depending on solvent
Pressurized liquid extraction (PLE)	Heat plus solvent under pressure	Automated advanced technique to conventional solvent extraction methods such as reflux, Soxhlet extraction, percolation or maceration	Reduced extraction time, reproducible, no filtering required	Possible degradation of thermo-labile compounds
Supercritical fluid extraction (SFE)	Pressure plus supercritical fluid	Use of liquefied CO_2 as the supercritical fluid for the extraction of bioactive molecules from solid matrices	Fast, high selectivity, extraction of thermo-labile substances	High cost of equipment, poor extraction of polar substances, many parameters to optimize
Pulsed electric field assisted extraction (PFE)	Electric field	Electroporation or electro-permeabilization, exposing the sample to short impulses of high intensity electric field	Use of green solvents, easy to scale up, extraction of thermo-labile substances, direct extraction from biomass	High cost of equipment, affected by air bubbles, efficiency highly dependable on medium conductivity
Ionic liquids assisted extraction (ILE)	Solvent contact	Tailor-made solvents for extensive extraction of natural compounds	Direct extraction from biomass, enhanced extraction yields	Cost for larger industrial use, limited availability, relative higher viscosity

[a]*Compiled from Medina-Torres et al. (2017), Kalra et al. (2020).*

microbial matrices. An alternative to traditional extraction by organic solvents is to accomplish the extraction by CO_2 based supercritical fluid extraction (SFE) method (Da Silva et al., 2016; Khaw et al., 2017). SFE has been recognized as a green sustainable technique for the selective isolation of molecules, including thermo-labile compounds. The employment of SFE for carotenoids extraction from diverse substrates from laboratory to the commercial scale have been reported (Kitada et al., 2009; Goto et al., 2015).

In addition, pressurized liquid extraction (PLE; also known as accelerated solvent extraction—ASE) (Lebeau et al., 2017), pulsed electric field (PEF)-assisted extraction (Martínez et al., 2020), and ionic liquids (IL)-assisted extraction (Mussagy et al., 2019) have been described as efficient and feasible green methods to improve the extraction yield of pigments from microbial biomass. All these techniques have pros and cons (**Table 2**) and despite the advances in extraction methodologies, improved green methods are still necessary.

Purification Protocols

Column chromatography and preparative thin layer chromatography are usual techniques for pigment purification. Polymeric resins and non-ionic adsorption resins have been used for the separation and purification of microbial pigments. The selected resin will adsorb the target pigment from the culture broth, making the process easier, with lower operational cost and solvent consumption. This method yielded a concentrated and partially purified pigment from *S. marcescens* with total recovery of 83%, which was much higher as compared to other conventional methods (Wang et al., 2004). A study using macroporous polymeric adsorption resins demonstrated the promising potential of HP-20 resins for the recovery and purification of prodigiosin from *Serratia marcescens* fermentation broth (Juang and Yeh, 2014).

Despite these methods can be adequate to reach the required purity for commercial applications, several technological advances are still necessary to improve the separation and purification of pigments from culture broth to reduce the energy and process costs (Wang et al., 2004; Venil et al., 2014).

HIGH-THROUGHPUT METHODOLOGIES IN MICROBIAL PIGMENT PRODUCTION

The emerging of "omics" tools for large-scale microbial examination at the molecular level have proven to be effective for bio-prospection and characterization of microorganisms and their metabolites (Luzzatto-Knaan et al., 2015). Although these methodologies have been widely applied to microbiological research, they are still underestimated for the study of pigment synthesis by microorganisms. Thus, high throughput technologies can be useful to provide advanced knowledge on biosynthetic pathways and discovery of new microbial pigments. A possible workflow considering the use of "omics" sciences coupled with the well-studied and advanced genetic methodologies is presented in **Supplementary Figure 4**.

Genomics and Metagenomics

Genomics approach may help the searching for gene clusters involved in pigment biosynthesis. The genomic analysis shows the microbial capacity to produce specific secondary metabolites even if they remain silent or criptic under laboratorial culture conditions. Indeed, approximately 90% of the biosynthetic gene clusters for secondary metabolites has been observed to be included into these categories (Baltz, 2017). Different recent works include the detection of pigment gene clusters (Liao et al., 2019; Xu et al., 2019; Mandakovic et al., 2020). The genome information given by the industrial strain *M. purpureus* YY-1, compared with closely related filamentous fungi, showed adaptation to starch-based foods. Moreover, correlated transcriptomics analysis revealed the highly expressed genes for pigments production on carbon starvation, providing useful insights for industrial applications (Yang et al., 2015). Pan-genomics analysis was used for identification of the tryptophan genes cluster as indispensable on the production of blue pigments by *Pseudomonas fluorescens* (Andreani et al., 2015). Moreover, genomics information becomes indispensable for improving microbial pigment production through genetic manipulation (Venil et al., 2020b), and supports the identification and/or confirmation of produced pigments by novel microorganisms (Varasteh et al., 2020).

Metagenomics analysis of assembled genomes from different microbial phyla, even from uncultured samples, permits to acquire information from unknown microorganisms. In this regard, next-generation sequencing platforms have given a strong contribution for the metagenomics study of microbial networks in a community (Jindal, 2020). This latter omics methodology permitted to overcome the problem of uncultivable microorganisms and to identify novel pigments from extremophile microorganisms, for example from hot springs (Thiel et al., 2019) or marine sources (Rambo et al., 2020). Metagenomics may overcome some limitations of normal laboratory methodologies to study microorganisms from these extreme environments (Stincone and Brandelli, 2020). Thus, metagenomics can be very useful to prospect novel microbial pigments.

Proteomics and Metabolomics

Proteomics is a powerful tool for identification of proteins on a large scale, providing a general overview of the total proteins expressed by an organism under determined conditions (Aslam et al., 2017). The comparative proteomics approach has been used to study proteins related to pigment production. Although works are mostly associated with plant pigments, more recently the microbial pigments production has been investigated by proteomics approach. These studies include the synthesis of pigments by *Monascus purpureus* under high ammonium chloride concentration (Zhou et al., 2020), the polyextremophilic bacterium *Deinococcus radiodurans* in response to oxidative stress (Gao et al., 2020), the pigmentation factors in *Pseudomonas fluorescens* ITEM 17298 (Quintieri et al., 2019), and the air-isolated *Aspergillus* sp. from International Space Station (Blachowicz et al., 2019).

Metabolomics include all the analytical profiling techniques that permit the identification of a large number of metabolites present in biological samples. Combining high-throughput analytical chemistry and multivariate data analysis, metabolomics offers a window on metabolic mechanisms (Manchester and Anand, 2017). In recent studies, untargeted metabolomics approach was selected as a good methodology to search and study the metabolic routes on pigments synthesis (Parrot et al., 2019; Fan et al., 2020). The combination of quantitative proteomics and metabolomics define correlations between abundance of natural products, such as pigments, and changes in the microbial protein poll, allowing the detection of biosynthetic enzyme clusters of the producing strains (Song et al., 2014; Du and Van Wezel, 2018).

Pigment Synthesis Through Genetic Engineering

Previous studies have shown that "omics" strategies can help in understanding the roadblocks in the production of pigments and to counter that, genetic engineering technologies can be used to increase pigment production for large scale applications (He et al., 2017; Lin et al., 2017). The overexpression of gene clusters associated with pigment biosynthesis can be achieved through strategies activating transcriptionally silent gene clusters and/or recombinant DNA technologies to increase the biosynthesis of secondary metabolites (Kjærbølling et al., 2019). The integration of "omics" results with genetic engineering approaches sometimes can support the analysis of the biosynthesis of microbial secondary metabolites and help on the development of a consolidate integrated strategy for the discovery of bioactive compounds (Palazzotto and Weber, 2018), including pigments.

A recent review detailed the recent advancements on engineered microbial systems contextualizing the possibility of using agri-food waste biomass as growth substrates (Usmani et al., 2020). From our point of view, this is a very interesting aspect to be exploited by pigmented microorganisms, eventually with bioactivities, by using organic wastes supporting a circular economy. Recent works have reinforced the use of metabolic engineered microorganisms for pigments production (Mohammad et al., 2020) and the application of these methodologies to produce microalgae biopigments (Saini et al., 2020). Different research groups around the world are directing the research to the use of metabolic and genetic engineering for pigment production, including the use in foods subjected to regulatory approval (Sen et al., 2019; Kalra et al., 2020; Venil et al., 2020b).

CONCLUSIONS AND PERSPECTIVES

Microbial pigments have huge potential applications in multiple areas, including health, since some of them display relevant biological activities. The advances in microbial biotechnology have been useful for improvement of cultivation protocols, allowing maximum pigment yields by growing dyed microorganisms on waste materials. The omics science can

helps on understanding of biosynthetic routes, thus providing important information that can be used to stimulate the production of these pigments for possible use in biotechnological scale. Future research should be conducted for improvement in methodologies for pigment extraction and purification, seeking for environmentally safe approaches reducing solvent use and energy inputs, easy methodologies that allows feasible scale-up. Regulatory challenges are associated with the use of microbial pigments in foods, nutraceuticals and cosmetics as the current legislation is often based on local and tradition. Although native pigment-producing microbes represent the greater regulatory and consumer acceptance, the main challenge to commercialize either native or non-native microbial pigments is the regulatory hurdles and associated consumers' preference. In addition, an adequate toxicity evaluation of promising bioactive pigments is necessary to warrant the delivery of such natural products with health benefits. Furthermore, a clear multidisciplinary aspect is associated with microbial bioactive pigments, connecting biotechnology, food and biomedical sciences to provide molecules with both colorant and nutraceutical functions, and potential health benefits.

AUTHOR CONTRIBUTIONS

MP-J and PS participate in bibliography research and writting of the manuscript. AB performed the conceptualization and writing. All authors revised the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Coffee (*Coffea arabica* L.) by-Products as a Source of Carotenoids and Phenolic Compounds—Evaluation of Varieties With Different Peel Color

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Analysis of pulp and peels of Arabica coffee varieties with different external fruit color allowed the identification of 16 phenolic compounds using high-performance liquid chromatography with diode array detection and electrospray ionization multi-stage mass spectrometry (HPLC-DAD-ESI-MS^n). Nine chlorogenic acids, three flavan-3-ols, the xanthone mangiferin, the flavonol rutin and two anthocyanins were tentatively identified and quantified. 5-*O*-Caffeoylquinic acid together with a putative (epi)catechin hexoside were the predominant phenolics detected in the coffee varieties analyzed in this work. Whereas, 3- and 4-caffeoylquinic acids, as well as 5-*O*-feruloylquinic acid were consistently found in higher quantities in the pulp than in the peels when individual varieties were compared, the opposite was found for the other phenolic compounds detected. Complementary, GC-MS after alkaline hydrolysis and trimethylsilylation permitted the identification of more than 30 constituents, including phenolic compounds and other benzenoids, caffeine, and diverse carboxylic acids. Detected anthocyanins were cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside. Anthocyanin contents in orange-colored berries were lower than those in red fruits, while no anthocyanins were found in yellow-colored fruit. Among non-anthocyanin pigments, we found β-carotene and lutein in all varieties, along with other chloroplast-specific carotenoids in some accessions. In addition, saponification evidenced the presence of several xanthophyll esters. Both, chlorophyll a and b, were detected in the peels of all varieties, while only chlorophyll b was observed in the pulp. Thus, the color of yellow-peeled varieties is due to carotenoids, while that of orange and red-peeled varieties is due to both carotenoids as well as low and high levels of anthocyanins, respectively. Present results point out to the

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potential use of by-products of particular coffee varieties with differences in the external fruit color as a source of distinctive bioactive compounds, including anthocyanins and carotenoids, with health benefits.

Keywords: anthocyanins, carotenoids, (epi)catechin hexoside, chlorogenic acids, *Coffea arabica*, coffee, cyanidin, lutein

INTRODUCTION

By-products accruing during coffee processing represent more than 50% of the coffee fruit dry weight (Esquivel and Jiménez, 2012). Considering an estimated world production of about 171.2 million 60-kg bags for 2018/2019 (USDA, 2018), a significant amount of biomass, which has been mainly considered as "waste" material by the coffee industry, is currently discarded without further valorization. In contrast to dry coffee processing, the wet procedure usually allows the recovery of non-degraded peels and pulp (Esquivel and Jiménez, 2019), a very interesting source of valuable bioactives, whose identification and quantification have sluggishly started to promote their utilization for nutritional and pharmaceutical purposes. Taking advantage of these by-products will certainly benefit from further studies and from the development of new methodologies to improve their extraction (Esquivel and Jiménez, 2012; Heeger et al., 2017; Saini and Keum, 2018; Torres-Valenzuela et al., 2020).

Phenolic compounds and carotenoids are ubiquitous constituents of higher plants. While the profile and concentrations of the former have been studied in detail in coffee seeds and brew, knowledge about their presence in coffee by-products (peel and pulp) is more limited (reviewed by Esquivel and Jiménez, 2012). Older publications mention that coffee processing by-products contain several isomeric caffeoylquinic acids, dicaffeolyquinic acids, feruloylquinic acids, and epicatechin, considered as potent antioxidants (Ramirez-Martinez, 1988; Clifford and Ramirez-Martinez, 1991). In addition to the aforementioned constituents, caffeine, diverse quercetin glycosides (+)-catechin, and procyanidin di-, tri-, and tetra-mers have been reported in coffee husk (Mullen et al., 2013). Furthermore, the anthocyanins cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-glucoside have been described (Prata and Oliveira, 2007), for which potential culinary applications have been proposed (Parra-Campos and Ordóñez-Santos, 2019). Regarding carotenoids, their contents have been only analyzed in whole berries of coffee, without separating the different fractions (i.e., pulp, peel, and bean), and their accumulation was associated with the expression of carotenoid biosynthetic genes (Simkin et al., 2010).

The chemical composition of coffee beans, used to prepare the coffee brew, varies depending on the genotype (Scholz et al., 2011; Tessema et al., 2011), which also seems to affect the composition of other fruit fractions (Ramirez-Martinez, 1988; Clifford and Ramirez-Martinez, 1991; González De Colmenares et al., 1994; Mullen et al., 2013). To the best of our knowledge, research on the compositional pattern of coffee by-products from different genotypes cultivated on the same site is scarce and has focused only on phenolic compounds (Ramirez-Martinez, 1988; Clifford and Ramirez-Martinez, 1991; Rodríguez-Durán et al., 2014), while carotenoids have not been considered. Aiming at increasing evidence about the potential use of coffee by-products as source of bioactive compounds, in this study we employed up-to-date analytical methods to characterize the phenolic compounds, including anthocyanins, as well as carotenoids in peels and pulp of five Costa Rican coffee (*Coffea arabica* L.) varieties growing at the same farm under the same agro-ecological conditions.

MATERIALS AND METHODS

Plant Material and Chemicals

Fully ripe *Coffea arabica* L. berries of the varieties Caturra Rojo, Caturra Amarillo, Anaranjado, Catuaí, and Arábica were manually collected from a commercial coffee farm located in Tres Ríos, Cartago, Costa Rica (9°54′42″N 83°59′09″W). While Caturra Rojo, Catuaí, and Arábica have red peel color, the exocarp is yellow- and orange-colored in Caturra Amarillo and Anaranjado varieties, respectively. Chemical standards were obtained from Sigma Aldrich Chemie (Taufkirchen, Germany). All further reagents or solvents were purchased from VWR International (Darmstadt, Germany), at least of analytical or HPLC grade. Deionized water was used throughout.

Color Measurements

CIE-L*a*b* color values from the peel of 10 freshly collected single fruits of each variety were measured at different positions using a colorimeter (Colorflex HunterLab, Reston, USA) operated by SpectraManager software. An observer angle of 10° and illuminant D_{65} were used. Hue angle ($h^\circ = \arctan(\frac{b^*}{a^*})$) and chroma ($C^* = \sqrt{a^{*2} + b^{*2}}$) were calculated from a* and b*.

Sample Preparation

After removing the seeds, peels and pulp were manually separated. Every sample was immediately frozen in liquid nitrogen and subsequently freeze-dried.

HPLC-DAD-ESI-MS^n Analysis of Phenolic Compounds

The protocol used for polyphenol extraction and analysis was modified from Kammerer et al. (2004), using 0.25 g of pulp and 0.5 g of freeze-dried peels. Polyphenols were extracted twice from each sample with MeOH/0.1% HCl (v/v), and the combined supernatants were evaporated to dryness *in vacuo* at 30°C. The residue was dissolved in 1 or 2 ml of deionized water (pH 3.0) for pulp and peels, respectively. Subsequently, samples were membrane-filtered (0.45 μm) into amber vials and stored at −20°C until analysis. For HPLC analysis, an Agilent HPLC series 1100 (Agilent, Waldbronn, Germany) equipped

with ChemStation software, G1379A degasser, G1312A binary gradient pump, G1313A autosampler, G1316A column oven, and G1315B diode-array detector (DAD), was used. The separation was conducted with a Phenomenex (Torrance, CA, USA) C18 Synergi® Hydro-RP (150 × 3.0 mm i.d., 4 µm particle size) column with a C18 ODS guard column (4.0 × 2.0 mm i.d.) operated at 25°C. LC/MS analyses were performed with the above HPLC system coupled online to a Bruker Esquire 3000+ ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) operating with an ESI source in negative and positive ion mode for phenolics and anthocyanins, respectively, as detailed by Kammerer et al. (2004).

For the analysis of phenolic acids, the mobile phase consisted of a mixture of 2% (v/v) acetic acid in water (eluent A) and 0.5% acetic acid in water and MeOH (10:90, v/v; eluent B). The gradient used started with 10% eluent B increasing to 35% B in 35 min, ramping from 35 to 75% B in 20 min and 75 to 100% B in 2 min, afterwards 100% B isocratic (5 min), from 100 to 10% B (2 min), 10% B isocratic (5 min). Total run time was 69 min at a flow rate of 0.4 ml/min. The injection volume was 10 µl. Detection wavelengths were 280 and 320 nm (Kramer et al., 2012).

A mixture of 5% (v/v) formic acid (eluent A) and MeOH/water/formic acid (80/10/10, v/v/v, eluent B) was used for analysis of anthocyanins and the gradient used ramped from 10 to 14% B (5 min), 14 to 23% B (25 min), 23 to 32% B (15 min), 32 to 62% B (15 min), 62 to 100% B (5 min), 100% B isocratic (5 min), 100 to 10% B (5 min), 10% B isocratic (5 min). Total run time was 80 min. The injection volume was 4 µl. Monitoring was performed at 520 nm and a flow rate of 0.4 ml/min was used.

Compounds were quantified by integration of the peak areas at the respective wavelength using calibration curves of corresponding standard compounds or related reference compounds (5-*O*-caffeoylquinic acid, epicatechin, *p*-coumaric acid, proanthocyanidin B1, ferulic acid, and cyanidin-3-*O*-glucoside). Limits of detection (LOD) and limits of quantitation (LOQ) were calculated based on the regression parameters of the calibration curve. In addition, the identity of mangiferin and cyanidin-3-*O*-rutinoside was confirmed by using authentic standards.

GC-MS Analysis of Phenolic Compounds and Other Metabolites

Extraction and SPE Purification

Methanolic crude extracts obtained as detailed above were subjected to purification by solid phase extraction (SPE). Briefly, 0.25 g of freeze dried and milled peel and pulp, was extracted using 3 × 5 mL of MeOH/0.1% HCl. The crude extract was evaporated to dryness, re-dissolved in 1 ml of H_2O and extracted with 5 × 2 mL of *n*-hexane for removal of lipids. The defatted extract was analyzed as detailed below or subjected to SPE as reported previously (Steingass et al., 2015).

GC-MS Analysis

GC-MS analysis was performed after alkaline hydrolysis of the methanolic crude extract and the SPE purified sample as detailed elsewhere (Steingass et al., 2015), including the following modifications. After the admixture of 3.5 mL of 1 M NaOH and 4.5 mg of ascorbic acid as an antioxidant (final concentration of 1%), the sample was stirred for 4 h at room temperature. Subsequently, a pH of 1.25 ± 0.1 was adjusted with 25% H_2SO_4, followed by liquid-liquid extraction with 3 × 2 mL of ethyl acetate. The combined ethyl acetate phase was dried with 2.5 g of Na_2SO_4 and evaporated to dryness. Successively, 4 × 3 mL of toluene evaporated *in vacuo* at 40°C was used to remove acetic acid. Then, the analytes were dissolved in 0.5 mL of water-free pyridine. An aliquot of 0.5 mL was transferred into a GC vial and 0.2 mL Sweeley reagent (10% v/v hexamethyldisilazane (HDMS): trimethylchlorosilane (TMCS) at a ratio of 2:1 v/v in pyridine) was admixed following heating to 45°C for 30 min. After centrifugation, the clear supernatant was analyzed by GC-MS.

GC-MS analysis was performed using a 6890 N gas chromatograph and a 5976 mass selective detector (both Agilent Technologies, Santa Clara, CA, USA). The injection volume applying the splitless mode was 1.0 µL. Chromatographic separation was achieved using a fused silica capillary column coated with 5% phenyl 95% polydimethylsiloxane (60 m × 0.25 mm i.d., film thickness $d_f = 0.25$ µm, HP-5 ms, Agilent J&W Columns, Santa Clara, CA, USA). The temperature program, source and transfer line temperature were set as reported previously (Steingass et al., 2015). The scan ranges were *m/z* 50–600 (scan frequency 2.7 Hz) between 4.6 and 35 min and *m/z* 50–800 (2.0 Hz) for the final segment, respectively.

Assignment of individual compounds was based on their electron impact (EI) mass spectra compared to Wiley 6N (Wiley and Sons, New York, NY, USA) and NIST 08 (National Institute of Standards and Technology, Gaithersburg, USA) libraries, linear retention indices calculated relative to *n*-alkanes (C8–C30), and authentic reference standards (Steingass et al., 2015).

HPLC-DAD-APcI-MS^n Analysis of Carotenoids

Carotenoid extraction and HPLC-DAD-APcI-MS^n analysis were performed using the instrumentation and system settings as described by Schweiggert et al. (2011).

For selected samples, saponification of carotenoid esters was performed as follows: Carotenoid extracts were evaporated to dryness *in vacuo* at 25°C, re-dissolved in 50 mL of petroleum ether, and subsequently 50 ml of methanolic 10% (w/v) KOH were added. The solutions were maintained in agitation at 300 rpm overnight under nitrogen atmosphere. After saponification, the organic phase was separated, washed twice with deionized water, evaporated to dryness, and prepared for HPLC analysis according to Schweiggert et al. (2011).

Identification of carotenoids was performed by comparing their UV/Vis and APcI(+)-MS1 spectra with data published previously (Britton, 1995; de Rosso and Mercadante, 2007; Maroneze et al., 2019).

Statistics

Determination of significant differences between concentration means was carried out with two biological replicates using one-way analysis of variance, followed by Tukey's test for mean

TABLE 1 | HPLC retention times (t_R), UV/Vis and ESI-MS spectral data of non-anthocyanin phenolic compounds from pooled samples of coffee peels and pulp.

No.	Proposed identity	t_R (min)	HPLC-DAD UV/Vis Absorption maxima (nm)	$[M-H]^-$ (m/z^a)	MS/MS fragment ions (m/z, % base peak intensity)	Reference(s)
1	3-*O*-Caffeoylquinic acid	15.2	300 sh, 325	353	191 (100), 179 (55), 173 (3), 135 (15)	Clifford et al., 2003; Weisz et al., 2009
2	5-*O*-Caffeoylquinic acid	23.7	247, 286, 343	707[a] (353)	[707]: 353 (87), 191 (8) [353]: 191 (100), 179 (3)	Gras et al., 2016
3	4-*O*-Caffeoylquinic acid	25.2	300 sh, 326	353	191 (40), 179 (46), 173 (100)	Clifford et al., 2003; Weisz et al., 2009
4	(Epi)catechin hexoside	27.0	274	451	289 (100), 245 (16)	Robbins et al., 2014
5	(Epi)catechin	29.2	279	289	245 (100), 205 (28)	Jaiswal et al., 2014
6	*p*-Coumaroylquinic acid	31.0	312	337	191 (100), 163 (8)	Weisz et al., 2009
7a	Mangiferin	33.7	n.d.[b]	421	403 (11), 331 (49), 301 (100)	Trevisan et al., 2016
7b	4-*O*-Feruloylquinic acid	33.7	–	367	191 (100)	Mullen et al., 2013
8	5-*O*-Feruloylquinic acid	36.5	243, 326	367	191 (100), 173 (6)	Weisz et al., 2009; Mullen et al., 2013
9	Catechin dimer	39.7	282	577	425 (80), 407 (100), 289 (24)	Sui et al., 2016; Rue et al., 2017
10	3,4-di-*O*-Caffeoylquinic acid	43.2	326	515	353 (100), 191 (10), 179 (13), 173 (20)	Weisz et al., 2009
11	3,5-di-*O*-Caffeoylquinic acid	44.1	326	515	353 (100), 191 (10), 179 (8)	Weisz et al., 2009
12	Rutin	45.4	256, 354	609	301 (100), 300 (30), 255 (10)	Mullen et al., 2013
13	4,5-di-*O*-Caffeoylquinic acid	46.7	327	515	353 (100), 203 (13), 173 (20)	Weisz et al., 2009

[a] *The MS^1 spectrum of no. 2 assigned to 5-O-caffeoylquinic acid displayed abundant dimeric ions ($[2M-H]^-$).*
[b] *The characteristic UV/Vis maxima of mangiferin at ca. 318 and 366 nm (Campa et al., 2012) were not found, presumably due to co-elution with a feruolylquinic acid. Identity of mangiferin was further confirmed by using an authentic standard.*
n.d., not detected.

comparison, using the statistical program Statistica 6.0 (StatSoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

HPLC-DAD-ESI-MS^n Analysis of Phenolic Compounds

Non-anthocyanin Phenolic Compounds

A total of 14 non-anthocyanin phenolic compounds were tentatively identified in pooled pulp and peel samples of the coffee fruit varieties by means of their UV spectra and mass fragmentation as well as the comparison to literature data. Nine of these compounds were free hydroxycinnamic acids (chlorogenic acids), including each three isomeric mono- (compounds 1–3 in **Table 1**) and di-caffeoylquinic acids (compounds 10, 11, and 13), in addition to *p*-coumaroyl- and feruloylquinic acids (compounds 6, 7b, and 8). All of these compounds are commonly found in green coffee beans (Clifford et al., 2003; Perrone et al., 2008) and, with the exception of *p*-coumaroylquinic acid, have been reported to be present in coffee pulp as well (Campa et al., 2012; Mullen et al., 2013; da Silveira et al., 2020).

The prevailing chlorogenic acid found in the peels and pulp of all five coffee varieties was 5-*O*-caffeoylquinic acid (**Table 2**). It displayed dimeric ions $[2M-H]^-$ (**Table 1**) as the most abundant species in the ESI(-)-MS1 spectrum. Similar to our results, Clifford and Ramirez-Martinez (1991) found higher concentration of 5-*O*-caffeoylquinic acid over the other chlorogenic acids measured in coffee pulp. This compound was differentiated from other caffeoylquinic acids by the distinctive fragmentation pattern (**Table 1**) according to Gras et al. (2016). Both, 3-*O*- and 4-*O*-caffeoylquinic acids (compounds 1 and 3 in **Table 1** and **Figure 1**) also exhibited $[M-H]^-$ precursor ions at m/z 353 but displayed different mass fragmentations as described previously (Clifford et al., 2003; Weisz et al., 2009). The deprotonated molecules $[M-H]^-$ at m/z 337 and 367 and the unique fragmentation patterns were considered for the identification of peaks 6 and 8 as *p*-coumaroylquinic and feruloylquinic acids, possibly 5-*O*-*p*-coumaroylquinic acid and 5-*O*-feruloylquinic acid, respectively (Weisz et al., 2009) (**Table 1**). The former could be only quantified in the peels, while the latter solely in the pulp, without a clear pattern between genotypes (**Table 2**). Some variation in the contents of individual chlorogenic acids in the pulp and husks of different coffee varieties, like in this report (**Table 2**), was previously described by Ramirez-Martinez (1988), Clifford and Ramirez-Martinez (1991) and Mullen et al. (2013) as well. Moreover, the concentrations in our work are within the range of concentrations they reported in the wet-processed coffee husks.

Additional phenolic compounds identified in coffee pulp and peels in our work include the monomeric flavan-3-ols (epi)catechin hexoside (epi)catechin and a catechin dimer (peaks 4, 5, and 9 in **Table 1**, respectively). Monomeric and dimeric aglycone flavan-3-ol forms have been previously reported in Arabica coffee pulp (Ramirez-Martinez, 1988; González De Colmenares et al., 1994; Ramirez-Coronel et al., 2004).

The putative (epi)catechin hexoside (peak 4, **Figure 1**) was one of the most conspicuous peaks and was found at concentrations ranging from 1,030 to 2,410 mg/kg. Although few differences were observed among genotypes, the yellow cultivar showed the highest contents in the peels, while the red Catuaí pulp had the lowest ones in the same fraction (**Table 2**). This peak displayed a deprotonated molecule $[M-H]^-$ at m/z 451 and

TABLE 2 | Concentration (mg/kg DM) of major phenolic compounds in pulp and peels of different coffee varieties[a].

Proposed identity	Anaranjado (orange)	Arábica (red)	Catuaí (red)	Caturra Rojo (red)	Caturra Amarillo (yellow)	Anaranjado (orange)	Arábica (red)	Catuaí (red)	Caturra Rojo (red)	Caturra Amarillo (yellow)
	Pulp					Peels				
Phenolic compounds										
3-*O*-Caffeoylquinic acid	242.2^{b}	259.9^{a}	220.8^{d}	227.5^{cd}	237.2^{bc}	133.2^{a}	136.9^{a}	65.3^{b}	71.1^{b}	156.0^{a}
5-*O*-Caffeoylquinic acid	1998.9^{ab}	2220.7^{a}	1738.2^{c}	1941.5^{bc}	2201.6^{a}	4526.1^{bc}	5693.4^{ab}	2745.6^{c}	4242.6^{bc}	8029.8^{a}
4-*O*-Caffeoylquinic acid	408.5^{a}	434.9^{a}	305.7^{b}	328.7^{b}	419.9^{a}	177.7^{b}	182.9^{b}	87.8^{c}	97.1^{c}	248.3^{a}
(Epi)catechin hexoside	1489.2^{ab}	1536.8^{ab}	1301.1^{b}	2028.6^{a}	1351.0^{b}	1865.9^{ab}	1837.3^{ab}	1029.8^{b}	1457.0^{ab}	2410.8^{a}
(Epi)catechin	369.8^{b}	398.5^{a}	346.5^{b}	166.7^{d}	292.2^{c}	498.5^{b}	507.0^{a}	288.0^{c}	408.5^{b}	517.8^{a}
p-Coumaroylquinic acid[b]	tr	tr	tr	tr	tr	79.7^{a}	74.9^{ab}	50.8^{b}	69.0^{ab}	89.2^{a}
5-*O*-Feruloylquinic acid[c]	9.7^{b}	11.2^{b}	11.8^{b}	57.6^{a}	16.4^{b}	tr	tr	tr	tr	tr
Catechin dimer[d]	tr	tr	tr	tr	tr	141.3^{b}	51.1^{cd}	32.1^{d}	80.6^{c}	238.4^{a}
3,4-di-*O*-Caffeoylquinic acid	420.1^{b}	514.6^{a}	353.9^{c}	288.8^{d}	382.7^{bc}	678.2^{b}	762.6^{a}	439.8^{c}	417.3^{c}	663.8^{b}
3,5-di-*O*-Caffeoylquinic acid	506.3^{ab}	575.5^{a}	450.5^{bc}	372.4^{c}	569.3^{a}	1537.3^{a}	1510.0^{a}	962.5^{a}	992.1^{a}	922.2^{a}
Rutin[e]	n.d.	n.d.	n.d.	n.d.	n.d.	1773.6^{a}	754.4^{bc}	492.8^{c}	698.0^{bc}	1063.4^{b}
4,5-di-*O*-Caffeoylquinic acid	280.1^{b}	326.3^{a}	266.1^{b}	235.3^{c}	281.4^{b}	367.2^{b}	401.8^{ab}	289.8^{c}	289.5^{c}	440.9^{a}
Anthocyanins										
Cyanidin-3-*O*-glucoside[f]	n.d.	n.d.	n.d.	n.d.	n.d.	4.9^{b}	22.8^{a}	15.9^{a}	16.8^{a}	n.d.
Cyanidin-3-*O*-rutinoside[g]	n.d.	n.d.	n.d.	n.d.	n.d.	10.2^{b}	97.7^{a}	62.9^{a}	75.4^{a}	n.d.

[a]Values represent means ± standard deviations (n = 2). Different letters within a row and fraction indicate significant differences of means (p < 0.05). DM: dry matter; [b]LOQ 6.7; [c]LOQ 4.8; [d]LOQ 12.7; [e]LOD 3.8; [f]LOD 0.7; [g]LOD 0.5; n.d., not detected; tr, trace; LOD, limit of detection; LOQ, limit of quantification.

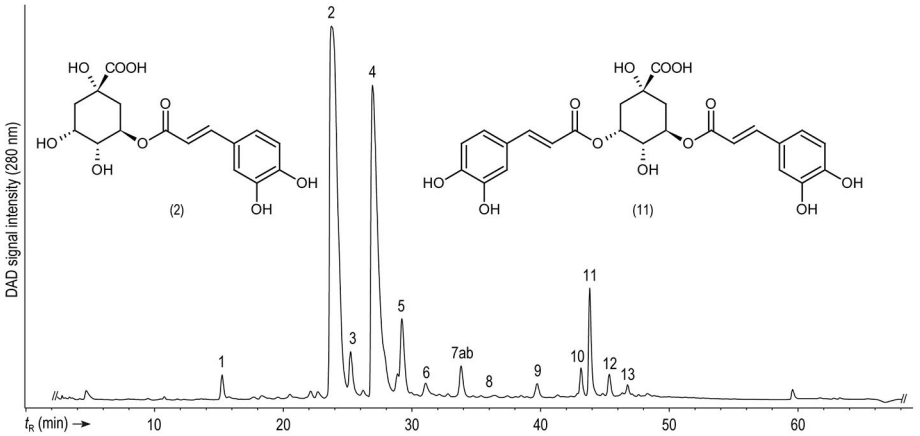

FIGURE 1 | Representative HPLC-DAD chromatogram showing the separation of non-anthocyanin phenolic compounds present in the peels of Caturra amarillo (yellow variety) monitored at 280 nm. For peak assignment, see **Table 1**.

a base peak fragment ion at *m/z* 289 generated by the neutral loss of 162 amu, resembling a dehydrated hexose moiety according to Robbins et al. (2014). (Epi)catechin (peak 5, **Figure 1**) with a precursor ion $[M\text{-}H]^-$ at *m/z* 289 and a base peak product ion at *m/z* 245 ($[M\text{-}H\text{-}CO_2]^-$) (Karar et al., 2014) was quantified at lower contents than those found for the (epi)catechin hexoside in peels and pulp showing less amount in the pulp of the red genotype Caturra Rojo than in the pulp of other genotypes. Peak 9 (**Figure 1**) was identified as a catechin dimer with a precursor ion $[M\text{-}H]^-$ at *m/z* 577 and product ions at *m/z* 425, 407, and 289 being characteristic for B-type procyanidins (Sui et al., 2016; Rue et al., 2017). The presence of proanthocyanidins has been previously reported by Ramirez-Martinez (1988), Ramirez-Coronel et al. (2004), and Mullen et al. (2013) in the pulp of *Coffea arabica*. Catechin and epicatechin levels in husks have previously shown differences according to the coffee species (*C. arabica* and *C. canephora*) and cultivation place (Mexico, India, or China), with much lower concentrations than the ones being reported in our work (Mullen et al., 2013). Conversely, higher levels of epicatechin have been previously reported in the pulp of eight coffee cultivars (Ramirez-Martinez, 1988) than those measured in this study, while similar values for catechin were described in the same report.

Furthermore, the *C*-glycosylated xanthone mangiferin (peak 7a, **Figure 1**), previously described as a compound with high potential as cancer chemopreventive (Gold-Smith et al., 2016), was identified by mass spectral data and using the corresponding standard. However, mangiferin quantitation was omitted due to a coelution with a feruloylquinic acid according to the mass fragmentation signals. Consistent with the elution sequence reported by Mullen et al. (2013), this compound could be 4-*O*-feruloylquinic acid (7b). Mangiferin has predominantly been found in mango (*Mangifera indica* L.) bark and fruits (Berardini et al., 2004, 2005; Nong et al., 2005), although it has been reported in leaves and fruits of diverse coffee species as well (Talamond et al., 2008; Campa et al., 2012; Trevisan et al., 2016). Therefore, its quantification in the coffee fruit byproducts of genotypes with different peel colors deserves more attention.

Finally, the flavonol rutin [quercetin 3-*O*-(6-*O*-rhamnosyl-glucoside)] (peak 12, **Table 1**), with a precursor ion $[M\text{-}H]^-$ at *m/z* 609 and a base peak product ion at *m/z* 301 ($[M\text{-}H\text{-}308]^-$) (Karar et al., 2014), was only detected and quantified in the fruit peels, but not in the pulp, of all studied coffee genotypes, predominantly in the orange one Anaranjado (**Table 2**). Previous works (Ramirez-Martinez, 1988; Heeger et al., 2017; Torres-Valenzuela et al., 2020) have reported lower levels of this compound in coffee husks. The fact that in our work rutin was only found in the peels could, at least partially, explain these differences to previous reports in which husk samples (containing pulp and peels mixed) were analyzed. The higher ratio of pulp to peels' mass could have caused a dilution in the rutin contents reported elsewhere. Because of the health beneficial properties of this flavonoid (antioxidant, antimicrobial, anti-inflammatory, anticancer, antidiabetic, antiallergic, etc.), there is an increasing interest in identifying new rutin sources, and residual biomass has been the focus of recent research (Sharma et al., 2013; Gullón et al., 2017; Junker-Frohn et al., 2019).

Anthocyanins

Two anthocyanins, namely cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside (peaks 1 and 2 in **Figure 2**, respectively), previously reported as the prevailing coffee anthocyanins (Prata and Oliveira, 2007), were identified by using the corresponding authentic standards (**Table 3**). Both cyanidins displayed molecular ions $[M]^+$ in the ESI(+)-MS1 spectra and product ions from the elimination of the sugar moieties. Chemical ionization dissociation (CID) of compound 1 with a $[M]^+$ at *m/z* 449 resulted in a single fragment ion from the elimination of a dehydrated hexose at *m/z* 287 ($[M\text{-}162]^+$) resembling cyanidin. CID of 2 with $[M]^+$ at *m/z* 595 generated fragment ions at *m/z* 449 ($[M\text{-}146]^+$) and 287 ($[M\text{-}308]^+$) from the neutral losses of the deoxyhexosyl (146 amu) and the

entire (deoxyhexosyl)hexosyl (308 amu) moiety, respectively (Faramarzi et al., 2015). Both anthocyanins were absent in the pulp of all varieties assessed and in the peel of the yellow coffee genotype. The concentrations of cyanidin-3-*O*-glucoside and of cyanidin-3-*O*-rutinoside in the orange sample amounted to 4.9 and 10.2 mg/kg, respectively. These values were clearly exceeded by the 15.9–22.8 and 62.9–97.7 mg/kg, respectively, as determined across all red-colored samples (**Table 2**), as can be expected according to the color of the peels. The same anthocyanins were detected previously in coffee husks and, likewise, cyanidin-3-*O*-rutinoside was detected in higher contents than cyanidin-3-*O*-glucoside (Prata and Oliveira, 2007; Murthy et al., 2012). Prevalence of cyanidin-3-*O*-rutinoside over cyanidin-3-*O*-glucoside has been consistently reported in several ripe palm fruits [e.g., *Euterpe oleracea* Mart., *Euterpe edulis* Mart. and *Bactris guineensis* (L.) H.E. Moore], although in much higher contents than the ones measured in coffee peels (Gordon et al., 2012; Vieira et al., 2017; Erşan et al., in press). Extraction of anthocyanins from the coffee fruit processing byproducts for their utilization in the food industry is under development and has a promising future (Murthy and Naidu, 2012; Punbusayakul et al., 2014; Parra-Campos and Ordóñez-Santos, 2019).

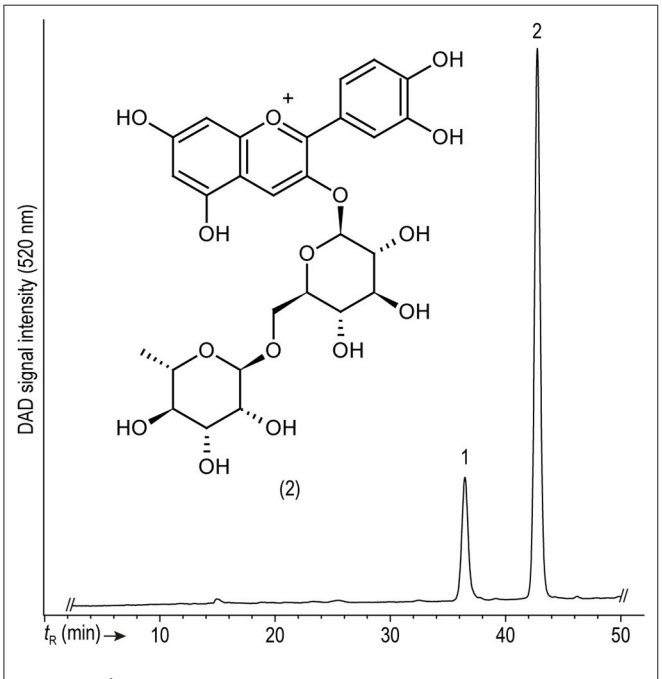

FIGURE 2 | Representative HPLC-DAD chromatogram showing the separation of anthocyanins present in the peels of Caturra Rojo (red variety) monitored at 520 nm. For peak assignment, see **Table 3**.

GC-MS Analysis of Phenolic Compounds and Other Polar Metabolites

GC-MS alkaline hydrolysis and trimethylsilylation was conducted to verify the compound assignments based on HPLC-DAD-ESI-MS^n analysis (**Table 4**). Abundant peaks were assigned to the hydroxycinnamic acids (*E*)-caffeic and (*E*)-*p*-coumaric acid, most likely resulting from the hydrolytic cleavage of their esters with quinic acid (chlorogenic acids). Their corresponding (*Z*)-isomers were detected as minor constituents and may represent possible workup artifacts (Steingass et al., 2015). In addition, quinic acid detected in the hydrolyzed extract may derive from these hydroxycinnamoyl esters. Two late eluting compounds detected at a low abundance were identified as epicatechin and catechin, thus substantiating their identification by LC-MS (**Table 1**).

Moreover, caffeine and diverse low molecular weight benzenoids were detected such as benzoic acid and derived hydroxybenzoic acids (hydroxybenzoic, protocatechuic, and vanillic acid) in addition to benzaldehyde, benzyl alcohol, eugenol, and phloretic acid. Noteworthy, the presence of protocatechuic acid and caffeine in coffee pulp has been previously reported (Ramirez-Martinez, 1988; Clifford and Ramirez-Martinez, 1991).

Further constituents detected by GC-MS comprised carboxylic acids from the citric acid cycle (citric, *cis*-aconitic, succinic, fumaric, and malic acid) ubiquitously occurring in plants. Glyceric acid has been previously reported in brewed coffee (Bähre and Maier, 1996). The retention indices and mass spectra of the coffee constituents compiled in **Table 4** represent a solid basis for continuative research and may be highly instrumental for a GC-MS based metabolite profiling, e.g., for the authentication of coffee varieties but also derived products.

HPLC-DAD-APcI-MS^n Analysis of Carotenoids and Chlorophylls

In this work, carotenoids were detected in the coffee fruit peels and pulp (**Table 5**). The much lower absolute signal observed in the latter fraction did not allow to assess potential differences between genotypes and, therefore, results are exemplified with the pulp of the yellow Caturra variety (**Table 5**). Regarding the peels, comparable profiles were observed in the yellow and orange coffee varieties analyzed here, both showing

TABLE 3 | HPLC retention times (t_R), UV/Vis and ESI-MS spectral data of anthocyanins from pooled samples of coffee peels and pulp.

No.	Proposed identity	t_R (min)	HPLC-DAD UV/Vis Absorption maxima (nm)	$[M]^+$ (m/z^a)	MS/MS fragment ions (m/z, % base peak intensity)	Reference
Anthocyanins						
1	Cyanidin-3-*O*-glucoside	36.5	280, 520	449	287 (100)	STANDARD
2	Cyanidin-3-*O*-rutinoside	42.7	280, 520	595	449 (19), 287 (100)	STANDARD

TABLE 4 | GC-MS analysis of methanolic extracts from coffee peel and pulp after alkaline hydrolysis, liquid-liquid extraction with ethyl acetate and trimethylsilylation.

t_R (min)	LRI	Proposed identity	Derivative[a]	$[M]^+$ (*m/z*)	$[M\text{-}CH_3]^+$ (*m/z*)	Other characteristic ions[b] (*m/z*, % base peak intensity)	Criteria[c]	Detected[d]	
								Ce	SPE
6.6	971	Benzaldehyde	–	106 (99)	–	105 (100), 77 (88), 51 (39)	MS, LRI, STD	Pe, Pu	Pe, Pu
10.7	1,157	Benzyl alcohol	1 TMS	180 (14)	165 (100)	135 (68), 91 (83)	MS, LRI, STD	Pe, Pu	Pe, Pu
12.0	1,206	Malonic acid	2 TMS	248 (1)	233 (8)	133 (4), 99 (2)	MS, LRI, STD	Pe, Pu	Pe, Pu
13.3	1,252	Benzoic acid	1 TMS	194 (8)	179 (100)	135 (40), 105 (57)	MS, LRI, STD	Pe	Pe, Pu
15.2	1,317	Succinic acid	2 TMS	262 (1)	247 (15)	172 (4), 133 (3), 129 (6)	MS, LRI, STD	Pe, Pu	Pe, Pu
15.9	1,340	Glyceric acid	3 TMS	322 (1)	307 (11)	292 (58), 205 (21), 189 (55), 133 (28), 103 (22)	MS, LRI	Pe, Pu	Pe, Pu
16.2	1,348	Fumaric acid	2 TMS	260 (<1)	245 (100)	217 (1), 155 (3), 143 (12), 133 (5), 115 (4), 83 (5)	MS, LRI, STD	Pe, Pu	Pe, Pu
20.1	1,480	Eugenol	1 TMS	236 (64)	221 (29)	206 (100), 179 (18)	MS, LRI, STD	-	Pe, Pu
20.8	1,501	Malic acid	3 TMS	350 (<1)	335 (7)	307 (5), 245 (19), 233 (33), 217 (6), 189 (11)	MS, LRI, STD	Pe, Pu	Pe, Pu
24.7	1,636	Hydroxybenzoic acid (unknown isomer)	2 TMS	282 (21)	267 (100)	230 (12), 223 (65), 217 (19), 193 (51), 105 (4)	MS	Pe	n.d.
28.1	1,760	*cis*-Aconitic acid	3 TMS	390 (<1)	375 (44)	285 (22), 229 (57), 211 (21)	MS, LRI, STD	Pe, Pu	n.d.
28.3	1,770	Phloretic acid	2 TMS	310 (28)	295 (8)	192 (64), 179 (100)	MS, LRI	Pe, Pu	Pu
28.5	1,775	Vanillic acid	2 TMS	312 (63)	297 (100)	282 (30), 267 (71), 253 (46), 223 (52), 193 (20), 165 (10), 135 (5), 126 (20)	MS, LRI, STD	Pe, Pu	n.d.
29.1	1,800	(*Z*)-*p*-Coumaric acid	2 TMS	308 (43)	293 (38)	249 (55), 219 (48), 203 (17), 191 (19), 179 (11), 131 (2)	MS	Pe, Pu	Pe, Pu
30.0	1836	Protocatechuic acid	3 TMS	370 (19)	355 (31)	311 (20), 281 (11), 267 (7), 223 (10), 193 (100), 165 (7)	MS, LRI, STD	Pe, Pu	Pe, Pu
30.2	1844	Citric acid	4 TMS	480 (<1)	465 (7)	375 (13), 363 (17), 347 (16), 273 (100)	MS, LRI, STD	Pe, Pu	Pe, Pu
30.7	1862	Caffeine	–	194 (100)	–	165 (6), 136 (4), 109 (42), 82 (19), 67 (25)	MS, LRI, STD	Pe, Pu	n.d.
31.5	1893	Quinic acid	5 TMS	552 (<1)	537 (2)	419 (5), 345 (100), 334 (8), 255 (33), 204 (10)	MS, LRI, STD	Pe, Pu	Pe, Pu
32.9	1,950	(*E*)-*p*-Coumaric acid	2 TMS	308 (78)	293 (100)	249 (48), 219 (88), 203 (7), 191 (6), 179 (16), 131 (2)	MS, LRI, STD	Pe, Pu	Pe, Pu
34.1	2,001	(*Z*)-Caffeic acid	3 TMS	396 (100)	381 (20)	307 (12), 293 (6), 249 (7), 219 (93), 191 (15)	MS, LRI, STD	Pe, Pu	Pe, Pu
37.7	2,155	(*E*)-Caffeic acid	3 TMS	396 (100)	381 (23)	307 (13), 293 (6), 249 (6), 219 (79), 191 (13)	MS, LRI, STD	Pe, Pu	Pe, Pu
52.0	2,897	Epicatechin	5 TMS	650 (6)	635 (<1)	368 (100), 355 (35), 280 (5), 267 (9)	MS, LRI, STD	n.d.	Pe, Pu
52.4	2,920	Catechin	5 TMS	650 (6)	635 (1)	368 (100), 355 (28), 280 (5), 267 (7)	MS, LRI, STD	n.d.	Pe, Pu

t_R, retention time; LRI, linear retention index; Pe, peels; Pu, pulp.

[a] *Trimethylsilyl (TMS) derivative detected after alkaline hydrolysis and partitioning with ethyl acetate. Ce, crude extract; SPE, sample purified by solid-phase extraction.*

[b] *Unspecific fragment ions at m/z 73 ($C_3H_9Si^+$), 75, and 147 ($C_6H_{15}SiO_2^+$) are not given herein.*

[c] *Identification criteria: Linear retention index (LRI) on a HP-5 ms column, mass spectrum (MS), and reference standards (STD).*

[d] *Detected in the defatted crude extract (CE) and the SPE extract (SPE) after alkaline hydrolysis and partitioning with ethyl acetate.*

the highest diversity of carotenoids. The red Caturra peels mainly showed lutein and β-carotene, but did not show quantifiable amounts neither of the xanthophylls violaxanthin and neoxanthin [both with protonated molecules $[M+H]^+$ at *m/z* 601], nor of α-carotene. β-Carotene and α-carotene displayed protonated molecules $[M+H]^+$ at *m/z* 537. The comparison of their distinctive Vis absorption spectra to those reported in literature (e.g., Britton, 1995) permitted assignment of these isobaric carotenes. Lutein and β-carotene were the major carotenoids detected in all varieties (peaks 4 and 8 in **Figure 3**, respectively). Lutein showed a protonated molecule $[M+H]^+$ at *m/z* 569 and an abundant, characteristic in-source fragment $[M+H\text{-}18]^+$ at 551, and UV/Vis absorption maxima of 420, 444, and 472 nm, which is in agreement with Britton (1995) and Rodriguez-Amaya (2001). Lutein was not baseline resolved from one of the two chlorophylls detected in the genuine extracts.

Chlorophylls a and b were identified in the ripe coffee peels of all varieties, while only the latter was present in the pulp. Presence of chlorophyll is not only evident in unripe coffee fruits because of the exocarp green color, but also due to their relevant photosynthetic capacity (Vaast et al., 2005). However, a progressive decrease in the chlorophyll content along ripening has been reported (Marín-López

TABLE 5 | HPLC retention times (t_R), Vis absorption maxima, APcI(+)-MS data and qualitative carotenoid and chlorophyll profiles in the peels of three coffee varieties and in the pulp of cv. Caturra Amarillo.

No.	Proposed identity	t_R (min)	Vis_{max} (nm)	$[M+H]^+$ (*m/z*)	Coffee variety and tissue analyzed*				References
					Caturra Rojo (red)	Anaranjado (orange)	Caturra Amarillo (yellow)	Caturra Amarillo (yellow)	
					Peels			pulp	
1	Violaxanthin	10.6	419/440/470	601	–	+	+	–	Britton, 1995
2	Neoxanthin	11.3	412/436/464	601	–	+	+	–	Britton, 1995
3	Chlorophyll b	22.1	466/650	906	+	+	+	+	Maroneze et al., 2019
4	Lutein	23.8	420sh/444/472	569, 551[a]	+	+	+	+	de Rosso and Mercadante, 2007
5	Chlorophyll a	29.5	432/666	892	+	+	+	–	Maroneze et al., 2019
6	n.i.	37.5	420sh/440/470	537	–	+	+	–	–
7	α-Carotene	43.6	422sh/446/474	537	–	+	+	+	de Rosso and Mercadante, 2007
8	β-Carotene	47.2	424sh/450/478	537	+	+	+	+	Britton, 1995

**Presence (+) or absence (–) of the compound.*
sh, shoulder in the Vis absorption spectrum.
[a]In-source elimination of water $[M+H-H_2O]^+$.
n.i., not identified.

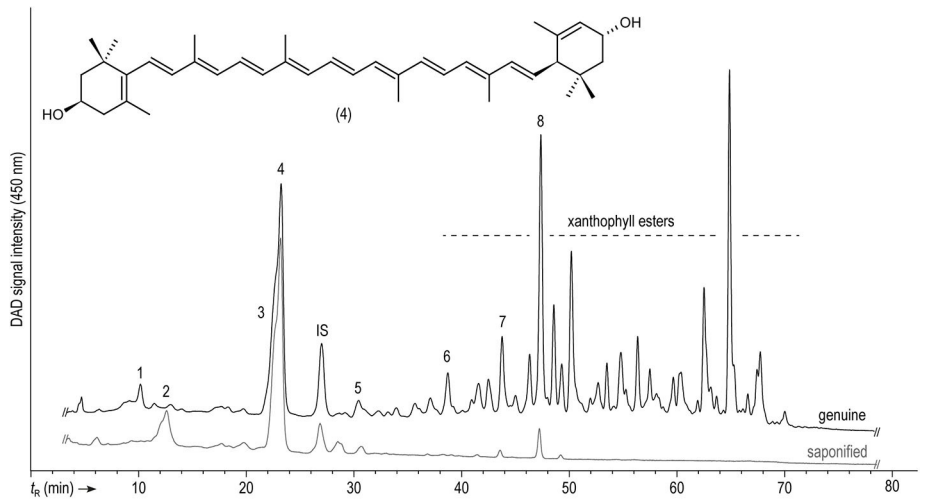

FIGURE 3 | Representative HPLC-DAD separation of carotenoids and chlorophylls in genuine and saponified extracts from the peels of the Anaranjado (orange) variety monitored at 450 nm. For peak assignment, see **Table 5**.

et al., 2003; de Castro and Marraccini, 2006). These primary photosynthetic pigments were identified in our work by their protonated molecules $[M+H]^+$ at *m/z* 892 and 906, respectively, and their corresponding absorption maxima, according to Maroneze et al. (2019).

Table 5 compiles the criteria for identification of the remaining carotenoids and the corresponding references. Comparison of chromatograms of genuine and saponified samples showed disappearance of several peaks in the latter, which points out to the presence of some carotenoid esters (**Figure 3**). This merits further investigation, especially, considering the increased bioavailability of esterified carotenoids over the free forms (Hempel et al., 2017; Schweiggert and Carle, 2017; Chacón-Ordóñez et al., 2019).

To the best of our knowledge, this is the first report on the characterization of carotenoids in different coffee fruit tissues (viz., pulp, and peels). In a previous work aiming at studying the profile and expression of carotenoid biosynthetic genes during coffee fruit development, Simkin et al. (2010) have analyzed complete fruits instead. They found the same pattern of carotenoids and chlorophylls we are reporting here. Similar to our results, these authors also found lutein to be the prevailing carotenoid in the ripe coffee fruits (in both *C. arabica* and *C. canephora* species). The carotenoid profile of coffee peel and pulp resembled that of a (degraded) chloroplast. These organelles ubiquitously occurring in green plant tissues contain chlorophylls a and b, in addition to a highly preserved carotenoid pattern, with prevailing constituents being lutein (40–45%) and

TABLE 6 | Color parameters of peels from different coffee genotypes.

Color parameter	Anaranjado (orange)	Arábica (red)	Catuaí (red)	Caturra Rojo (red)	Caturra amarillo (yellow)
L*	37 ± 0^{b}	25 ± 2^{c}	26 ± 1^{c}	21 ± 1^{d}	43 ± 2^{a}
C*	38 ± 2^{a}	27 ± 1^{b}	26 ± 2^{b}	21 ± 3^{c}	41 ± 2^{a}
h°	56 ± 2^{b}	30 ± 4^{cd}	35 ± 2^{c}	26 ± 4^{d}	75 ± 2^{a}

[a]*Values represent means ± standard deviations (n = 2). Different letters within a row indicate significant differences of means (p < 0.05).*

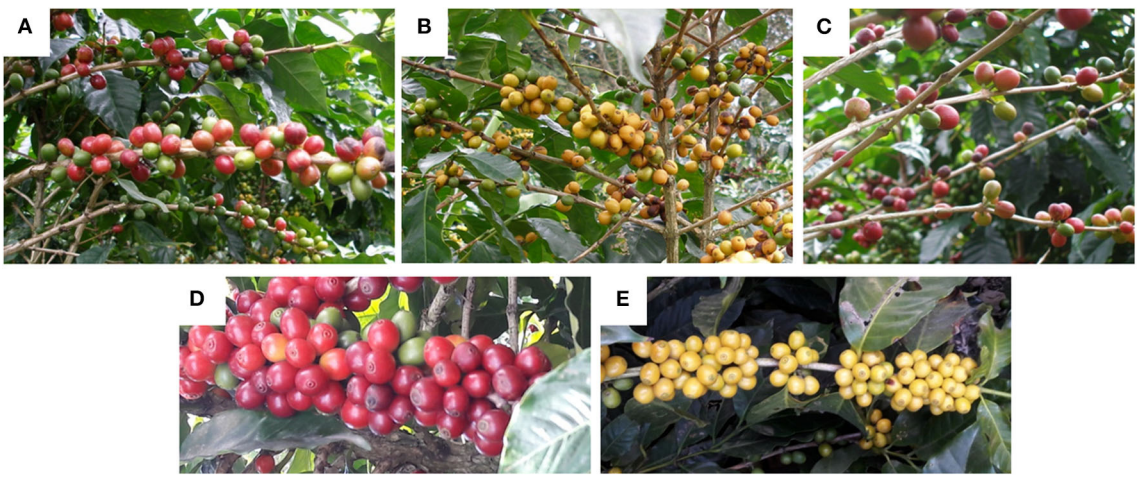

FIGURE 4 | Photographs of the ripe coffee berries analyzed in this work: **(A)** Caturra Rojo (red), **(B)** Anaranjado (orange), **(C)** Catuaí Rojo (red), **(D)** Arábica (red), **(E)** Caturra amarillo (yellow).

β-carotene (20–25%), as well as violaxanthin and neoxanthin (both 10–15%) (Schweiggert and Carle, 2017).

Lutein, together with zeaxanthin, are the only carotenoids of about 20–30 found in the human tissues, that accumulate in the macula lutea, which is the most sensitive region of the retina of humans and other primates. These carotenoids are believed to be important for the prevention of age-related macular degeneration, being a cause of impaired vision and blindness in elderly people. Furthermore, frequent lutein intake has been associated with improved cognitive functions like verbal fluency in senior citizens (Johnson, 2012; Eisenhauer et al., 2017). In addition, β-carotene, the other predominant carotenoid found in the peels and pulp of coffee berries according to results in **Figure 3**, is well-known for its provitamin A activity. Deficiency of this vitamin is an important drawback for deprived populations in various regions of the world and has been related to eye health issues and increased severity and death rates due to infectious diseases (Wiseman et al., 2017). Therefore, looking for affordable lutein and β-carotene sources has been the aim of considerable research, and coffee by-products might be considered for the food industry in the framework of the application of circular economy strategies. Further investigation regarding the effect of environmental and genetic conditions on carotenoid accumulation, as well as evaluation of extraction methods, to attain high recovery and reduce prospective antinutritional factors, are necessary to take advantage of this potential source of functional compounds (Janissen and Huynh, 2018; Kumar et al., 2018; Saini and Keum, 2018).

Color Analysis

Color parameters of the peels are displayed in **Table 6** and pictures of the studied coffee cherries are shown in **Figure 4**. In agreement with the visual perception, L* values corroborated higher lightness for yellow- and orange-colored fruits (L* of 43 and 37, resp.) when compared to the red ones (L* of 21–26). Moreover, yellow- and orange-colored coffee cherries also showed higher chroma (C*) values when compared to the other materials. As expected, hue (h°) values of 75 in the yellow colored berries agreed with the yellow tonalities (h° at ∼70–90), orange colored fruits with hue values of ∼56, also neared the expected orange tonalities (h° at ∼40–70). Hue values ranging between 26 and 35 were measured for the red colored fruits, also in accordance with red tonalities (h° at ∼10–30).

The profile of anthocyanins, together with that of carotenoids, explains the observable colors of the coffee peels in the different genotypes. The relationship between both pigment groups in terms of quantity, profile and distribution in the different tissues has been pointed out as determinant for generating the observable color. This has been described previously in several tropical fruits such as bananas, cashew apples, mango, and nance (Schweiggert et al., 2016; Fu et al., 2018; Irías-Mata et al., 2018;

Ranganath et al., 2018). In flowers (Lewis et al., 2003) and banana fruits (Fu et al., 2018), the deposition of anthocyanins in external cell layers has been documented, whereas carotenoids are sometimes located in more internal ones (sub-epidermis and mesophyll) and, thus, their yellow color might be less dominantly visible than the red color of anthocyanins. In any case, the color of anthocyanins often overlays with the color of carotenoids and, eventually, chlorophylls and, therefore, according to the pigments available, different colorations are noticeable. Knowing whether differential deposition in layers also occurs in the coffee peels might be the subject of further research. Nevertheless, clearly, the absence of anthocyanins in the yellow genotype (Caturra Amarillo) (**Table 2**) allows the sole appearance of a yellow color caused by lutein and β-carotene, the most conspicuous carotenoid peaks observed (**Figure 3**), and widely known for their yellow and orange colors, respectively (Meléndez-Martínez et al., 2007). Orange pigmentation in *Zantedeschia* (calla lily) and red pigmentation in bananas have been found to result from the visual appearance of having both pigment groups (carotenoids and anthocyanins) in different cell layers, and not because of their mixture (Lewis et al., 2003; Fu et al., 2018). Something similar might occur in the orange coffee variety (Anaranjado), a genotype that contains a lower concentration of anthocyanins compared to the red ones (**Table 3**). Nevertheless, in the red-colored varieties, the much higher concentration of anthocyanins apparently had totally masked the yellow background color of the more internal carotenoids, hiding their appearance, either simply due to their contents or because of their localization in the outer cell layers.

CONCLUSIONS

5-*O*-Caffeoylquinic acid together with a putative (epi)catechin hexoside were consistently the prevailing phenolic compounds among 16 detected in the pulp and peels of coffee berries belonging to different varieties with distinct exocarp color. Moreover, the xanthone mangiferin, a cancer chemopreventive agent, was found in all samples and its study deserves more attention. GC-MS analysis after alkaline hydrolysis and trimethylsilylation substantiated the assignment of the individual phenolic compounds by HPLC-DAD-ESI-MS^n and, moreover, permitted the identification of additional plant metabolites, thus providing a solid basis for ongoing research. Anthocyanins were found in red-colored fruit peels and in lower contents in orange-colored ones, but were absent in the yellow-colored berries. On the other hand, carotenoid peel profiles can be divided into two groups, with the red genotype on the one side and the yellow and orange ones clustered together on the other. The latter showed additional carotenoid compounds to the ones found in the former. These results point out to the potential that coffee berry by-products have for their utilization as a source of health beneficial compounds, including natural pigments.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

PE, EG, RC, and VJ designed research. MV, CS, MG, and RS conducted research. PE, MV, CS, RS, and VJ analyzed data. PE, MV, CS, EG, RC, RS, and VJ discussed the data. PE, MV, CS, RS, and VJ wrote the paper. All authors read and approved the final manuscript.

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Marine Bacteria Is the Cell Factory to Produce Bioactive Pigments: A Prospective Pigment Source in the Ocean

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The course of investigations of bioactive compounds like bacterial pigments from the marine environment has greatly expanded in the recent decades. Despite the huge concern in secluding and collecting marine bacteria, microbial metabolites are progressively alluring to science due to their wide ranging applications in various fields, particularly those with distinctive color pigments. This review is a short appraisal of the studies undertaken over the past 5 years on the bacterial pigments sourced from the marine environment. Herein, we have reviewed the potential of different bacterial species isolated from marine environment in diverse studies that are producing bioactive pigments that have potential commercial applications.

Keywords: production, pigments, bioactive, cell factory, bacteria, marine

INTRODUCTION

Marine environments are one of the most important eco-systems of our earth that are yet to be explored to understand their full potential. They contain immeasurable and uncountable undiscovered mysterious black boxes holding colossal information that is to be decoded scientifically and exploited for the benefit of mankind. Marine environments are home to a multitude of organisms ranging from whales that are to a height of a building to microorganisms that are nano-scale life forms. The marine microorganisms are renowned to maintain and regulate the Bio-geo chemical cycle in the ocean environment and the interactions of the microbial community in the ocean to maintain such a biogeochemical cycle extraordinarily; metabolomics studies by profiling the variety of molecules reveal that the organisms produce to interact (Sogin et al., 2019). More than 6,000 and 10,000 species of prokaryotes and protists have been described so far (Aryal et al., 2015); yet the ocean hides an even more complex diversity of microorganisms that we are yet to fathom by appropriate investigations and explorations. Currently, the marine bacteria are being explored for their production of clinically and industrially important secondary metabolites; the pigments produced by marine bacteria as a result of quorum sensing are of current interest due to their anti-microbial, anti-cancer, photoprotective, anti-parasitic, and immunosuppressive activities (Ramesh et al., 2019). The modification of current natural compounds to synthetic compounds seems to be slow compared to the exponential evolution of resistant organisms which forces our hands to look for alternatives where secondary metabolites from marine bacteria seem to be the answer (Andryukov et al., 2019). Bacterial

pigments are also used as fluorescence-based indicators for labeling antibodies, screen several reactions, and also to cure the damage caused by free radicals (DeLange and Glazer, 1989). The marine bacteria produce various pigments like carotene, melanin, phenazine, pyrrole, violacein, and quinones (**Figure 1**). This review evaluates the advancements that were made in the studies of marine bacterial pigments in the past 5 years and their prospective applications in various fields (**Table 1**).

PHENAZINE

Phenazines are redox-active, small nitrogen-containing aromatic compounds produced by a diverse range of bacterial genera, including *Streptomyces* (terrestrial), *Pseudomonas* (ubiquitous), *Actinomycetes* (terrestrial and aquatic), *Pelagibacter* (aquatic), and *Vibrio* (aquatic), under the control of quorum sensing and also a nitrogenous aromatic compounds of reverse redox potential converting molecular oxygen into toxic reactive oxygen species and are used in a broad spectrum of antibiotics, anti-viral, insecticidal agent, anti-cancerous, antiprotozoal agents (Pierson and Pierson, 2010; Soliev et al., 2011; Guttenberger et al., 2017). Phenazine also functions as a respiratory pigment which is pyocyanin (blue) and *Pseudomonas* sp., has been highly researched (**Figure 1**). The biosynthesis of phenazine is catalyzed by five enzymes PhzE, PhzD, PhzF, PhzB, PhzG where PhzE converts chorismic acid to 2-amino-2-deoxyisochorismic acid (ADIC) by diffusion of NH3 from GATase1 to active MST domain of the enzyme by ligand binding and end with Ψ face ofC2 chorismate and converted to DHHA by PhzD by acid/base catalytic system and is isomerized by PhzF by utilizing glutamate and catalyze by proton shift into AOCHC; but it is still unstable by forming aminoketone AOCHC and goes into a second condensation reaction of AOCHC into a secondary molecular structure which is accelerated by PhzB and by conjugation HHPDC is produced which is unstable and undergoes oxidative decarboxylation resulting in THPCA which is a PhzG substrate producing PCA and if PhzG binds to HHPDC oxidation takes place producing PDC the two final core phenazine compounds (Blankenfeldt and Parsons, 2014). Liang et al. (2017) isolated six phenazines from *Streptomyces* sp., 182SMLY with three new phenazine compound (-)- streptophenazines M–O and three already known phenazine 1-carbomethoxyphenazine; (-)-streptophenazines A; streptophenazines B where it was tested against the proliferation of glioma cell lines but didn't have any inhibitory effect on proliferating glioma cells and it was also screened against *E. coli* and MRSA, but only streptophenazines B was effective against MRSA compared to control norfloxacin which inhibited both *E. coli* and MRSA. Phenazine compound pyocyanin has been isolated from marine *P. aeruginosa* by Li et al. (2018), and demonstrated to be acting as an anti-chlamydial agent with a dose of IC50 (0.02 μM) inhibiting its infectivity by directly targeting Elementary body (EB) but low doses didn't seem to increase the host ROS. The IC50 dose of pyocyanin did not show any immune suppressant activity and performed equally to the IC50 dose of Tetracycline against MRSA supporting the clinical use of pyocyanin for treatment. Patil et al. (2016a,b), reported that GS-33 marine P. aeruginosa produced Phenazine-1- carboxylic acid which inhibited the charcoal root rot caused by M. phaseolina, promotes plant growth, and even conferred resistance of plants toward saline conditions by producing NH3 and solubilizing phosphate. The GS-33 isolate PCA was also reported to inhibit skin melanoma in low doses in human cell melanoma cell lines SK-MEL-2 and when combined with the commercial SPF lotions showed a synergistic increase of around 10–30% UV-B protection. Cytotoxic studies revealed that concentration up to 100 ppm PCA was safe which showed hemolysis around permissible levels. The limited commercial availability of substituted phenazines indicates that their synthesis presents a challenge for the synthetic chemist. To date, no efficient and generally applicable synthesis of substituted phenazines exists (Laursen and Nielsen, 2004).

CAROTENE

Carotene is synthesized mainly by plants followed by other microorganisms like filamentous fungi, yeast, and bacteria which have been reported to display Pro-vitamin Activity and displays strong antioxidant activity. Carotene is used as food colorants, cosmetics, and in feed industries where their color ranges from yellow to red (Da Costa Cardoso et al., 2017). Carotene is a C40 Polyunsaturated hydrocarbon compound a resultant of condensation of C20 compounds (Liang et al., 2006). The biosynthesis of carotene takes place by two core terpenoids Isopentenyl pyrophosphate and dimethylallyl pyrophosphate which is synthesized from the MEP pathway and DXP pathway where the IPP and DMAPP are condensed into GGPP and FPP and converted to phytoene by phytoene synthase and metabolized into lycopene by lycopene synthase and finally to β-carotene by Lycopene β-cyclase and further undergoes five intermediates to produce astaxanthin (Zhang et al., 2020). Afra et al. (2017) has reported the isolation of red-colored carotenoid pigment from marine *Arthrobacter* sp., G20 which displayed excellent anti-oxidant activity where its EC50 scavenging activity was equivalent to β-carotene and α-tocopherol. Mild tumor-suppressive activities were observed in esophageal cancer cells but specific anti-proliferative activities were observed in the KYSE30 cell line where the cells were found to be round a characteristic feature of apoptotic cells. But, the extracted carotene pigment didn't show any appreciable anti-microbial activity. Kallscheuer et al. (2019) reported two planctomycetes strain *R. rubra* LF2T and *R. brasiliensis* Gr7 producing three carotenoid compounds saproxanthin, flexixanthin, 2′-isopentenyldehydrosaproxanthin and established biosynthetic pathway for the production of carotenoids. Asker (2017) have testified the production of carotenoids astaxanthin, 2- hydroxyastaxanthin, and 2,2′-dihydroxyastaxanthin by *Brevundimonas* sp. strain N-5 which was isolated and characterized by high-throughput sequencing. Hegazy et al. (2020) have demonstrated that haloarchea *Natrialba* sp., displayed antiproliferative and apoptotic activity against colon, breast, liver, and cervical cancer cells by interfering and inhibiting the MMP-9pathway and displayed anti-viral activity

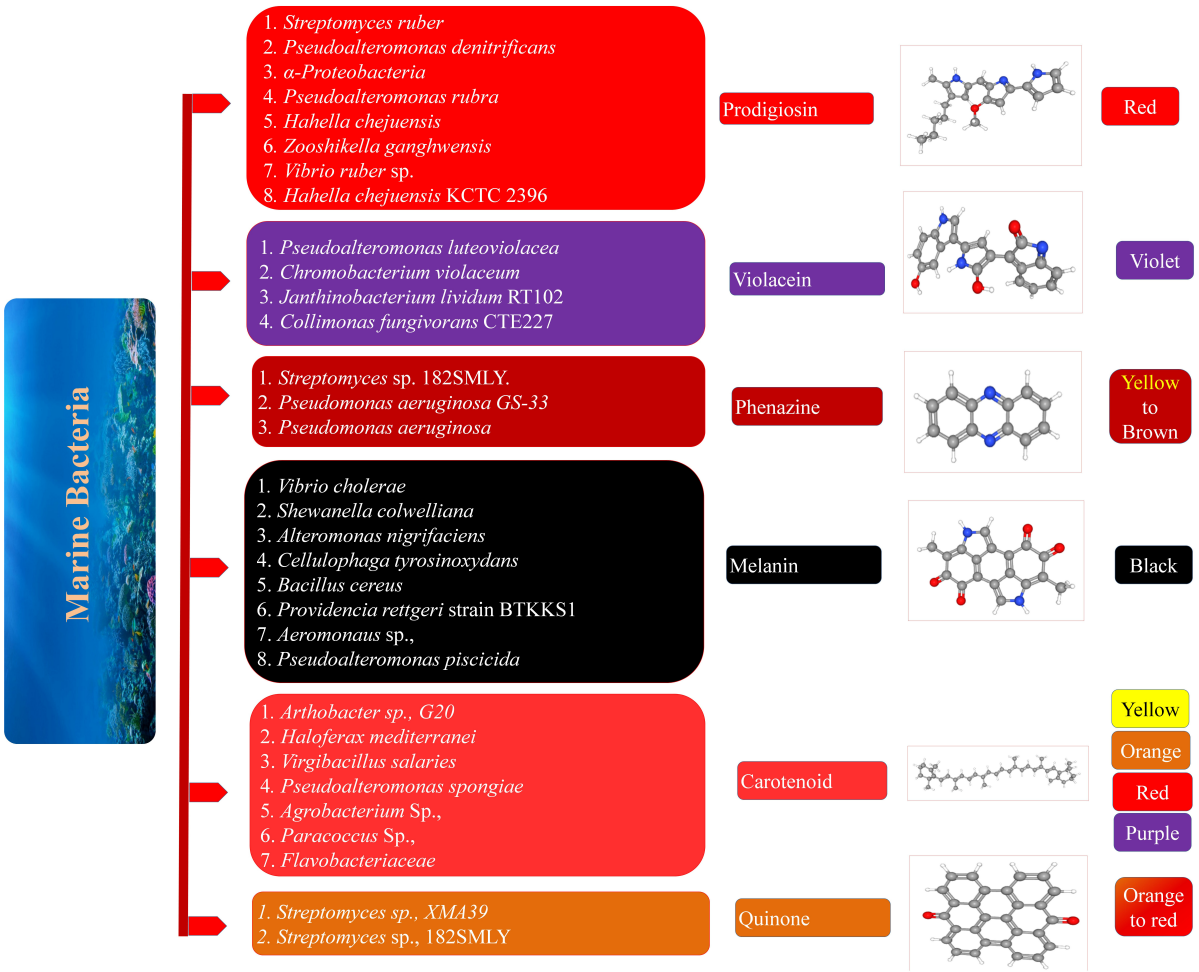

FIGURE 1 | Types of marine bacterial pigments.

by suppressing replication against HCV and HBV by inhibiting HCV NS5B polymerase and HBV-DNA dependent DNA polymerase. Earlier, synthetic β,β-carotene was produced on an industrial scale and used as a feed and food dye. Of the nearly 700 naturally occurring carotenoids, only a few are synthesized on an industrial scale. Among them are lycopene, canthaxanthin, astaxanthin, β,β-carotene, β-apo-8′-carotenal, β-apo-8′-carotene, and cytranaxanthin. Wittig reactions of Grignard compounds are used to obtain carotenoids (Alvarez et al., 2014; Bogacz-Radomska and Harasym, 2018).

MELANIN

Bacterial Melanin is a product of biosynthesis of L-tyrosine via the enzyme tyrosinase oxidizing it into L-3, 4-dihydroxyphenylalanine which is further metabolized into dopachrome and finally converted to melanin by oxidoreduction where the color usually ranges from black-brown in color (El-Naggar and El-Ewasy, 2017). Traditional melanin nanoparticle synthesis usually takes around 12 h, but Wang et al. (2020) demonstrated the production of melanin nanoparticle synthesis in bacteria within 30 min by controlling the growth of cell-free conditions by changing the salinity concentration of the medium and incubation period. This demonstrates the exciting potential of bacteria as melanin pigment factories and melanin has a broad range of usages such as anti-oxidative, anti-cancer, anti-bacterial, anti-viral properties also displaying thermal resistance, radiation damage by absorption of wide range electromagnetic spectrum, and even chemical resistance (Narsing Rao et al., 2017). Vijayan et al. (2017) isolated 156 sponge-associated bacteria out of which 14% displayed melanin production and its extraction from the sponge made the sponge colorless hinting the synergy between bacteria and the sponge for its photoprotective role and out of the 14% melanin-producing isolates 56% were observed to be *Vibrio* sp., and the remaining isolates were *Providencia* sp., *Bacillus* sp., *S. algae, S. sciuri, P. maritimus, S. roseus, G. creatinolyticus*; but the *Vibrio* sp., were selected for its high pigment production and the extracted pigment was observed for the photoprotective role from UV where highest efficiency was noted at 200 ppm displaying 65% protective effect in mouse

TABLE 1 | Microorganisms and its corresponding pigments.

Organism	Pigment	References
Shewanella algae	Melanin	Vijayan et al., 2017
Staphylococcus sciuri	Melanin	Vijayan et al., 2017
Planococcus maritimus	Melanin	Vijayan et al., 2017
Salinicoccus roseus	Melanin	Vijayan et al., 2017
Glutamicibacter Creatinolyticus	Melanin	Vijayan et al., 2017
Providencia sp.,	Melanin	Vijayan et al., 2017
Bacillus sp.,	Melanin	Vijayan et al., 2017
Pseudomonas stutzeri	Melanin	Manirethan et al., 2018, 2020
Pseudolateromonas lipolytica	Melanin	Kurian and Bhat, 2018
Chromobacterium violaceum	Violacein	Füller et al., 2016
Janithobacter lividum	Violacein	Füller et al., 2016
Psudoalteromonas amlolytica	Violacein	Wu et al., 2017
Streptomyces sp., XMA39	Quinone	Liang et al., 2016
Streptomyces sp., 182SMLY	Quinone	Jiang et al., 2018
Zooshikella sp.,	Prodigiosin	Ramesh et al., 2020
Streptomyces sp.,	Prodigiosin	Ramesh et al., 2020
Vibryoruber DSM 14379	Prodigiosin	Danevčič et al., 2016
Actinomyces sp.,	Prodigiosin	Abdelfattah et al., 2019
Hahella sp., KA22	Prodigiosin	Abdelfattah et al., 2019
GS-33 marine Pseudomonas aeruginosa	Phenazine	Patil et al., 2016a,b
pseudomonas aeruginosa	Phenazine	Li et al., 2018
streptomyces sp.,	Phenazine	Liang et al., 2017
Arthrobacter sp., G20	Carotene	Afra et al., 2017
Rhodopirellula rubra LF2T	Carotene	Kallscheuer et al., 2019
Rubinisphaera brasiliensis Gr7	Carotene	Kallscheuer et al., 2019
Natrialba sp.,	Carotene	Hegazy et al., 2020
Brevundimonas sp.,	Carotene	Asker, 2017

fibroblast L-929 cells and brine shrimp compared to the control at 30% but levels higher than 200 pmm of melanin didn't show much efficiency or any cytotoxic effects up to 500 ppm due to the transfer of extra energy to oxygen species producing Reactive oxygen species (**Figure 1**). Melanin extracted from bacteria has also been reported in heavy metal treatment, prevent fouling and formation of biofilm. Manirethan et al. (2018, 2020) used the melanin pigment extracted from gram-negative bacterium *P. stutzeri* for the absorption of heavy metals like copper, mercury, chromium, lead, and Arsenic where the absorption may be due to the presence of the COOH, NH, phenolic OH groups where maximum efficiency was absorbed in a pH ranging from mildly acidic to neutral. As a twist Melanin impregnated with copper and iron on its surface proved to be efficient in removing arsenic by chemisorption and it was also noted that melanin exhibited thermostability up to 120°C and showed peak absorption of arsenic at pH of 4–6 where XPS studies showed that arsenite before binding to Cu-, Fe-Impregnated melanin oxidized to arsenate and finally removing the Cu and Fe by Hcl treatment for the recycling of melanin and again impregnating it with fresh Cu and Fe element for absorption which showed 99% efficiency to 4 absorption and desorption cycles. *P. stutzeri* BTCZ10 strain was reported to play a photoprotective role by displaying SPF value equivalent to sunscreen showed by Kurian and Bhat (2018). Zeng et al. (2017) reported the hyperpigmentation of pyomelanin which is a complex of polyphenolic heteropolymer produced under elevated temperature by marine hmgA mutant *P. lipolytica* displaying anti-fouling activity. Kiran et al. (2017) confirmed the anti-microbial activity against *S. aureus, B. subtilis, E. coli,* and *P. aeruginosa* displayed strong anti-biofilm activity against MDSA by nano-melanin synthesized by sonication of *Pseudomonas* sp., isolated from marine sponge *T. citrine* where the efficiency of nano-melanin was observed to be higher than the traditional melanin.

PRODIGIOSIN

Prodigiosin is blood-red colored compounds that display strong anti-microbial, cytotoxic, and immune suppressive activities by a range of mechanisms like decoupling H/Cl- transporters modulating the pH of the cell and cleaves the DNA in the presence of copper (Huryn and Wipf, 2008). Prodigiosin biosynthesis undergoes a bifurcation pathway consisting of two systems a unique maltose-binding protein (MBP) and a common monolayer-protected cluster (MPC) biosynthesis. The 2-methyl-3-n-amyl-pyrrole (MAP) biosynthesis utilizes three genes pigB, pigD and pigE whereas the 4-methoxy-2,2′-bipyrrole-5-carbaldehyde (MBC) biosynthesis uses seven genes pigA, pigFpigG, pigH, pigI, pigJ, pigL, and pigM and the products MAP and MBP are condensed together to form prodigiosin (Sakai-Kawada et al., 2019). Ramesh et al. (2020) isolated 17 different strains from south Andaman which produce pigments ranging from brown, yellow, red, and orange (**Figure 1**) where strain *Zooshikella* sp S2.1 and *Streptomyces* sp., BSE6.1 producing red pigment were confirmed to be prodigiosin was selected for further application in the use as food colorants and displayed effective antibacterial activity against *S. aureus* at concentrations of 150 to 400 μg/mL. Marine *V. ruber* DSM 14379 isolated by Danevčič et al. (2016) exhibited the production of prodigiosin which showed strong bactericidal activity during the exponential phase of *B. subtilis* and bacteriostatic activity during stationary growth where it interferes with the cytoplasmic membrane and it also induces autolysin activity enhancing the killing of *B. subtilis*; however it was observed that the decrease of autolysin resulted in the abolished activity of prodigiosin. Abdelfattah et al. (2019), testified the antioxidant and anti-inflammatory activity of prodigiosin extracted from *Actinomyces* sp., isolated from sponge *S. mastoidea* against HCl/ethanol gastric lesion by over-expressing HO-1 resulting in elevated mucous production antioxidant activity, increasing HSP activity, apoptosis inhibition, and stabilizing cellular membrane thus preventing gastric injury. Prodigiosin has also been reported as an algicidal agent. Zhang et al. (2016) confirmed the algicidal activity of *P. globosa* by prodigiosin from *Hahella* sp., KA22 where prodigiosin inhibits photosynthesis by increasing ROS activity and finally necrotizes due to oxidative damage of the cells.

VIOLACEIN AND QUINONE

Violacein an indole derivative is a purple-colored pigment exhibiting anti-tumor, anti-microbial properties mainly produced by *C. violaceum* and *J. lividum* (Masuelli et al., 2016). The biosynthesis of violacein starts from the oxidation of the precursor molecule tryptophan into indole-3-pyruvic acid by the flavoenzyme VioA, where VioB couples the two IPA imine molecule forming the intermediate Imine dimer and in the presence of Violacein biosynthesis protein VioE the dimer is converted to protodeoxyviolaceinic acid where VioD hydroxylates forming proto violacein ic acid and VioC hydroxylates and undergoes oxidative decarboxylation producing violacein (Durán et al., 2007; Füller et al., 2016). Wu et al. (2017) described a novel-violacein producing organism and characterized it which was isolated from the surface of seawater of the Arabian sea and by 16S typing, the strains JW1T and JW3 were closely related to *Pseudoalteromonas* sp., and displayed independent lineage where there seem to be a display phylogenetic and chemotaxonomic differences, phenotypic properties and hence the name *P. amylolytica* was proposed. Currently, *C. violaceum* is being used as a bioassay strain for quorum sensing and quorum quenching activity for violaceum production to understand the QS or QQ activity of the desired strain (Liu et al., 2018; Balakrishnan et al., 2020; Singh et al., 2020). Quinone displays the anti-viral, anti-cancer, anti-microbial and insecticidal activities where the color ranges from yellow to red (Soliev et al., 2011). Liang et al. (2016) reports the suppression of glioma cell lines by two polycyclic quinones N-acetyl-N-demethyl rapamycin and strepto anthraquinone A at IC50 extracted from marine *Streptomyces* sp., 182SMLY which induced apoptosis in glioma cells. N-acetyl-N-demethylmayamycin was shown to be highly effective against MRSA. Quinones are also reported to display anti-fungal activities. Jiang et al. (2018) extracted medermycin-type naphthoquinones -strepoxepinmycin A to D and medermycin from *Streptomyces* sp., XMA39 where C and D showed cytotoxicity against HCT-116 and PC-3 cell lines and moderate inhibition of ROCK2 kinase was also observed. Collectively all the five compounds exhibited antagonistic activity against *E. coli, S. aureus*, and *Candida* sp. (**Figure 1**).

FUTURE PERSPECTIVE

Recently, a number of review papers have appeared in the literature, and they give an outline of all findings of the marine environment and its isolates. However, in this mini-review, we focus exclusively on the active marine bacterial pigmented compounds and its potential applications have been stated. The secondary metabolites of marine bacteria are gaining importance for its remarkable potential as anti-cancer, anti-microbial, anti-parasitic, insecticidal, anti-fowling, and anti-biofilm properties. The continuous evolution of the resistance observed in pathogenic microbiota poses a significant threat corresponding to the time taken for the discovery of new drugs. The gap of altering the present product into a synthetic drug could be bridged by utilizing the full potential of the microorganism in the marine environment as the marine organisms for its survival produces secondary metabolites for competing with organisms in the community and when extracted it is of great potential. Due to the growth of high-throughput sequencing, metagenome, and the rapidly growing field of metabolomics study enables us to cultivate bacteria that are usually viable but non-cultivable. Despite the enormous difficulty in isolating and harvesting marine bacteria, significant progress has been achieved in this field, and investigations of bioactive compounds produced by these species are rapidly increasing. It has given us the set of tools to view from a distinct perspective helping us to understand the synergy that takes place in the marine ecosystem. Microbial pigments are of great interest due to its long fetching application from industry to pharmaceutical companies. Further large-scale studies are required to understand the interaction and evolution of these microorganisms that enable them to produce these pigments. Bacteria as pigment production units are of great interest due to its rapid production (or) fermentation of pigments. From the time consuming traditional synthesis of pigments bacterial pigment production could be achieved in a matter of minutes to hours by manipulating the cell-free condition, growth, and nutrients in the growth medium. This enables us to elect marine bacteria as a superior source factory for bioactive pigment production.

AUTHOR CONTRIBUTIONS

PV and CV: conceptualization, original draft preparation, and writing. LD: writing, review, and editing. AV: review. All authors contributed to the article and approved the submitted version.

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frontiers in Sustainable Food Systems

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Characterization of Prodiginine Pathway in Marine Sponge-Associated *Pseudoalteromonas* sp. PPB1 in Hilo, Hawai'i

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Interest in bioactive pigments stems from their ecological role in adaptation, as well as their applications in various consumer products. The production of these bioactive pigments can be from a variety of biological sources, including simple microorganisms that may or may not be associated with a host. This study is particularly interested in the marine sponges, which have been known to harbor microorganisms that produce secondary metabolites like bioactive pigments. In this study, marine sponge tissue samples were collected from Puhi Bay off the Eastern shore of Hilo, Hawai'i and subsequently were identified as *Petrosia* sp. with red pigmentation. Using surface sterilization and aseptic plating of sponge tissue samples, sponge-associated microorganisms were isolated. One isolate (PPB1) produced a colony with red pigmentation like that of *Petrosia* sp., suggesting an integral relationship between this particular isolate and the sponge of interest. 16S characterization and sequencing of PPB1 revealed that it belonged to the *Pseudoalteromonas* genus. Using various biological assays, both antimicrobial and antioxidant bioactivity was shown in *Pseudoalteromonas* sp. PPB1 crude extract. To further investigate the genetics of pigment production, a draft genome of PPB1 was sequenced, assembled, and annotated. This revealed a prodiginine biosynthetic pathway and the first cited-incidence of a prodiginine-producing *Pseudoalteromonas* species isolated from a marine sponge host. Further understanding into the bioactivity and biosynthesis of secondary metabolites like pigmented prodiginine may uncover the complex ecological interactions between host sponge and microorganism.

Keywords: *pseudoalteromonas*, marine sponge, prodiginine, prodigiosin, pigments, marine bacteria

INTRODUCTION

Interest in bioactive pigment production and isolation has been on the rise due to their versatile uses in cosmetics, food supplements, pharmaceuticals, and textile dyes (Tuli et al., 2015; Hernández-Almanza et al., 2017; Ramesh et al., 2019b). Focus has shifted from synthetic pigments to natural pigments for industrial application because of their sustainability and high yield in production, non-toxic nature, and low-impact on the environment (Ramesh et al., 2019a,b). These natural pigments are derived from various biological sources including invertebrates (e.g., carotenoids, indigotins), plants (e.g., carotenoids, anthocyanins), microorganisms (e.g., prodiginines, violacein), and contribute key ecological roles such as defense against environmental adaptations and protection against predation (Bandaranayake, 2006; Venil et al., 2014; Leong et al., 2018; Tan et al., 2020).

Marine sponges are an essential component of coral reefs, providing structural support and biodiversity as well as contributing to nutrient cycling (Diaz and Rützler, 2001). Sponges have been known to harbor microorganisms that produce a variety of biologically active compounds, including bioactive pigments (Bowman, 2007; Webster and Taylor, 2012). Although sponges lack an overall muscular and nervous system, they are still able to sense and respond to changes within their environment. This may be due to their siliceous spicule system, which has been hypothesized to work as a potential networking system in place of the nervous system through the use of cryptochrome-based photoreception (Perović et al., 1999). Current spongology classifies sponges based on one of three types of spicule composition: (i) calcium carbonate (Calcarea), (ii) glass, siliceous (Hexactinellida), or (iii) spongin (Demospongiae) (Pallela et al., 2011).

With the growing interest in preserving coral reefs, more studies have been performed to investigate the sponge's role within the reef ecosystem. Sponges have been found to not only contribute to the biodiversity and biomass but also provide sources for nutrient and silicon cycling (Diaz and Rützler, 2001; Maldonado et al., 2005). In addition, they offer shelter for invertebrates and are often a link between benthic and pelagic communities.

Marine sponges are greatly impacted by climate change and the new environmental stressors imposed on them, two of which include ocean warming and acidification. Thermal stress in particular can decrease efficacy of defense mechanisms, leading to increased mortality due to pathogens and diseases. In addition, ocean warming can lead to species invasion, shifts in species' latitudinal ranges, and bleaching (Carballo and Bell, 2017).

Bleaching causes deterioration of inner sponge tissue and leads to eminent sponge mortality (Angermeier et al., 2011). Major causes for bleaching include an influx of visible and ultraviolet radiation, freshwater dilution, sedimentation, and introduction of pollutants (Webster, 2007). Aside from the many abiotic factors, the sponge must also deter predators, such as fish (Dunlap and Pawlik, 1996), molluscs (Pawlik et al., 1988), echinoderms (Waddell and Pawlik, 2000), and urchins (Ayling, 1981), as well as compete for space in a constant battle of arms against other marine organisms within the reef ecosystem (Alino et al., 1992; Aerts, 2000). Due to the sponge's simplistic anatomy, it is limited in its capability to protect itself from many of these environmental constraints (Bramhachari et al., 2016).

Sponges have not developed specialized tissues due to its simplistic evolutionary structure, which limits its capability to form a sophisticated immune system. Current studies have shown a spotlight on the sponge innate immunity, demonstrating its use of toll-like receptors or nucleotide-binding domain and leucine-rich repeats for recognition of foreign materials (Wiens et al., 2005, 2007; Yuen et al., 2013). However, there has been no indication of adaptive immunity, suggesting an alternate mechanism of protection against infections.

In recent years, emphasis has been placed on the sponge microbiome and the crucial role it has in host defense (Hentschel et al., 2002; Goldberg, 2013). However, the question has been how these sponges select and obtain many of their symbionts. Three models have been proposed in microbial acquisition: (1) vertical transmission, (2) horizontal transmission, and (3) leaky vertical transmission or mixed mode of transmission, which utilizes a combination of vertical and horizontal transmission (Schmitt et al., 2008; de Oliveira et al., 2020). Vertical transmission describes the microbial acquisition primarily from parent to progeny, horizontal transmission acquires their microbiome from the environment, whereas leaky vertical transmission acquires their microbial community from both their parent and environment.

The associated microorganisms compete for space and resources within the sponge tissue while providing their host the tools necessary to protect itself from disease, predation, and UV irradiation. The bacterial genus, *Pseudoalteromonas* has been associated with harboring within various hosts as well as with producing numerous bioactive pigments (Bowman, 2007; Ramesh et al., 2019b; Sakai-Kawada et al., 2019). One example includes *Pseudoalteromonas luteoviolacea* which was isolated from a marine sponge, *Iotrochota protea,* and was shown to produce the pigment, violacein (Sakai-Kawada et al., 2016). Violacein has demonstrated both antibiotic and antiprotozoal activities that could benefit both itself and its host (Soliev et al., 2011).

This study focused on a marine sponge that was collected in Puhi Bay, Hilo, Hawai'i on June 5, 2013 from a depth of 3 m (**Figure 1**). The marine sponge tissue expressed a red-purple hue and was characterized as belonging to the genus, *Petrosia* sp. (**Figure 2**). Various microbial colonies were isolated from the sponge tissue after surface sterilization. One isolate (PBB1) expressed a red pigment under certain conditions. Through 16S rRNA sequencing and characterization this isolate clustered closely with the genus *Pseudoalteromonas*.

The aims of this study were (i) to assay the antibacterial and antioxidant biological activity of PPB1 and (ii) to characterize various biosynthetic gene clusters involved in secondary metabolite and pigment production.

MATERIALS AND METHODS

Bacterial Isolation and Culture Conditions

All media, glassware, and other reagents were sterilized before use. Approximately 5 g of sponge tissue was surface sterilized

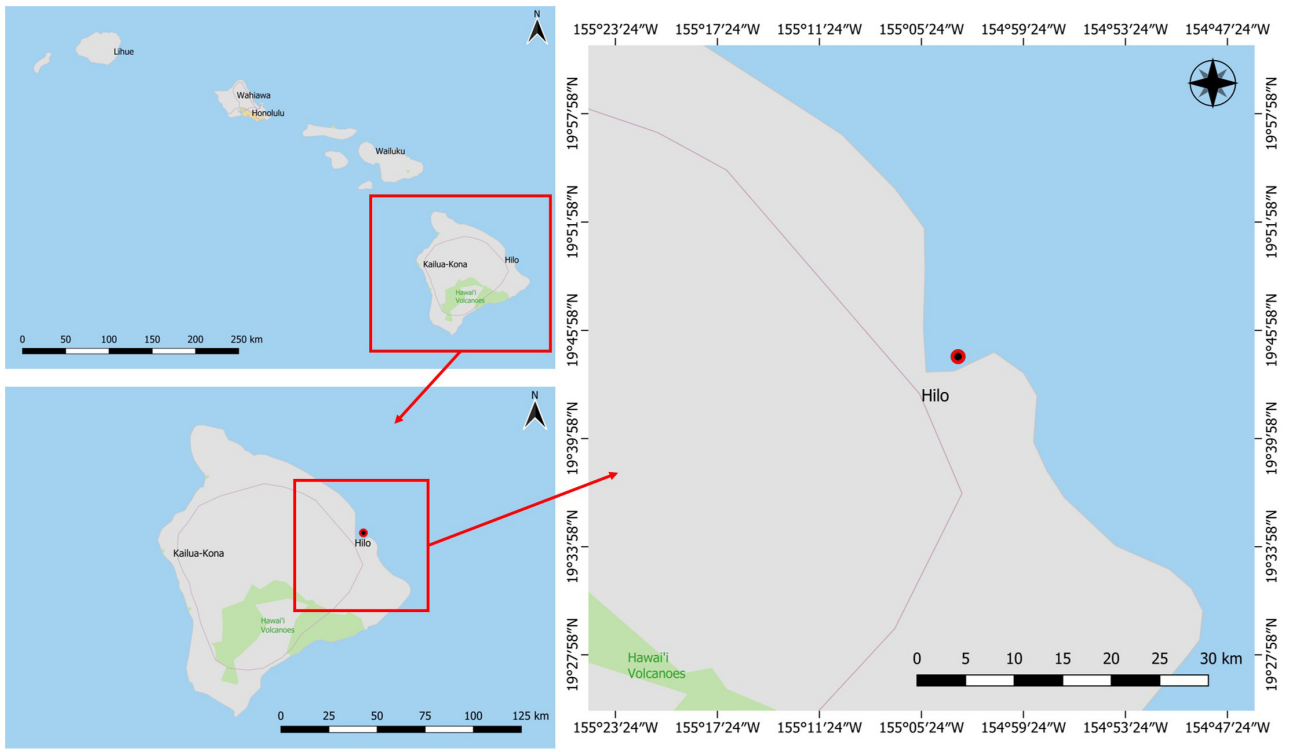

FIGURE 1 | Marine sponge (*Petrosia*) collection site in Puhi Bay on Hawai'i Island.

with 70% ethanol (EtOH) and air-dried. Each 5-g sample was sliced to ∼1 cm in thickness and set on marine agar (MA) (Difco) to incubate for 7 days at ambient lab temperature (22–25°C). In addition, ∼5 g of sponge tissue was surface sterilized with 70% EtOH, air-dried, and the sponge surface was spread on MA plates as a negative control for outside bacteria growth. Sponges were observed for different microbial growth—based on color, colony morphology, and other distinguishing characteristics.

Bacterial strains used in this study are described in **Table 1**. Unless stated otherwise, *Pseudoalteromonas* sp. PPB1 was grown and maintained on agar or in liquid marine media containing 0.5% peptone in prepared artificial seawater. All *B. cereus, E. coli,* and *S. aureus* strains were grown and maintained on LB agar or LB broth (1% peptone, 0.5% yeast extract, 0.5% NaCl).

Electron Microscopy

PPB1 cells were grown on MA and resuspended in sterile autoclaved ddH_2O, centrifuged, and decanted with ddH_2O 3 times. Cells were fixed with 4% glutaraldehyde in 0.1 mol/L sodium cacodylate with 0.35 mol/L sucrose, pH 7.6, for 1 h. The cell mixture was loaded onto a 0.2 μm filter and the liquid was filtered through. Cells were washed in 0.1 mol/L sodium cacodylate with 0.44 mol/L sucrose, two times for 20 min. Cells were then dehydrated in a graded EtOH series of 30, 50, 70, 85, and 95%, two times for 3 min at each dilution. Cells were dried with 100% EtOH for 10 min, three times. Cells were dried to a critical point with hexamethyldisilazane. The filter containing the prepared cells was mounted on conductive carbon tape that was mounted on an aluminum disc. The silver polish was dotted from the edge of the disk to the edge of the filter. The cells were gold sputter-coated for 60 s in a vacuum chamber. Cells were visualized using Hitachi S-3400N Variable Pressure Scanning Electron Microscope.

TA Cloning and Determination of 16S rRNA Gene Sequence

Genomic DNA was isolated using the UltraClean microbial DNA isolation kit (Mo Bio) according to the provided protocol. Polymerase chain reaction (PCR) amplification of the 16S rRNA was performed using primers 27F and 1492R (**Table 2**) (Kennedy et al., 2009). The PCR was carried out under the following conditions: initialization at 94°C for 15 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and elongation at 72°C for 2 min, followed by a final extension at 72°C for 10 min and hold at 10°C for 11 min.

The PCR product was run on a 1.5% agarose gel run at 90 V for ∼75 min to confirm the presence of the gene product. The PCR product was cleaned up using the Wizard SV gel and PCR clean-up system according to the provided protocol. The PCR product was then ligated into a pGEM-T easy vector and incubated overnight at 4°C.

TOP10 cells were incubated in LB broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.5) at 37°C and shaken

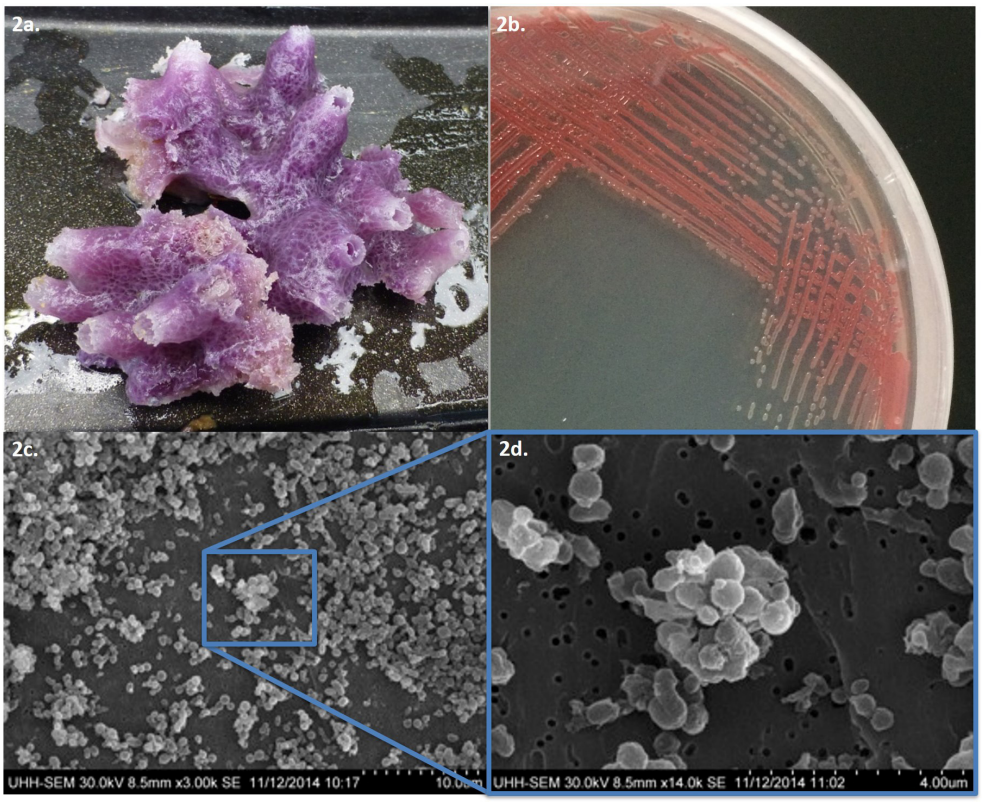

FIGURE 2 | (a) Tissue sample of *Petrosia* species and **(b)** pigmented microbial isolate PPB1. **(c)** Scanning electron microscope images of *Pseudoalteromonas* PPB1 field of bacteria and **(d)** magnified cluster of bacteria.

at 16.8 rad/s overnight. Hundred μL of the overnight grown TOP10 cells were inoculated in 10 mL of fresh LB broth and incubated at 37°C in a shaker at 16.8 rad/s for ∼3 h—until the OD_{600} = 0.5. The cells were placed on ice for 15 min and spun at 523.5 rad/s for 10 min at 4°C. The supernatant was discarded, and the cells were resuspended in an equal volume of 0.1 mol/L $CaCl_2$ at 4°C by gently shaking. The cells were spun again at 523.5 rad/s for 10 min and resuspended in one-tenth the volume with 0.1 mol/L $CaCl_2$ at 4°C by gently shaking. Two μL ligation reaction was added to 50 μL of competent cells and mixed gently. The cells were placed on ice for 20 min and submerged in a water bath at 42°C for 50 s. The cells were immediately returned to the ice for 2 min. Five-hundred μL of LB broth was added and incubated for 1 h at 37°C and shaken at 16.8 rad/s.

Transformants were selected using blue-white screening on LB agar supplemented with 15 mg/L ampicillin and 0.06% v/v Chromomax overnight at 37°C (Green and Sambrook, 2019). White colonies were selected and incubated overnight at 37°C at 16.8 rad/s in LB broth supplemented with 15 mg/L ampicillin. The plasmid was isolated from the transformant cells using the Promega PureYield plasmid miniprep system according to the provided protocol. The plasmid was digested using EcoRI and ran on a 1.5% agarose gel to confirm the presence of the ligated gene product.

Non-ribosomal Peptide Synthase (NRPS) and Polyketide Synthase (PKS) Screening

PPB1 was screened for NRPS, PKS I, PKS II genes using primers A3F, A7R, K1F, M6R, KS_{α}, and KS_{β} (**Table 2**). gDNA was isolated using the UltraClean microbial DNA isolation kit (Mo Bio) according to the provided protocol and quantified using NanoDrop ND-1000 spectrophotometer. PCR was set to the following parameters for touchdown: initialization at 95°C for 5 min, 10 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s with 2° decrease each cycle, elongation at 72°C for 90 s, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 40°C for 30 s, final extension at 72°C for 7 min, and held at 4°C (Kennedy et al., 2008). PCR products were verified on a 1.5% agarose gel run at 80 V for ∼30–60 min and using Promega 100 bp ladder. *P. aeruginosa* was used as a positive control. *E. coli* and nuclease-free water served as negative controls.

Pigment Extraction

PPB1 liquid cultures were centrifuged at 523.5 rad/s for 10 min and the supernatant was discarded. The pelleted cells underwent EtOH extraction (Patil et al., 2011). The EtOH extract was then syringe-filtered (0.2 μm pore size), dried, and stored until further application.

Disc Diffusion Assay

Twenty-five μL of the following solutions were spotted onto sterile 6 mm diameter disks (Whatman) that were six filters thick: 10,000 μg/μL PPB1 extract in dimethylsulfoxide (DMSO), DMSO, 400 μg/μL ampicillin in DMSO, and 400 μg/μL kanamycin in DMSO. The DMSO disc served as a negative control. The ampicillin and kanamycin disks served as positive controls.

B. cereus ATCC 14579, *E. coli* K12, and *S. aureus* ATCC 25923 were grown in 10 mL LB broth, while *P. arabiensis*, *P. denitrificans*, *P. rosenbergii*, and *V. harveyi* were grown in 10 mL marine broth (Difco). All cultures were grown at 25 °C for ∼24 h, shaking at 12.5 RPM. The final concentration of cells was $\sim 10^6$ CFU/mL. Hundred μL lawn of each culture was spread on Mueller-Hinton agar—for *B. cereus*, *E. coli*, and *S. aureus*—and MA—for *P. arabiensis*, *P. denitrificans*, *P. rosenbergii*, and *V. harveyi*. The plates were left to air dry in a laminar flow cabinet. Disks were aseptically placed onto the surface of the inoculated agar plate and incubated for 20 h. at 37 °C (Kennedy et al., 2009). Zones of inhibition were measured and recorded.

TABLE 1 | Bacterial strains used in this antibacterial assay and TA cloning.

Strain	Characteristics	References
PPB1	Wild-type strain *Pseudoalteromonas* sp. isolated from *Petrosia* sp.	This study
ATCC 14579	Non-virulent strain *Bacillus cereus*	Hayrapetyan et al., 2015
ATCC 25923	Non-virulent strain *Staphylococcus aureus*	Treangen et al., 2014
K12	Non-virulent strain *Escherichia coli*	Bachmann, 1972
PB 4-1 (B1)	Wild-type strain *Pseudovibrio denitrificans* isolated from *Petrosia* sp.	This study
PB 4-1 (B2)	Wild-type strain *Photobacterium rosenbergii* isolated from *Petrosia* sp.	This study
PB 4-2 (B1)	Wild-type strain *Vibrio harveyi* isolated from *Petrosia* sp.	This study
PB 4-2 (C2)	Wild-type strain *Pseudoalteromonas arabiensis* isolated from *Petrosia* sp.	This study
TOP10	Chemically competent *Escherichia coli* F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Chan et al., 2013

Flow Cytometry

S. aureus was inoculated in 10 mL nutrient broth (NB) at 37 °C for ∼12 h with shaking (16.8 rad/s). After 12 h, triplicates of each 100 μL treatment were placed in 5 mL NB inoculated with 50 μL *S. aureus*. The negative controls were the untreated and DMSO treatment. The positive controls were the antibiotic treatments: 10 mg/mL ampicillin in DMSO (amp10) and 30 mg/mL chloramphenicol in DMSO (cam30). The sample treatments were 1,000 mg/kg PPB1 extract in DMSO, 750 mg/kg PPB1 extract in DMSO, and 500 mg/kg PPB1 extract in DMSO. The treatments in *S. aureus* were incubated at 37 °C for ∼12 h with shaking (160 RPM).

After 12 h, 1 mL of each treatment was incubated with 0.5 μL of 5 mmol/L SYTO BC green-fluorescent nucleic acid stain (Invitrogen) and 0.5 μL of 1.5 mmol/L propidium iodide (PI) for 20 min at room temperature.

In a 96-well plate, 8 μL of each treatment and 72 μL dH_2O was aliquoted into each well, making a total of 80 μL. Each set of triplicates was separated by a well of dH_2O to clean the flow cytometer in between samples.

TABLE 2 | Primer sequences.

Primer	Sequence	Gene of Interest	References
27F	5′-AGAGTTTGATCMTGGCTCAG-3′	16S rRNA	Lane, 1991
1492R	5′-TACGGYTACCTTGTTACGACTT-3′	16S rRNA	Lane, 1991
A3F	5′-GCSTACSYSATSTACACSTCSGG-3′	NRPS Adenylation domain	Ayuso-Sacido and Genilloud, 2005
A7R	5′-SASGTCVCCSGTSCGGTAS-3′	NRPS Adenylation domain	Ayuso-Sacido and Genilloud, 2005
K1F	5′-TSAAGTCSAACATCGGBCA-3′	PKS-1 Ketosynthase—acyltransferase domains	Ayuso-Sacido and Genilloud, 2005
M6R	5′-CGCAGGTTSCSGTACCAGTA-3′	PKS-1 Ketosynthase—acyltransferase domains	Ayuso-Sacido and Genilloud, 2005
KS_α	5′-TSGCSTGCTTGGAYGCSATC-3′	PKS-2 Ketosynthase domain	Metsä-Ketel et al., 1999
KS_β	5′-TGG AANCCG CCGAABCCTCT-3′	PKS-2 Ketosynthase domain	Metsä-Ketel et al., 1999

Flow cytometry was performed with a C6 Flow Cytometer (BD Accuri). The green fluorescence of the SYTO BC dye (FL1) was collected using a 533 nm ± 30 nm optical filter; the red fluorescence of the PI dye (FL3) was collected using a 585 nm ± 40 nm optical filter. SYTO BC (maximum at 500 nm) is a nucleic acid stain for both gram-positive and gram-negative bacteria. PI (maximum at 617 nm) uptake indicates ruptured cell membranes and cell death (Gunasekera et al., 2000; Ben-Amor et al., 2005).

Using the 96 deep well plate template, the flow rate was set at 14 μL/min, the threshold was set at FSC-H < 10, and run limits were set at 15 μL for sample wells and 10 μL for dH_2O wells. During the run, there was one wash cycle per well and the plate was agitated for two cycles per well.

Sorting criteria were defined by drawing gates in a bivariate dot plot of FL3-H (PI red fluorescence height) vs. FL1-H (SYTO BC green fluorescence height). All parameters were measured using logarithmic amplification.

The total count and the number of counts in each quadrant for all samples were recorded. The adjusted total count was calculated by taking the difference between the total count and the count in the lower-left quadrant (Ben-Amor et al., 2005). The percentage of viable (upper-left quadrant), injured (upper-right quadrant), and dead (lower-right quadrant) cells was calculated with the adjusted total count. A triplicate average for the adjusted total count and the percentage of viable, injured, and dead cells per treatment. Statistical analysis of the viable, injured, and dead percentages before averaging were done with a one-way ANOVA and with Tukey's multiple comparisons.

Ferric Reducing Antioxidant Power (FRAP) Assay

The components of the FRAP reagent were 10:1:1 with 15 mL of 300 mmol/L acetate buffer (pH 3.6), 1.5 mL of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution, and 1.5 mL of 20 mmol/L $FeCl_3 \cdot 6H_2O$ (stored at 4°C). Before use, the FRAP reagent was heated to 37°C for 30 min. In a 96-well plate, 150 μL of working FRAP reagent was placed into wells and 150 μL of dH_2O were placed into separate wells and a blank reading at 595 nm was taken. Twenty μL of samples, positive control (ascorbic acid), negative control (EtOH), and standard (1,000 μmol/L $FeSO_4 \cdot 7H_2O$) were added to the FRAP reagent wells in triplicates. Twenty μL of only the samples were added to the dH_2O wells in triplicates. After 8 min, a second reading at 595 nm was taken. FRAP values for each sample were determined by taking the difference of the final reading with their respective blank reading of the FRAP reagent and their respective averaged pigment reading. A two-sample *T*-test was performed between the PPB1 crude extract-treated and the untreated samples (Moon and Shibamoto, 2009).

Genomic DNA Isolation, Library Preparation, and Genome Sequencing

gDNA was isolated using the UltraClean microbial DNA isolation kit (Mo Bio) according to the provided protocol. Genome library was prepared using the New England Biolabs Fast DNA fragmentation and library prep set for Ion Torrent, selected to a target length of 480 bp. The Kapa Biosystems Ion Torrent library quantification kit and Agilent high sensitivity DNA kit were used to determine the library dilution factor and assess the library size distribution, respectively. Emulsion PCR was performed using the Ion PGM Hi-Q OT2 kit−400 on the Ion OneTouch 2 System. The percent templated Ion Sphere particles from unenriched samples was measured with the Ion Sphere quality control kit on the Qubit Fluorometer 3.0. Template enrichment was performed on the Ion OneTouch Enrichment System. The sample was loaded into an Ion 318 chip v2 and sequenced using the Ion PGM Hi-Q sequencing kit on the Ion PGM system (Sakai-Kawada et al., 2016).

Genome Assembly and Annotation

The data quality for the high throughput raw sequences was assessed using FASTQC. A De Bruijn graph assembly was performed using SPAdes assembler v. 3.5.0. The assembly quality was assessed using MUMmer, ABySS, and FRC. Genome annotation was performed using MAKER, PGAP, RAST, RNAmmer, and CRISPRfinder (Grissa et al., 2007; Lagesen et al., 2007; Aziz et al., 2008; Overbeek et al., 2014). Secondary metabolite gene clusters were characterized using antiSMASH—a bioinformatic tool for rapid genome-wide analysis of antibiotics and secondary metabolite biosynthesis, BAGEL4—a bioinformatic tool that mines for bacteriocins and bacterial RiPPs, NaPDoS, NP.searcher, and PRISM. The latter three are bioinformatic tools that rapidly detect NRPS and PKS domains (Altschul et al., 1990; Steinbeck et al., 2003; Li et al., 2009; Finn et al., 2011; Medema et al., 2011; Prlić et al., 2012; Ziemert et al., 2012; Blin et al., 2019).

RESULTS

Physical Characterization and Identification of Pigment-Producing Bacteria

Isolate PPB1 displayed a bright to deep red color on agar plates and within broth (**Figure 2**). Growth on agar began unpigmented and then red pigmentation developed as the colony grew over time. The bacterium grew within temperatures ranging from 18 to 27°C. Electron microscopy revealed coccoid and short rods forms of PPB1 (**Figure 2**). 16S rRNA characterization revealed the closest neighbor as *Pseudoalteromonas rubra* strain SCSIO 6842 (Accession: CP013611.1) at 93% identity (**Figure 3**).

NRPS and PKS Screening and Antimicrobial Activity

NRPS and PKS screening showed that PPB1 was positive for both NRPS and PKS genes. Upon initial screening for an antimicrobial activity via disc diffusion assay, PPB1 crude extract showed zones of inhibition against Gram-positive bacteria, *B. cereus* and *S. aureus* at 4.0 mm ± 0.7 mm and 5.4 mm ± 2.1 mm diameters, respectively. PPB1 crude extract did not show antimicrobial activity against Gram-negative bacterium, *E. coli* (0.0 mm) (**Table 3**). PPB1 also inhibited the growth of other marine bacteria from the *Petrosia* sponge tissue and included

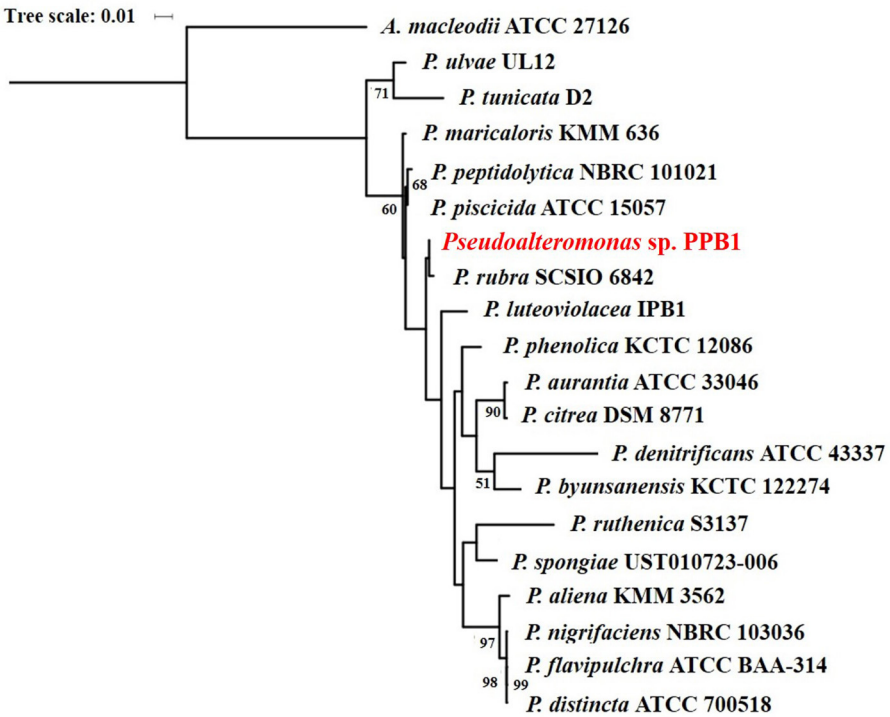

FIGURE 3 | Maximum likelihood phylogenetic tree of pigmented *Pseudoalteromonas* species based on 16S rRNA gene sequences.

TABLE 3 | Disc diffusion assay of PPB1 extract.

Test organism	Growth inhibition zone activity			
	PPB1 extract (250 mg)	Ampicillin (10 mg)	Kanamycin (10 mg)	DMSO
B. cereus[a]	+ (4.0 mm ± 0.7 mm)	+	+	-
E. coli[a]	− (0.0 mm)	+	+	-
S. aureus[a]	+ (5.4 mm ± 2.1 mm)	+	+	-
P. rosenbergii[b]	+ (1.1 mm ± 0.5 mm)	+	+	-
P. arabiensis[b]	+ (1.0 mm)	+	+	-
P. denitrificans[b]	variable (1.3 mm ± 1.1 mm)	-	+	-
V. harveyi[b]	variable (0.8 mm ± 0.4 mm)	-	+	-

[a]*Assay performed on Mueller-Hinton media.*
[b]*Assay performed on marine agar (Difco).*

P. rosenbergii (1.1 mm ± 0.5 mm) and *P. arabiensis* (1.0 mm). On the other hand, PPB1 demonstrated variable antimicrobial activity against *P. denitrificans* (1.3 mm ± 1.1 mm) and *V. harveyi* (0.8 mm ± 0.4 mm) (**Table 3**).

Quantitative antimicrobial assays were conducted via flow cytometry to further investigate the effects of the crude extract on *S. aureus* (**Figure 4A**). The viability of *S. aureus* cells decreased from 67.8% to 25.2% when the concentration of the crude extract was increased from 0 mg/kg to 500 mg/kg (**Table 4**). However, there was no significant difference in response to crude extract treatments among viable (25.2–28.8%), injured (69.4–72.7%), and dead cells (1.4–2.1%).

Antioxidant Activity

The FRAP assay analyzed the antioxidant potential of a test sample by measuring how readily it reduces ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). As a result, the ferrous ions formed a complex with the $TPTZ_2$ working reagent and produced blue coloration, which were measured and quantified. The PPB1 crude extract showed significant ferric reducing antioxidant potential (1.395 ± 0.123; $p \leq 0.05$) in comparison to the EtOH negative control (0.004 ± 0.001) (**Table 5**). This response was more than the 1 mmol/L $FeSO_4 \bullet 7H_2O$ standard (1.052 ± 0.056), which had iron present in the already reduced (Fe^{2+}) form, but less than ascorbic acid control (1.814 ± 0.040) (**Figure 4B**).

Genome Sequencing and Assembly

Upon sequencing the genome, a total of 8,297,550 raw reads were produced. After accounting for enrichment, clonal amplification error, test fragments, adapter dimers, and other quality assessments, 68% of those reads remained usable, yielding a total of 5,632,815 reads with an average read length of 307 bp and a median read length of 345 bp, resulting in 1.73 Gb of sequenced data.

SPAdes assembled a genome containing 121 contigs with a total size of 5,915,516 bp (283-fold draft coverage), an N_{50} contig length of 192,396 nucleotides, and a mean GC-content of 47.4% (**Table 6**).

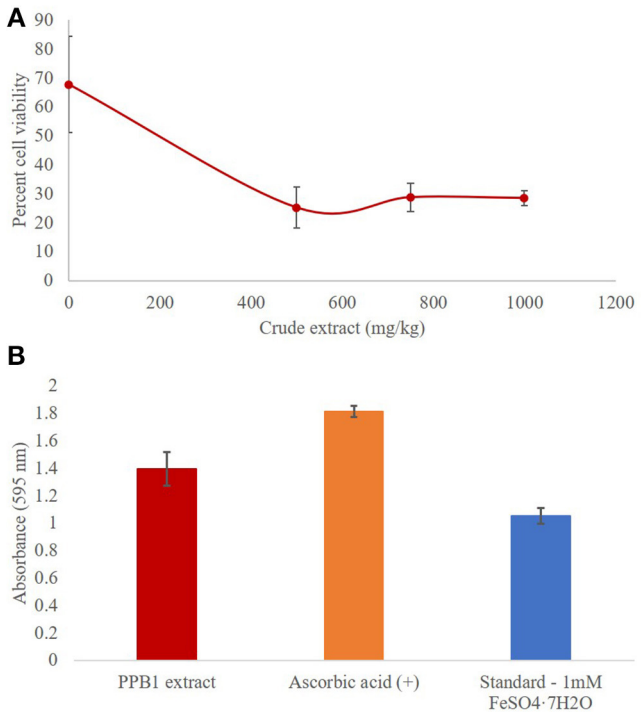

FIGURE 4 | (A) Antimicrobial activity of PPB1 crude extract against *S. aureus*. PPB1 crude extract demonstrated a significant decrease in cell viability at 500 mg/kg (25.2 ± 2.6), 750 mg/kg (28.8 ± 4.8), and 1,000 mg/kg (28.5 ± 7.1). Error bars represent the standard deviation of the samples. **(B)** Antioxidant activity of PPB1 crude extract via FRAP assay. The PPB1 crude extract demonstrated significant ferric-reducing potential (1.395 ± 0.123; $p \leq 0.05$) in comparison to the EtOH negative control (0.004 ± 0.001). Error bars represent the standard deviation of the samples.

Genome Mining and Annotation

RAST predicted 5,201 gene-coding sequences with 2,082 features within the RAST subsystem. Of those subsystem features, 15 were characterized as bacteriocin and ribosomally-synthesized antibacterial peptides, and 16 were characterized as secondary metabolism (**Table 6**).

Upon analysis of secondary metabolism and natural product biosynthesis, several gene clusters and domains of interest were found. Analysis using antiSMASH resulted in 29 hits (Blin et al., 2019). NaPDoS returned 80 hits. Of those, 12 were KS-domains and 68 were C-domains. NP.searcher returned 11 hits, seven modular NRPSs, and three mixed modular NRPS/PKSs. PRISM, on the other hand, returned 10 hits, one PKS, 14 NRPS, and four NRPS/PKS clusters. Furthermore, BAGEL4 resulted in 4 hits belonging to bottromycin and lanthipeptide classes (**Table 7**).

The presence of a potential indigoidine pathway was particularly intriguing, since the bacterial isolate PPB1 did not express a blue or purple pigment on agar plates or in liquid media. The NRPS encoding indigoidine synthetase (*indC*) in *Streptomyces chromofuscus* showed a 40% sequence similarity with a region of the PPB1 genome on node 23. On the other

TABLE 5 | Antioxidant activity of PPB1 crude extract via FRAP assay ($n = 3$).

Treatment	Average absorbance at 595 nm (Standard deviation)
PPB1 extract	1.395 (0.123)
EtOH (negative control)	0.004 (0.001)
Ascorbic acid (positive control)	1.814 (0.040)
1 mM $FeSO_4 \bullet 7H_2O$ (standard)	1.052 (0.056)

TABLE 4 | Average cell viability of *S. aureus* in various treatments ($n = 3$).

Treatment	Average adjusted total count	Average viability count (%)		
		Viable cells (SD)	Injured cells (SD)	Dead cells (SD)
No treatment	2.65×10^4	30.4 (1.9)	69.5 (1.9)	0.1 (0.01)
amp10	1.30×10^3	13.8 (1.5)	81.0 (2.0)	5.3 (0.7)
cam30	1.42×10^3	16.0 (8.4)	79.0 (8.0)	5.0 (0.5)
0 mg/kg ext.	4.24×10^4	67.8 (16.6)	32.0 (16.9)	0.1 (0.1)
500 mg/kg ext.	4.80×10^4	25.2 (2.6)	72.7 (2.7)	2.1 (0.2)
750 mg/kg ext.	4.40×10^4	28.8 (4.8)	69.8 (5.2)	1.4 (0.8)
1,000 mg/kg ext.	3.09×10^4	28.5 (7.1)	69.4 (7.2)	2.1 (0.2)

TABLE 6 | Nucleotide and gene count levels of the genome of *Pseudoalteromonas sp.* PPB1.

Attribute	Value
Genome size (bp)	5,915,516
DNA G + C (%)	47.4
DNA contigs	121
N_{50} (bp)	192,396
L_{50} (bp)	11
Draft coverage	283
Total predicted genes	5,201
Number of RNAs	109
rRNA operon count	12
Genes in subsystem features	2,082
CRISPR count	9

TABLE 7 | Secondary metabolite gene clusters via various bioinformatic tools.

Tool	Hits	Class
antiSMASH	29	amonabactin P750, kalimantacin A, indigoidine, rhizomide A-C, NRPS, NRPS-like, T1PKS, T3PKS, transAT-PKS, bacteriocin, lanthipeptide, hserlactone
BAGEL4	4	bottromycin; lanthipeptide class I; lanthipeptide class IV
NaPDoS	80	12 ketoacyl synthase domains; 68 condensation domains
NP.searcher	11	Seven modular NRPSs; three mixed modular NRPS/PKSs; 1 non-mevalonate terpenoid (*mep*) genes
PRISM	10	1 polyketide cluster; 14 non-ribosomal peptide clusters; 4 non-ribosomal peptide/polyketide clusters

hand, the marine sponge host, *Petrosia* had an observed red-purple coloration. It is inferred that the reddish hue is caused by the abundance of prodiginine within PPB1; however, no other pigmented microorganism was isolated from the sponge tissue.

PPB1 Genome Contains a Prodiginine Biosynthetic Gene Cluster and Monooxygenase-Like Enzyme

Genome mining and annotation characterized the presence of a prodigiosin biosynthetic gene cluster and verified the presence of prodigiosin and its analogs. This study also observed the heterogeneity of the gene cluster across various prodiginine-producing species and genera (**Table 8**, **Figure 5**). Thirteen open reading frames (ORFs) on node 3 (204,016–224,268) in PPB1 were aligned against the *pig* gene cluster in *P. denitrificans, P. rubra,* and *S. marcescens* and the *red* gene cluster in *S. coelicolor*.

P. denitrificans, P. rubra, and *S. marcescens* shared both the MAP and MBC biosynthetic pathway with PPB1. Out of those three species, the *pig* gene cluster from *P. rubra* aligned the closest. The percent identity of each ORF ranged from 88.58 to 97.18% with a mean and median of 93.61 and 94.30%, respectively. Three of those ORFs (ORF 2, ORF 4, ORF5) aligned with the MAP biosynthetic pathway ranging from 91.21 to 95.34% with a mean of 93.79% and a median of 94.83%. Nine ORFs (ORF1, ORF6-13) aligned with the MBP biosynthetic pathway ranging from 88.58 to 97.18% with a mean of 93.61% and a median of 94.30%. One ORF aligned with a gene responsible for terminal condensation of MAP and MBC to form prodigiosin and had a percent identity of 93.09%. The *pig* pathways in *P. denitrificans* and *S. marcescens* aligned loosely with the ORFs in PPB1. When PPB1 was aligned with *S. marcescens*, the percent identity of each ORF ranged from 47.83% to 73.33% with a mean and median of 63.59 and 64.52%, respectively. Interestingly, when PPB1 was aligned with *P. denitrificans*, there was a slightly lower percent identity ranging from 45.39 to 62.25% with a mean of 54.58% and a median of 54.29%.

Meanwhile, *S. coelicolor* shared only the MBC biosynthetic pathway and the gene responsible for terminal condensation with PPB1; however, there was high heterogeneity ranging from 17.19 to 48.82% with a mean and median of 33.44 and 31.44%, respectively.

In addition, ORF14, on node 13 (46,438–47,706) closely aligned to a putative oxidase with PRUB680 in *P. rubra* at 95.27% and loosely aligned with *redG* in *P. denitrificans* and *S. coelicolor* at 42.65 and 43.44%, respectively (**Table 8**, **Figure 5**). *redG* is a Rieske oxygenase responsible for cyclization of undecylprodigiosin to form streptorubin B (Salem et al., 2014). ORF14 and *redG* were also homologous to a putative oxidase (*tamC*) in *P. tunicata*, which was known to facilitate the cyclization of tambjamine YP1 (Burke et al., 2007). This may explain the formation of cycloprodigiosin and other cyclic prodiginine analogs in the PPB1 strain.

DISCUSSION

PPB1 demonstrated potential of biological activities including antibacterial and antioxidant activity. The data suggests that the PPB1 crude extract targets structures within Gram-positive bacteria. Previous studies have shown that prodigiosin and its various derivatives demonstrated antimicrobial activity against a wide-range of terrestrial bacterial species (Sakai-Kawada et al., 2019). In this study, 250 mg of a crude extract was used to observe the holistic effects of PPB1 against various bacterial species. Although the concentration of the crude extract in this study was higher than in previous studies, the zones of inhibition against species such as *B. cereus*, *E. coli*, and *S. aureus* were notably narrower (Ramesh et al., 2019a). This study expanded those tests to several marine isolates to understand how PPB1 interacts within its marine environment such as *P. rosenbergii*, *P. arabiensis*, *P. denitrificans*, and *V. harveyi*. Testing revealed that PPB1 had variable bioactivity against several other species of bacteria harboring within the *Petrosia* marine sponge, which may suggest PPB1 was competing for space and resources within

TABLE 8 | Annotated prodiginine biosynthetic gene cluster and their associated homologs.

ORFs (PPB1)	Predicted gene function	*P. denitrificans*		*P. rubra*		*S. marcescens*		*S. coelicolor*	
		pig genes	Percent identity	*pig* genes	Percent identity	*pig* genes	Percent identity	*red* genes	Percent identity
ORF1	L-prolyl-PCP dehydrogenase (MBC)	*pigA*	59.22	*pigA*	94.55	*pigA*	65.63	*redW*	43.57
ORF2	H_2MAP oxidase/dehydrogenase (MAP)	*pigB*	51.46	*pigB*	91.21	*pigB*	61.58	-	-
ORF3	Terminal condensing enzyme	*pigC*	58.07	*pigC*	93.09	*pigC*	67.99	*redH*	39.14
ORF4	3-acetyloctanal synthase (MAP)	*pigD*	58.58	*pigD*	95.34	*pigD*	68.90	-	-
ORF5	Putative class III aminotransferase (MAP)	*pigE*	60.76	*pigE*	94.83	*pigE*	71.80	-	-
ORF6	HBC *O*-methyl transferase (MBC)	*pigF*	62.25	*pigF*	95.98	*pigF*	73.33	*redI*	21.81
ORF7	Peptidyl carrier protein (MBC)	*pigG*	54.03	*pigG*	97.18	*pigG*	64.52	*redO*	17.19
ORF8	HBM synthetase (seryl transferase) (MBC)	*pigH*	58.47	*pigH*	94.30	*pigH*	67.55	*redN*	48.82
ORF9	L-prolyl-AMP ligase (MBC)	*pigI*	50.31	*pigI*	94.26	*pigI*	58.44	*redM*	38.70
ORF10	Pyrrolyl-β-ketoacyl ACP synthase (MBC)	*pigJ*	49.16	*pigJ*	92.19	*pigJ*	60.53	*redX*	31.44
ORF11	Hypothetical protein (MBC)	*pigK*	54.29	*pigK*	96.19	*pigK*	64.44	*redY*	46.15
ORF12	Phosphopantetheinyl transferase (MBC)	*pigL*	45.39	*pigL*	89.24	*pigL*	47.83	*redU*	22.84
ORF13	HBC dehydrogenase (MBC)	*pigM*	47.60	*pigM*	88.58	*pigM*	54.07	*redV*	30.47
ORF14[a]	Putative oxidase	*redG*-like	42.65	PRUB 680	95.27	-	-	*redG*	43.44

[a] *denotes outside of prodiginine biosynthetic gene cluster, but directly involved in prodiginine biosynthesis.*

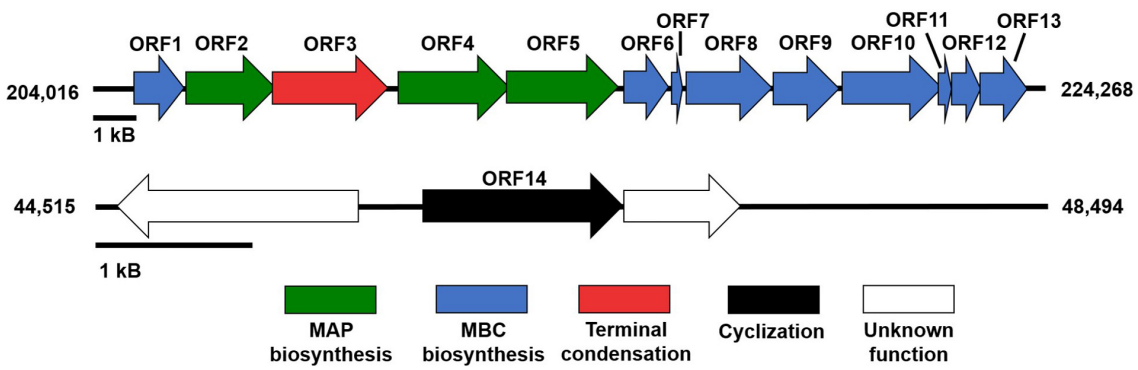

FIGURE 5 | Prodiginine biosynthetic gene cluster composed of MAP pathway (green), MBC pathway (blue), and terminal condensation (red). Gene involved with cyclization (black) was located outside of prodiginine biosynthetic gene cluster, but was directly involved in prodiginine biosynthesis.

its host. To obtain more comparable results with previous studies a bioassay-directed chemical purification may be performed to isolate the pigmented compounds and test their antibacterial properties separately.

Algal blooms frequently occur within coral reef systems (Schupp et al., 1999; Kim et al., 2008). The resulting ROS byproducts can cause oxidative stress via oxidation of lipid molecules and nucleic acids within the cell and ultimately lead to damaging effects on marine sponge tissue. Prior prodiginine studies suggested that the pigment stimulated the production and affected the influx of ROS (Danevči et al., 2016; Kimyon et al., 2016; Zhang et al., 2017). Sajjad et al. (2018) performed

2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay followed by a lipid peroxidation inhibition assay using prodigiosin extracted from *Streptomyces* and obtained comparable results. Antioxidant activity along with previously recorded algicidal activity could help mitigate harmful algal blooms and provide much-needed protection for the *Petrosia* species.

Without a sponge tissue cell line, there is no way to observe the direct interaction between the host sponge and harboring bacteria. Beyond testing the various prodiginine analogs and other metabolites, a proper sponge tissue cell line must be created to accurately assess the effect of PPB1 and its secondary metabolites on *Petrosia*.

The characterization of a prodiginine biosynthetic gene cluster and the monooxygenase-like enzyme involved in cyclization confirms the presence of prodiginine pigments in PPB1. Although the gene cluster was annotated, various prodiginine analogs may still be synthesized in PPB1. ORF14 was annotated on a separate node over 150 kB away from the rest of the biosynthetic gene cluster. Other genes involved in prodiginine analog biosynthesis may be found distant from the primary prodiginine biosynthetic gene cluster. Other than cycloprodigiosin, the genes for prodiginine analog biosynthesis have yet to be determined, which makes finding additional genes a difficult task (de Rond et al., 2017).

de Rond et al. (2017) and Kimata et al. (2017) demonstrated a directed approach by analyzing prodigiosin-like compounds (e.g., roseophilin and streptorubin B) and their respective pathways (e.g., *rph* and *red* pathways). This was followed by mining for homologous genes within their species of interest. Although the gene of interest (PRUB680) showed low sequence similarity with the *rph* and *red* genes, it demonstrated a similar function and was characterized as the gene responsible for the cyclization of prodigiosin.

The prodiginine biosynthetic pathway characterized in PPB1 already showed high heterogeneity between itself and other species such as *P. denitrificans*, *S. marcescens*, and *S. coelicolor*. Although the genes diverged between genera, their overall function remained the same (Williamson et al., 2006). *P. rubra* showed the closest association with the PPB1 isolate; however, there was still some variability within the gene cluster.

Petrosia had an observed red-purple coloration. One notable peptide-based metabolite that was mined from the genome was indigoidine, a bipyridyl deep blue-purple pigmented metabolite (Ramesh et al., 2019a). This pigment has been found in many different genera of bacteria and its biosynthesis was described in *Streptomyces*. Indigoidine biosynthesis is facilitated by a multimodule NRPS initiated by L-glutamate synthetase (*glnA*), which is driven by ATP and utilizes L-glutamate and ammonia to form L-glutamine, followed by indigoidine synthetase (*indC*), which facilitates the condensation and cyclization of two glutamine molecules to synthesize indigoidine (Xu et al., 2015). However, the pigment was not observed within the cultured samples. Two possible explanations for this could be: (i) the presence of a recalcitrant pigment-producing microorganism that is unable or extremely difficult to grow in the laboratory setting or (ii) PPB1 is unable to produce the pigment at the same abundance under laboratory conditions. It is possible that indigoidine was produced, but only at low amounts. Furthermore, prodiginine was produced at high levels and thus could mask the expression of indigoidine. Rather than compete with the expression of prodiginine in PPB1, heterologous expression of the proposed indigoidine gene cluster from PPB1 into an unpigmented, manageable, and manipulatable microorganism like *E. coli* would confirm the presence and function of the pathway.

It is of note that mining looks only at the sequence similarity, but molecular manipulation and proper metabolomics are needed to confirm the presence of these bioactive compounds and their secondary metabolite gene clusters. Also, genome mining presents only previously discovered gene clusters but does not report any novel gene sets. Further molecular analysis is required to find unique gene sets that may be specific to a species or strain.

CONCLUSION

The production of bioactive secondary metabolites within host-associated microorganisms suggests their role in providing environmental adaptation for its host. This study highlighted the antibacterial activity of PPB1 crude extract toward marine bacterial species, suggesting a competitive and protective role this microorganism has within its host environment. In addition, the antibacterial activity of PPB1 crude extract toward common terrestrial bacterial pathogens emphasized the potential that bioactive pigments have as future natural pharmaceuticals. The antioxidant activity of PPB1 crude extract also suggests that microorganisms can mitigate the harmful effects of ROS produced in marine ecosystems. Further studies need to be done in order to fully understand the complex relationship between host sponge and microorganisms and how they interact through secondary metabolites; further understanding of bioactive pigments, in turn, can allow us to develop natural products for various biotechnological applications to replace potentially harmful synthetic compounds with those that are safer to consume.

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Certain commercial equipment, instruments, or materials are identified in this paper in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by NIST, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

DATA AVAILABILITY STATEMENT

The annotated draft genome sequence was deposited in DDBJ/EMBL/GenBank under accession no. JACEQV000000000. The version described in this paper is version JACEQV000000000.1.

AUTHOR CONTRIBUTIONS

FS-K wrote the first draft and conducted experiments involved the identification of microbe, antimicrobial and antioxidant assays, genome sequencing, mining, and annotation. CI conducted antimicrobial assays (disc diffusion, flow cytometry), antioxidant assay, and genome sequencing. KH was involved in sampling of sponge, isolation of microbe, and antioxidant assay. C-JY was involved in genome sequencing, assembly, and submission. H-YN was involved in microbial isolation, electron microscopy, antimicrobial assay (disc diffusion), NRPS and PKS screening. MH conducted genome assembly and assembled the phylogenetic tree. EA was involved in genome assembly and submission. JA was the project advisor and facilitated all experiments performed in this study. All authors contributed to revision, read, and approved the submitted version of the manuscript.

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REVIEW
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Impact of High-Pressure Homogenization on the Extractability and Stability of Phytochemicals

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High-pressure homogenization (HPH) and high-pressure processing (HPP) are emerging technologies for the food industry. Both technologies employ high pressure to preserve foods. However, the principal mechanism of HPH is based on shear stress distribution in a material instead of a decrease in volume due to an increase in pressure as occurring in HPP. HPH can be used in extraction or preservation of bioactive compounds and phytochemicals. This review first describes the mechanism of HPH processing. Next, this review discusses the impact of HPH on extractability and stability of phytochemicals such as carotenoids, vitamin C, polyphenols, and anthocyanins in various food matrices. In general, the use of HPH slightly improved or maintained the extractability of the phytochemicals. Similarly, HPH slightly reduced or maintained the stability of the phytochemicals but this is dependent on the food matrix and type of phytochemical. HPH has a great potential to be used to improve the extractability and maintaining the stability of these phytochemicals or to be used together with milder thermal processing. Besides understanding the impact of HPH on the extractability and stability of phytochemicals, the impact of HPH on the nutritional quality of the food matrices needs to be thoroughly evaluated.

Keywords: anthocyanin, ascorbic acid, carotenoid, carotene, extraction, lycopene, phenolic acid, vitamin C

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INTRODUCTION

Phyto is a Greek word that means "plant." Hence, phytochemicals are chemicals derived from plants. They are secondary metabolites of plants that provide certain color, flavor, or for protection against pests and pathogens (Puri et al., 2012). Phytochemicals are small molecules in plant that constitutes about 10% or less of the plant matrix. They can be recovered from flowers, fruits, vegetables and herb using various extraction techniques (Harjo et al., 2004). Phenolic compounds belong to a large class of compounds with great diversity in their structures. Examples are simple phenolic acids that contain one hydroxyl group attached to an aromatic ring such as vanillin and caffeic acid. Besides simple phenols, there are polyphenols such as flavonoids and stilbenes in which the molecule bear a minimum of two phenolic rings. Example of these polyphenols include anthocyanin, proanthocyanins, and gallotannins (Cheynier, 2012). Carotenoid is a natural, lipid-soluble pigment occurring in plants, algae, and certain fungi. Carotenoid is located in the chloroplast and chromoplast of plants or bounded to a macromolecule such as fiber or protein (Serment-Moreno et al., 2017). Carotenoid contributes to yellow, orange and red color. The base structure of carotenoid consists of a branched five-carbon unit which is called isoprenoid (Boon et al., 2010).

Conventional extraction techniques for the recovery of phytochemicals can be carried out using solid-liquid extraction method. These include maceration, infusion, Soxhlet extraction, and steam distillation method (Brennan et al., 2013). Soxhlet extraction is conventionally used for recovery of phytochemicals from plants and is frequently used as the reference technique to evaluate other conventional and non-conventional extraction techniques (Wang and Weller, 2006). Conventional techniques usually employ the use of polar or non-polar organic solvents except for steam distillation and infusion technique that employ water as solvent (Haroen et al., 2013; Dhanani et al., 2017).

Extraction yield of conventional solvent extraction methods are usually high but the usage of solvents in these techniques limits the application of the extracted phytochemicals in foods as solvents may pose harmful effects when ingested (Selvamuthukumaran and Shi, 2017). This issue may be solved by using food grade solvents but there are other challenges as well. These include long duration of extraction, high cost to use high purity solvent, and the use of heat treatment which will result in degradation of thermo labile phytochemicals (Azmir et al., 2013). Therefore, alternative approaches involving non-thermal or solvent-free extraction methods are highly desirable for application of phytochemicals in the food industry. Pulsed-electric field, supercritical fluid extraction and high-pressure processing are examples of non-conventional techniques (Azmir et al., 2013).

High pressure processing (HPP) applies pressure uniformly and transmit the pressure to a sample using a pressure transmitting medium at ambient or sub-ambient temperature for several minutes, without inducing a shearing effect. Although many food products such as fruit and vegetable beverages are commercially being treated with HPP for pasteurization, this process is not a continuous process and therefore only allowing a relatively low processing volume. On the other hand, high-pressure homogenization (HPH), also known as high-pressure valve homogenization or dynamic high-pressure homogenization, is an emerging continuous flow process technology that enables homogenization and pasteurization or in certain cases, sterilization of fluids in one single step (Levy et al., 2020). HPH can be used in the food industry to inactivate spoilage or pathogenic microorganisms, prepare emulsions, reduce particle size and improve the rheological properties of food products. Research on the use of HPH to extract or increase the functionality of bioactive compounds has been increasing since the year 2010. It was suggested that this trend is due to increasing interest in the food industry to improve nutritional and organoleptic quality of foods, besides concern for food waste valorization. HPH technology is a green technology as it does not require the use of polluting solvents, short processing times, low energy consumption and carbon dioxide emissions (Mesa et al., 2020). In this context, HPH technology has huge potential for applications in the food industry. One of the emerging applications is the use of HPH technology for extraction of phytochemicals. However, information on the application of HPH technology for extraction of phytochemicals has to be derived from individual studies conducted. Thus, the application of HPH technology for extraction of phytochemicals such as carotenoids and polyphenols, and its impact on their extractability is organized and discussed in this review. Phytochemicals are key components in foods that provide benefits to human health. As the main focus of most studies is either on the impact of HPH technology on microbial inactivation, physical stability or stability of macromolecules, this review also discusses the impact of HPH on the stability of various phytochemicals. The mechanism of HPH is also discussed.

TABLE 1 | Summary of distinguishing high-pressure homogenization from high-pressure processing.

Description	High-pressure homogenization (HPH)	High-pressure processing (HPP)
Principle	- Distribution of shear stress across products - Changes in the structure of the product	- Equal distribution of pressure in all directions in products (isostatic principle and principle of *Le Chatelier*) - Non-porous food will maintain their original shape
Pressure level	100–350 MPa	400–600 MPa
Temperature rise	15–18°C/100 MPa - Irreversible	3–8°C/100 MPa - Reversible upon decompression
State of product	Liquid	Liquid and solid
Mode of operation	Continuous	Batch and semi-continuous
Number of industrial applications	Few	Many

Adapted from Martinez-Monteagudo et al. (2017) and Augusto et al. (2018).

MECHANISM OF HPH

HPH is a homogenization technology that results in the alteration of the physical structures and microbial and enzyme inactivation of food products. Both HPH and HPP technology are able to achieve microbial and enzyme inactivation in food products. However, the application of HPP depends on the pressure whereas the application of HPH is dependent on the distribution of shear stress across a product (Augusto et al., 2018). The difference between HPH and HPP is summarized in **Table 1**. HPH functions at an elevated pressure of more than 100 MPa and is not limited to a single configuration. **Figure 1** shows a general representation of HPH. With its homogenization pumps, valve and accessories, many configurations can be allowed but due to the particularities of the equipment such as valve design, only fluidic products and not particulate products can be used in HPH. Therefore, HPH process is considered as a continuous process (Augusto et al., 2018).

The main components of HPH consists of a homogenization valve and a high-pressure pump. A pressure of 34 MPa was considered as high-pressure homogenization in the early days; but 300 MPa or more can be achieved (Diels and Michiels, 2006). HPH was defined by Harte (2016) as homogenization processes whereby the pumps can reach at least 100 MPa to

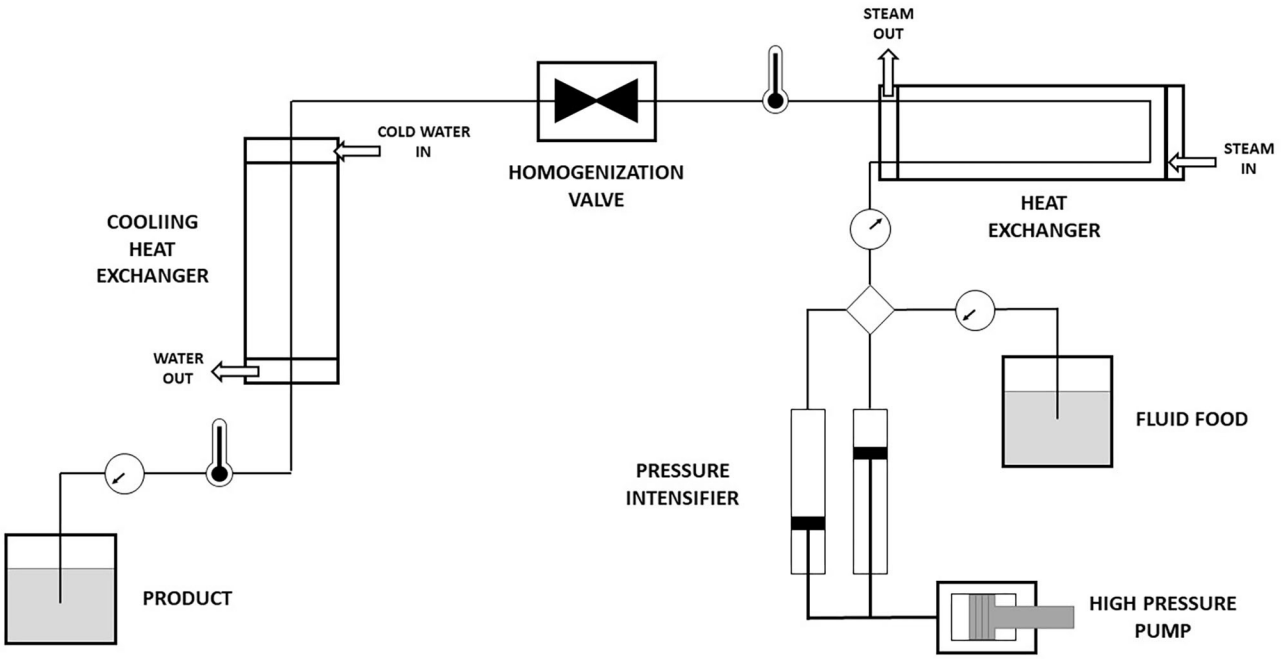

FIGURE 1 | Schematic representation of high-pressure homogenization technology.

a liquid food. The HPH process carried out at upper pressure range of 200 MPa or more is called an ultra-HPH (Marszałek et al., 2017). It should, however, be noted that the cut-off point between HPH and ultra-HPH can differ between authors. This is because HPH technologies are kept on evolving (Harte, 2016). Pressure is first intensified by the high-pressure pump that pressurizes a product that results in a fluid pressurization system. The high pressure generated can be up to 400 MPa and acts as the driving force that results in the flow of the fluid through and beyond the homogenization valve (Georget et al., 2014; Marszałek et al., 2017; Augusto et al., 2018). Although the high pressure is the driving force of the homogenizer, the main work occurs at the homogenization valve. The homogenization valve consists of a small orifice (in order of micrometers) in between the valve and the valve seat (**Figure 2**). At the orifice of the valve, a fluid is subjected to shearing action as its motion is disrupted by this orifice (Sanguansri and Augustin, 2006). Different phenomena such as cavitation, turbulence, collision and impingement contributed to these shearing effects (Martinez-Monteagudo et al., 2017). However, this review focuses on cavitation and turbulence.

SHEARING EFFECTS OF HPH

Cavitation

Due to abrupt reduction in pressure of the flowing liquid, cavitation occurs (Carlton, 2012) whereby cavities are formed followed by subsequent collapse within the liquid (**Figure 3**). This is a result of vaporized fluid condensation (Martinez-Monteagudo et al., 2017). At the orifice of the valve, a continuous decrease in pressure is occurring due to fluid acceleration. When the fluid vapor pressure is reached, this allows vaporization which is the transition of liquid into vapor. When the fluid leaves the valve, an increase in the flow area for the fluid decreases its velocity. Thus, the pressure at the exit increases back to atmospheric pressure. Due to this, a great amount of energy is released that causes great shear stress because of the condensation of vaporized fluid (Augusto et al., 2018).

Turbulence

Turbulence is a phenomenon that occurs when the motion of a fluid is suddenly reduced by about 100–1,000 times around the valve. As the area for flowing fluid is abruptly reduced, velocity increases according to the law of mass conservation. With increasing velocity around the valve and high magnitude of velocity gradients, there is non-uniform fluid motion that gives rise to turbulent flow (Martinez-Monteagudo et al., 2017). Turbulent flow (**Figure 3**) enhances diffusivity of mixing as well as improving mass and heat transfer. Turbulent flow also causes generation of heat through dissipation of kinetic energy (Rosa, 2006). The dissipative nature of turbulence is important as sufficient energy is required to breakup particle and cause the formation of droplets (Martinez-Monteagudo et al., 2017).

Heat of Homogenization

Even though HPH is considered as a non-thermal process, increase in the temperature of food materials is unavoidable because of adiabatic heating. Turbulence as well as shearing and mixing effects of liquid during homogenization produces adiabatic heating. Therefore, it is important to control and

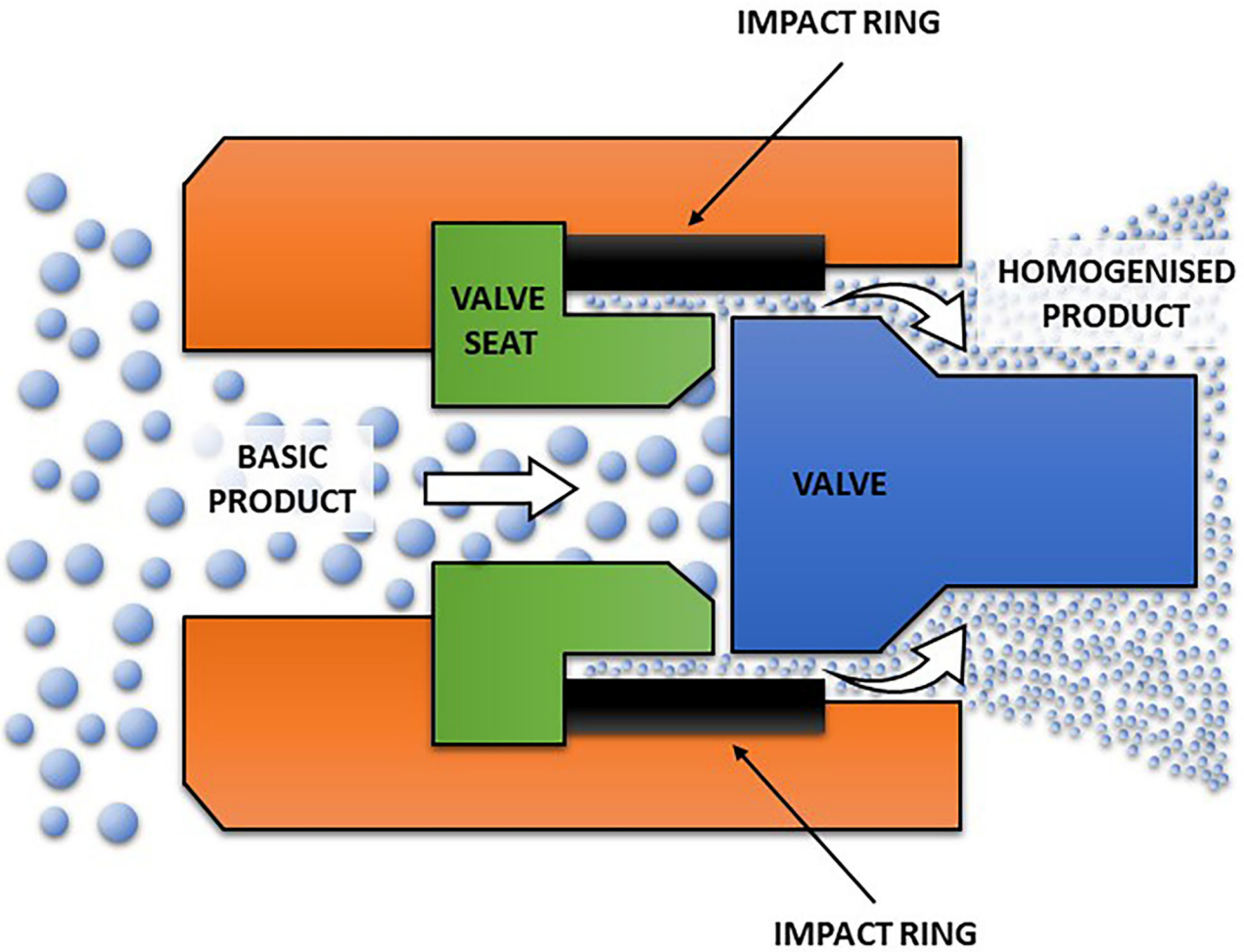

FIGURE 2 | Schematic diagram a simple flow path for homogenizing valve of high-pressure homogenization technology.

monitor the temperature of food materials after homogenization for prevention of over-heating and subsequent loss of heat-sensitive molecules (Diels and Michiels, 2006; Dumay et al., 2013). Heat exchanger (**Figure 1**) following the homogenization valve (Augusto et al., 2018) is generally used to control the temperature of HPH.

IMPACT OF HPH ON EXTRACTABILITY OF PHYTOCHEMICALS

Pre-treatment of phytochemicals with HPH before extraction is a relatively new approach. Hence, studies on the effect of HPH treatment on the extractability of phytochemicals is lesser studied in various food systems than studies on the stability of phytochemicals. Conventionally, phytochemicals are recovered from plant materials using solid-liquid extraction techniques but newer non-thermal and solvent-free approaches such as high-pressure extraction is being increasingly used for the extraction of phytochemicals (Cardoso et al., 2013; Casquete et al., 2014). The hypothesis is that the use of HPH process may increase the extractability of any compound such as phytochemicals (Martinez-Monteagudo et al., 2017). **Table 2** summarizes various conditions used in HPH and the effects of HPH on phytochemicals in several food matrices. The model, valve type and scalability of the HPH used are mentioned as reported by the authors of these studies. Current knowledge on HPH valves in terms of design, flow rate, operating pressure and scalability has been discussed in the review by Martinez-Monteagudo et al. (2017).

Phenolic Compounds

There are limited studies on the impact of HPH on the extractability of phenolic compounds. Zhu et al. (2016) investigated the effect of HPH at a pressure of 158.58 MPa and alkaline treatment for the extraction of phenolic acids from potato peels. With HPH treatment, an increase of 27.4% in extraction yield was obtained compared to that without HPH treatment. Combination of HPH and alkaline treatment in the extraction of phenolic acids further improved the extraction yield

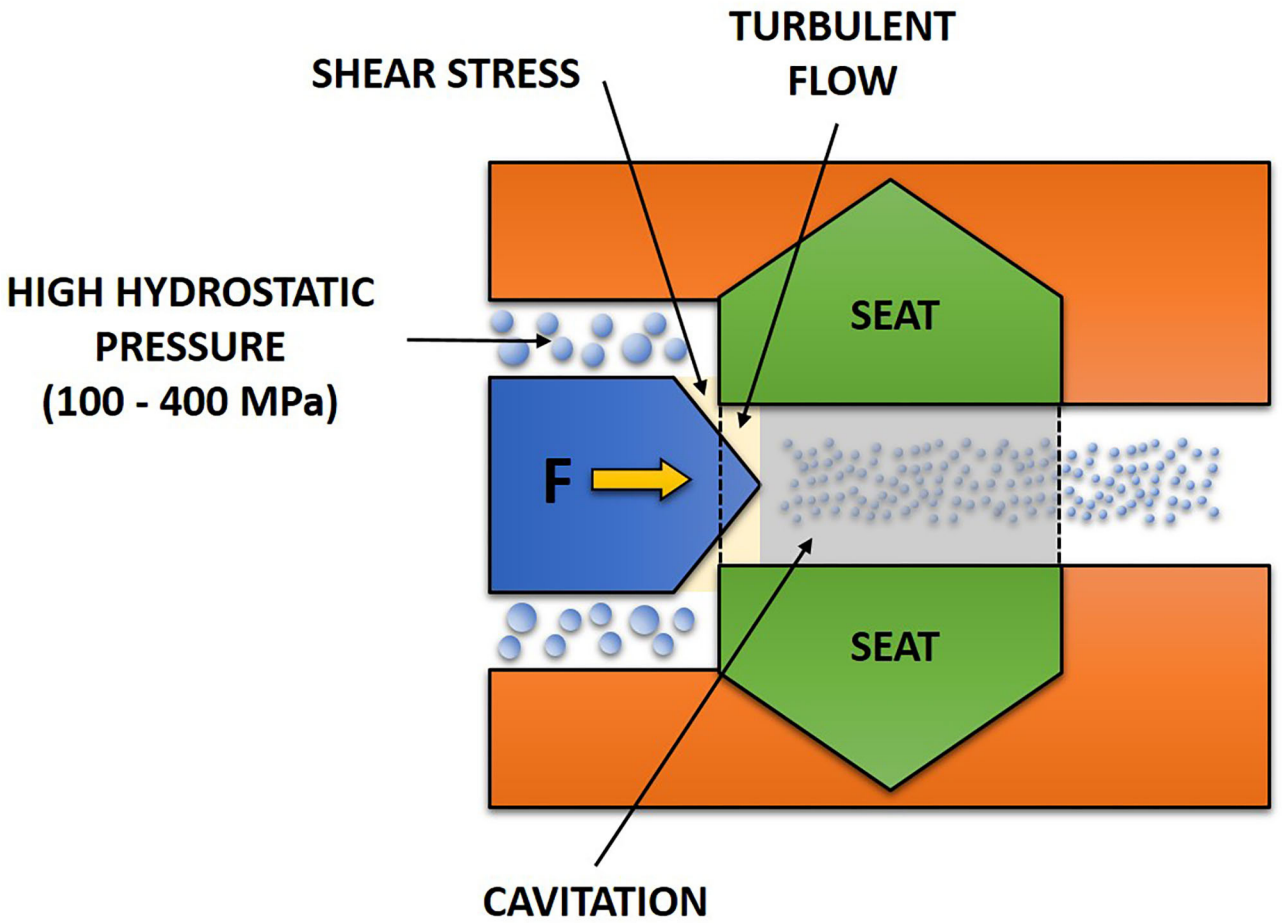

FIGURE 3 | Occurrence of turbulent flow and cavitation in a fluid during high-pressure homogenization.

by 44.4%. In these potato peels, the most abundant compounds are the free or bound phenolic acids (Samarin et al., 2012). HPH was suggested to cause structural changes to the cells by weakening the cell walls and releasing bound phenolic acids (Mattila and Kumpulainen, 2002).

HPH at 100 MPa for 1–10 passes with water as the process medium were used by Jurić et al. (2019) to extract valuable compounds from tomato peels. HPH treatment for 10 passes resulted in an increase in the extraction of polyphenols by 32.2% and was better than high-shear mixing at 5 min at 20,000 rpm. This was attributed to the release of intracellular compounds such as polyphenols is dependent mainly on the extent of cell disruption. These studies demonstrated positive effect of HPH on the extraction of phenolic acids from different food matrices, but they only utilized one pressure. Increasing the pressure of the HPH system or number of HPH cycles or passes is most likely going to increase the extractability of phenolic acids.

Carotenoid

The release of carotenoid by HPH processing is achieved through the mechanical disruption of plant cell walls. Plant cell walls are not able to withstand high shear stress occurring at the valve (**Figure 3**) of a high-pressure homogenizer (Colle et al., 2010a). Extraction of carotenoids from tomato peels into aqueous phase using HPH at 100 MPa for 1–10 passes was investigated by Jurić et al. (2019). Lycopene was found to be the main carotenoid in the pellet and supernatant of the aqueous phase after centrifugation. Interestingly, the pellet from aqueous phase treated by high-shear mixing at 5 min at 20,000 rpm contained higher amounts of lycopene than that by 5 HPH passes whereas the supernatant from the aqueous phase treated by 5 HPH passes contained higher amount of lycopene than that of high-shear mixing. It was suggested that lycopene can be extracted from the tomato peel structure into the aqueous phase using HPH. In addition, the yields of extraction of lycopene in this study was found to be comparable with other studies using solvent extraction assisted by cellulase and pectinase enzymes or ultrasound-assisted extraction but higher than those using pulsed electric field-assisted solvent extraction, supercritical carbon dioxide extraction, pressurized water extraction as well as conventional solvent extraction from dried tomato peels using various combinations of solvents (Jurić et al., 2019). Varying the

TABLE 2 | The impact of high-pressure homogenization on phytochemicals.

Food Matrix	Compound	Equipment	Conditions	Effect	References
Tomato pulp	- Lycopene	- Panda 2K, Gea Niro Soavi, Mechelen, Belgium - Two homogenizer valves with a spherical impact head	- Pressure: 8–132 MPa	1. Lycopene content after HPH ranged from 87 to 102% 2. Lycopene isomerization was not observed after HPH	Colle et al., 2010a
Mandarin juice	- Carotenoid	- Panda 2 K, GEA Process Engineering Inc., Italy	- Pressure: 30–120 MPa - Flow rate: 7 L/h - T_{in}: 15 and 30°C	1. HPH maintained carotenoid content	Carreño et al., 2011
Apple juice	- Polyphenol - β-Carotene - Vitamin C	- Model/DRG No. FPG 11300:400 Hygienic Homogenizer, Stansted Fluid Power Ltd., Harlow, U.K. - A ceramic valve able to withstand 400 MPa	- Pressure: 100, 200, and 300 MPa - Flow rate: 100 L/h - T_{in}: 4 and 20°C	1. HPH resulted in loss of polyphenol 2. HPH resulted in loss of β-carotene 3. HPH maintained vitamin C content	Suárez-Jacobo et al., 2011
Mango nectar	- Vitamin C	- Model FPG 7400H:350, Stansted Fluid Power Ltd., Essex, U.K.	- Pressure: 200 MPa - Flow rate: 270 mL/min	1. HPH resulted in significant degradation of vitamin C	Tribst et al., 2011
Fruit juices	- Vitamin C	- A bench-scale high-pressure homogenizer (nm-GEN 7400 series by Stansted Power Fluids, UK) - A high-pressure disruption valve	- Pressure: 50–250 MPa - Flow rate: 0.7 L/h	1. HPH resulted in significant degradation of vitamin C	Maresca et al., 2011
Carrot puree	- β-Carotene	- Panda 2K; Gea Niro Soavi, Mechelen, Belgium	- Pressure: 10, 50 and 100 MPa - Cycle: 1 - T_{in}: 4°C	1. HPH showed no significant influence on β-carotene content	Knockaert et al., 2012a
Tomato puree	- Lycopene	- Panda 2K; Gea Niro Soavi, Mechelen, Belgium	- Pressure: 10 MPa - T_{in}: 4°C	1. HPH resulted in no significant change in lycopene content but isomerization occurred after HPH	Knockaert et al., 2012b
Mango puree	- β-Carotene	- Panda 2 K, Gea Niro Soavi, Mechelen, Belgium - Homogenizer valve with a spherical impact head	- Pressure: 30, 60, 90 and 130 MPa - T_{in}: 4°C	1. Minimal isomerization of β-carotene was observed after HPH	Lemmens et al., 2013
Tomato pulp	- Lycopene - Lutein - Carotene	- Panda 2K; Gea Niro Soavi, Mechelen, Belgium	- Pressure: 20, 50 and 100 MPa - T_{in}: 4°C	1. HPH resulted in inconsistent carotenoid content	Panozzo et al., 2013
Orange juice	- Flavonoid - Carotenoid - Vitamin C	- FPG 11300:400, Stansted Fluid Power Ltd., Essex, U.K. - A ceramic valve that was able to withstand up to 400 MPa	- Pressure: 100, 200 and 300 MPa - Flow rate: 120 L/h - T_{in}: 10 and 20°C	1. HPH increased extractability of phytochemicals	Velázquez-Estrada et al., 2013
Mulberry juice	- Anthocyanin - Vitamin C	- A bench-scale high-pressure homogenizer (JC-10C series by Guangzhou Juneng biology and technology Co., Ltd., Guangdong, China - A high pressure disruption valve	- Pressure: 200 MPa - Flow rate: 10 L/h - T_{in}: 4°C	1. HPH significantly reduced anthocyanin content 2. HPH significantly reduced vitamin C content	Yu et al., 2014
Soya milk	- Polyamines - Tocopherols - Phytosterols	- Model FPG11300, Stansted Fluid Powder Ltd., Essex. U.K. - Ceramic UHPH valve (Stansted Series FPG 9080)	- Pressure: 200, 300 MPa - T_{in}: 55, 65, and 75°C	1. HPH maintained the levels of polyamines 2. HPH resulted in 20–50% of losses in the tocopherol contents 3. HPH increased the total phytosterol extractability	Toro-Funes et al., 2014a
Almond beverage	- Polyamines - Tocopherols - Phytosterols	- Model FPG11300, Stansted Fluid Powder Ltd., Essex. U.K. - Ceramic UHPH valve (Stansted Series FPG 9080)	- Pressure: 200, 300 MPa - T_{in}: 55, 65, and 75°C	1. HPH maintained the levels of polyamines 2. HPH resulted in 80–95% of losses in the tocopherol contents 3. HPH increased the total phytosterol extractability	Toro-Funes et al., 2014b
Bovine milk	- Vitamin C - α-tocopherol	- Stansted Benchtop HPH nG12500, Stansted Fluid Power Ltd., Essex, UK - A ceramic valve that was able to support 350 MPa	- Pressure: 300 MPa - Flow rate: 8 L/h - T_{in}: 45°C	1. HPH retained 81% of vitamin C 2. HPH maintained the level of α-tocopherol	Amador-Espejo et al., 2015

(Continued)

TABLE 2 | Continued

Food Matrix	Compound	Equipment	Conditions	Effect	References
Strawberry juice	- Polyphenol	- Nano Disperser—NLM 100, South Korea	- Pressure: 60 and 180 MPa - Passes: 2 and 5	1. HPH maintained total phenolic content at 60 MPa but increased the total phenolic content at 180 MPa	Karacam et al., 2015
Almond milk	- Vitamins B1 and B2	- MRI in-house HPH system - Dunze Hochdrucktechnik GmbH, Hamburg, Germany, Type 402-60-2, nominal valve size DN, 2.8 mm	- Pressure: 350 MPa - Flow rate: 0.5 mL/s - T_{in}: 85°C	1. HPH maintained the levels of vitamins B_1 and B_2	Briviba et al., 2016
Potato peel	- Phenolic acid	- Brand name was not provided by authors	- Pressure: 158.8 MPa - Cycles: 2	1. HPH improved the extraction of phenolic acids	Zhu et al., 2016
Apple juice Grape juice Orange juice	- Phenolic acid	- Nano homogenize machine, ATS Engineering Inc., Canada	Pressure: 250 MPa	1. HPH decreased the total phenolic content of apple juice 2. HPH increased the total phenolic content of grape and orange juices	He et al., 2016
Tomato juice	- Carotenoids	- A pilot-scale HPH (FPG11300:350, Stansted Fluid Power Ltd., Essex, UK) - Two-stage valves	- Pressure: 246 MPa - Flow rate: 90 L/h - T_{in}: 99°C	1. HPH maintained β-carotene and total lycopene content	Yan et al., 2017
Brocolli	- Vitamin C	- HOMOLAB, FBF, Italy	Pressure: 10–100 MPa	1. HPH increased the extraction yield of vitamin C by 152.8% for floret and 44.1% for stalk	Yang et al., 2018
Tomato peels	- Lycopene - Polyphenols	- An orifice valve assembly (orifice diameter of 150 μm)	- Pressure: 100 MPa - T_{in}: Below 24°C - Passes: 10	1. HPH enabled recovery of lycopene up to 56.1% of the initial peel content 2. HPH increased the extraction of polyphenols by 32.2%	Jurić et al., 2019
Carrot juice	- Carotenoids	- JN02HC, Guangzhou Juneng, China	- Pressure: 20, 60, and 180 MPa - T_{in}: 25, 50, and 70°C - Passes: 1, 2, and 3	1. HPH increased total carotenoid bioaccessibility	Liu et al., 2019c
Carrot beverage	- Carotenoids	- JN-02HC, Guangzhou Juneng, China	- Pressure: 60, 120, and 180 MPa - T_{in}: 25 and 60°C - Passes: 1 and 3	1. HPH resulted in better total carotenoid content during storage	Liu et al., 2019b
Carrot juice	- Carotenoids	- JN02HC, Guangzhou Juneng, China	- Pressure: 20, 60, 100, 150, and 180 MPa - T_{in}: 25, 50, and 70°C - Passes: 1, 2, and 3	1. HPH maintained total carotenoid content	Liu et al., 2019ca
Strawberry nectar	- Anthocyanin - Polyphenol	- PFK FPG12800, Stansted Fluid Power, U.K.	- Pressure: 50, 100, 150, and 200 MPa - Passes: 1, 3, and 5 - T_{in}: 25°C	1. HPH slightly affected anthocyanin content 2. HPH maintained total phenolic content	Moscovici Joubran et al., 2019
Kiwi juice	- Polyphenol	- PANDA (GEA, Parma, Italy) - A R-type valve	- Pressure: 200 MPa - T_{in}: 4°C - Cycles: 2 and 3	1. HPH increased total phenolic content	Patrignani et al., 2019
Rosehip nectar	- Carotenoid	- Two-stage HPH (GEA Niro Soavi-Panda Plus 2000 Homogenizer, Parma, Italy)	- Pressure: 75, 100, and 125 MPa - Passes: 1, 2, and 3	1. HPH increased total carotenoid content with increasing passes	Saricaoglu et al., 2019
Lettuce waste	- Polyphenol	- A continuous lab-scale high-pressure homogenizer (Panda Plus 2000, GEA Niro Soavi, Parma, Italy) - Two PS type valves	- Pressure: 80 and 150 MPa - Flow rate: 10 L/h - Cycles: 1 and 10	1. HPH partially maintained phenol content	Plazzotta and Manzocco, 2019
Peach juice	- Ascorbic acid - Polyphenol	- Two-stage HPH, SPX Flow Technology, soeborg, Denmark	- Pressure: ∼55 MPa - T_{in}: 45°C	1. HPH decreased ascorbic acid content 2. HPH increased total phenolic content	Yildiz, 2019
Pomelo and kiwi juices	- Polyphenol	- ATS Engineering Inc., Canada	Pressure: 250 MPa	1. HPH increased total phenolic content	Quan et al., 2020
Mixed carrot, apple, and peach juices	- Polyphenol	- JN-02HC series, Guangzhou Juneng, China	- Pressure: 140 MPa - T_{in}: 25°C	1. HPH increased total phenolic content	Wellala et al., 2020

pressure of the HPH system or number of HPH cycles or passes is most likely going to affect the extractability of carotenoids.

IMPACT OF HIGH-PRESSURE HOMOGENIZATION ON THE STABILITY OF PHYTOCHEMICALS

HPH process is conventionally utilized mainly for enzyme and microbial inactivation in the food industry (Welti-Chanes et al., 2009; Carreño et al., 2011). The decrease of microbial count in HPH-treated food material is usually not accompanied by a loss in nutritional value (Hayes et al., 2005; Calligaris et al., 2012). As HPH is a non-thermal process, it is usually regarded that HPH would cause minimal impact on the stability of phytochemicals. The following sections discussed the impact of HPH on the stabilities of vitamin C, phenolic compounds and carotenoid in several food matrices.

Vitamin C

The stability of L-ascorbic acid is influenced by the presence of light, oxygen and temperature (Volf et al., 2014). The retention of L-ascorbic acid in mango nectar with combination of HPH at 200 MPa and heat treatment (61.5 and 75.5°C) was investigated by Tribst et al. (2011). In addition, HPH treatments without combination with heat treatment at 200 MPa and 300 MPa were also investigated. The retention of L-ascorbic acid in mango nectar was found to be low. HPH treatment resulted in ∼50% losses of L-ascorbic acid. However, this is in contrast with the study of Pérez-Conesa et al. (2009), who reported that HPH at 10, 15 and 20 MPa did not reduce the L-ascorbic acid content in tomato. This was most likely due to the low pressure that was employed in the HPH treatment (Zhou et al., 2017). Besides, the presence of oxygen in the mango nectar and increase in temperature during HPH could result in degradation of L-ascorbic acid (Tribst et al., 2011). Hence, deaeration to remove oxygen is recommended prior to HPH processing.

The effect HPH treatment from 50 to 250 MPa on commercial orange, red orange, pineapple fruit juices and Annurca apple juice was investigated (Maresca et al., 2011). The vitamin C concentration of the HPH treated juice was reported to be similar to the fresh juices. It was suggested that the HPH treatment did not significantly cause the degradation of vitamin C in the juices (Maresca et al., 2011). The effect of HPH at 100, 200, and 300 MPa on L-ascorbic acid content in orange juice in comparison to thermal pasteurization (90°C for 1 min) was investigated by Velázquez-Estrada et al. (2013). There was a gradual decrease of L-ascorbic acid content (1.7, 4.6, and 10.7%) at pressures of 100, 200, and 300 MPa, respectively. However, HPH-treated juices retained L-ascorbic acid better than pasteurized juice with a decrease of 20.1%. This study is in accordance with another study using apple juice (Suárez-Jacobo et al., 2011). In contrast, the degradation of L-ascorbic acid due to a gradual increase in HPH pressure is not in accordance with the study by Welti-Chanes et al. (2009) in which the stability of L-ascorbic acid in orange juice remained stable after HPH treatment at 50–250 MPa. The degradation of L-ascorbic acid during storage is deduced to be more influenced by oxygen, temperature and light instead of the initial HPH treatment (Sharabi et al., 2018). The presence of trace metals in the processing equipment could also promote the degradation of L-ascorbic acid (Ball, 2006; Tribst et al., 2011).

The effect of HPH treatment at 75, 100, and 125 MPa on the ascorbic acid content of rosehip nectar was investigated by Saricaoglu et al. (2019). HPH treatment was found to decrease the ascorbic acid content of rosehip nectar as compared to the control. There were no significant differences between the ascorbic acid content of rosehip nectar treated at 75 and 100 MPa but the lowest ascorbic acid content in rosehip nectar was obtained using 125 MPa (Saricaoglu et al., 2019). HPH treatment at approximately 55 MPa for 3 min with an inlet temperature of 45°C of peach juice was found to be better than heat treatment at 72°C for 15 s in retention of ascorbic acid and total phenolic contents. However, ultrasonic homogenization at a frequency of 20 kHz was better than HPH treatment in retention of ascorbic acid and phenolic compounds of the peach juice (Yildiz, 2019). Overall, ascorbic acid is susceptible to degradation during HPH treatment.

Phenolic Compounds

The stability of phenolic compounds after isolation from their respective plant matrix is crucial as they are susceptible toward degradation in the presence of thermal stress or oxygen from the external environment (Karaaslan et al., 2013). The stability of anthocyanin in bilberry juice upon HPH and thermal treatment was investigated (Frank et al., 2012). Encapsulation of bilberry juice in monolayers of triglycerides was first carried out followed by thermal treatment, HPH at pressures from 30 to 150 MPa or combination of both thermal and HPH treatment. The anthocyanin content in the bilberry juice after HPH treatment remained relatively similar to untreated juice but thermal treatment caused degradation of the anthocyanin content. This study shows that the stability of anthocyanin is maintained even after mechanical stress induced by HPH treatment (Patras et al., 2010; Marszałek et al., 2017).

The impact of HPH on the polyphenol content of apple juice was investigated (Suárez-Jacobo et al., 2011). Fresh apple juices were subjected to HPH treatment at pressures of 100, 200, and 300 MPa or thermal treatment at 90°C for 4 min. The total polyphenol content of apple juices decreased (10.6, 6.0, and 1.4%) when subjected to HPH treatment under pressure of 100, 200, and 300 MPa, respectively, as compared to pasteurized juices. This indicates that the polyphenols were more stable with increasing pressure. Nevertheless, polyphenols in apple juice are generally not stable due to the presence of polyphenol oxidase, an enzyme that can cause degradation of polyphenols (Buckow et al., 2009). It was suggested that polyphenol oxidase remained stable at low pressures lower than 200 MPa and oxidized the polyphenols. However, the high pressure of 300 MPa must have inactivate the enzyme (Schilling et al., 2008), hence, there was no significant impact of HPH on total polyphenol content of apple juice. On the contrary, 300 MPa was reported in other studies to unable to inactivate PPO in apple juices (Saldo et al., 2009; Mckay et al., 2011).

Velázquez-Estrada et al. (2013) investigated the effect of ultra HPH and thermal pasteurization on the properties of orange juice. Orange juice was heated at 90°C for 1 min for thermal pasteurization whereas for HPH processing, orange juice was pressurized to 100, 200, and 300 MPa. The polyphenol content decreased by 0.77% (100 MPa), 1.54% (200 MPa), and 6.61% (300 MPa). Although polyphenol content was reduced because of HPH treatment, it was significantly lower than that of thermal pasteurization which showed a decrease of 19.0%. This study showed that the degradation of polyphenols was minimized under non-thermal treatments (Oancea et al., 2018).

There was no effect of HPH treatment at 60 MPa on the total phenolic content of strawberry juice. However, HPH treatment at 100 MPa resulted in an increase on the total phenolic content of strawberry juice. Increasing HPH passes from 2 to 5 also resulted in in an increase on the total phenolic content of strawberry juice (Karacam et al., 2015). HPH treatment at 250 MPa for 10 min decreased the total phenolic content of apple juice but increased the total phenolic contents in grape and orange juices. It was suggested that HPH treatment ruptured the cellular structure of grape and orange fruits, favoring the release of bound phenolic substances from these fruit cells and hence enhancing their phenolic content but for apple juice, size reduction of plant tissues might have led to the interaction of cytoplasmic polyphenol oxidase and phenolic compounds in the vacuoles and result in oxidative degradation of the phenolic compounds (He et al., 2016).

The effect of HPH processing (200 MPa and thermal pasteurization (95°C for 1 min) on the polyphenol content of mulberry juice was investigated by Yu et al. (2014). HPH caused a reduction in the anthocyanin content, cyanidin 3-rutinoside and cyanidin-3-glucoside by 33.2 and 38.8%, respectively, in mulberry juice. This was due to the presence of PPO. This study is in accordance with the study on apple juice in which a pressure of 200 MPa was found to be insufficient to inactivate PPO (Suárez-Jacobo et al., 2011). HPH processing may promote the oxidation of PPO through the exposure of active sites as a result of a change in its conformation (Liu et al., 2009; Bot et al., 2018). However, the pressure used in HPH processing has to be high enough ($\geq$300 MPa) to result in inactivation of PPO.

The inactivation of oxidative enzymes by the use of blanching prior to HPH produces a color-stable juice but blanching can cause significant reduction in phenolic content. This is because the applied heat and leaching effect in the water used in blanching. Ground lettuce waste was firstly homogenized at 40 MPa before proceeding to HPH treatments at 80 and 150 MPa. The combination of HPH with a blanching pre-treatment resulted in a homogeneous lettuce juice with partially maintained polyphenolic content (Plazzotta and Manzocco, 2019). Rosehip nectar treated at 125 MPa resulted in higher total phenolic content than that treated at 75 MPa. This was attributed to the decreasing particle size and releasing of materials from the cells (Saricaoglu et al., 2019). The total phenolic content of kiwifruit juices with ultra HPH treatment at 200 MPa for 3 cycles significantly increased in comparison to the control from 35 to 42 mg/100 mL of juice. These results indicate that an increase in the availability of phenolic compounds (Patrignani et al., 2019).

The effect of HPH treatment up to 200 MPa on the total phenolic content of strawberry nectar was investigated by Moscovici Joubran et al. (2019). The anthocyanin content was only slightly affected by the HPH pressure levels. The total phenolic content was not affected by HPH pressure levels but significantly increased up to 30% following number of HPH passes. This was attributed to more extraction of polyphenols from the achenes and pulp of strawberry (Moscovici Joubran et al., 2019). HPH treatment at 140 MPa, 25°C was found to enhance the total phenolic content of carrot, apple and peach mixed juices. This was attributed to the release of more polyphenols from the vacuoles of different fruits as a result of intensive cell disruption (Wellala et al., 2020). There were no significant differences between HPH-treated (250 MPa for 10 min) and thermally treated (80°C for 30 min and 90°C for 30 s) kiwi and pomelo juice in terms of total phenolic content. Both treatments showed an increase of 10.6–17.5% in total phenolic acids. This was attributed to both treatments were able to damage plant cell walls that assisted in the release of phenolic compounds (Quan et al., 2020). Overall, HPH processing affects the stability of phenolic compounds in juices but depending on the pressure that was applied and number of HPH passes.

Carotenoid

Carotenoid content in fruits is depending on their stage of developmental and environmental growth conditions. Lutein is the most representative carotenoid at the green stage of fruit, which reflects the characteristic of chloroplastic tissues, followed by β-carotene, violaxanthin and neoxanthin (Choo, 2019). Carotenoids contain a conjugated system of double bonds that are susceptible to oxidation and isomerization (Boon et al., 2010). These chemical reactions occur frequently during thermal processing due to high heat and exposure to oxygen. HPH processing may maintain the stability of carotenoid as HPH is non-thermal technology.

The effect of HPH has been investigated in tomatoes, carrots and mango fruits. It was found that the effect of HPH process on tomatoes varied according to the homogenization pressure (8.4–132.7 MPa). The total lycopene content ranged between 87 and 102% following these different pressure treatment (Colle et al., 2010a). This variation in the lycopene content may be due to the variability between the tomatoes as well as ripeness of the tomatoes (Martínez-Valverde et al., 2002). Knockaert et al. (2012a) compared the equivalent thermal and high pressure sterilization processes of HPH-treated tomato puree with olive oil. No significant differences were found between the thermal and high pressure sterilization processes. Panozzo et al. (2013) investigated the effect of HPH at pressures of 20, 50, and 100 MPa on carotenoid content in red, orange and yellow tomato cultivars. The type of carotenoid in each tomato differed according to the types of tomatoes. Lycopene is the predominant carotenoid in red tomatoes whereas lutein was the predominant carotenoid in yellow tomatoes. Carotene was found predominantly in orange tomatoes (Rizk et al., 2014; Chaudhary et al., 2018). This variability in the type of predominant carotenoid was also due to the ripeness of the tomatoes (Zeng et al., 2015). Lutein in the globular chromoplast

was found to be more resistant to rupture than the lycopene-residing crystalloid ones. The crystalline state of lycopene is more fragile and can be easily ruptured by mechanical stress (Schweiggert et al., 2012). Panozzo et al. (2013) found that tomato cells were completely distrupted with HPH treatment at 20 MPa which ease the release of cellular content including lycopene, lutein and carotene. The increase in homogenization pressure further disrupted the cell material with only fragments of cells were observed for HPH treatment at 100 MPa (Panozzo et al., 2013).

The effect HPH treatment on the isomerization and degradation of lycopene in tomato puree was investigated by Knockaert et al. (2012b). The lycopene content of HPH treated tomato puree was similar to the untreated puree. It was suggested that HPH at 10 MPa was insufficient to result in the disruption of the cell walls of tomato puree (Knockaert et al., 2012b; Kubo et al., 2013; Zhou et al., 2017). A pressure >50 MPa was recommended. In addition, the lycopene in HPH treated tomato puree remained as the stable *trans*-lycopene at 85.4% and *cis*-lycopene was at 14.6%. There was no formation of *cis*-lycopene in untreated tomato puree. In tomato pulp, intense thermal pasteurization caused elevation of *cis*-lycopene from 14.6 to 26.8% (Colle et al., 2010b). Furthermore, 13-*cis*-lycopene was the predominant degradation product of lycopene (Khoo et al., 2011; Lemmens et al., 2013).

The stability of carotene in mango puree upon HPH and thermal treatment (80–150°C for 20 min) was investigated by Shi et al. (1999). At 30, 60, 90, and 130 MPa, the ratio of β-carotene/total β-carotene was found to be 0.65, 0.63, 0.62, and 0.55, respectively. Isomerization of the stable *trans*-β-carotene was minimal as well. Isomerization of *trans*-β-carotene into *cis*- β-carotene would reduce its bioactivity and increase the susceptibility toward oxidation (Ball, 2006). The impact of HPH on the degradation and isomerization of carotenoids is depending on the pressure that was applied during HPH processing.

Carreño et al. (2011) studied the effect of HPH at pressure range of 0–120 MPa on the total carotenoid content of mandarin juice as compared to thermal pasteurization (90°C for 10 s) or HPP at 0–450 MPa. There was a decrease in total carotenoid content observed using HPH and HPP processing at 1.66 and 5.54%, respectively. However, thermal processing was shown to increase the total carotenoid content by 6.80%. Increasing pressure resulted in greater loss of carotenoid although all three treatments did not cause significant decrease of carotenoid content. The release of carotenoid from HPH processing is due to the mechanical disruption of plant cells within the chromoplast (Colle et al., 2010a; Palmero et al., 2016a,b).

Yan et al. (2017) investigated the impact of two high pressure techniques (HPH treatment at 246 MPa, 99°C, <1 s and HPP at 600 MPa, 46°C, 5 min) and compared to that of thermal processing at 90°C, 90 s of tomato juice. Total lycopene content and isomerization in the tomato juice were not significantly affected by the HPH and HPP treatment. Similar results were obtained for β-carotene content. Thermal processing resulted in significant decrease in β-carotene content of tomato juice. It was proposed that these two pressure-based technologies have the potential for application in tomato juice processing with good carotenoid retention (Yan et al., 2017).

HPH treatment assisted by moderate inlet temperature (180 MPa, 1 pass and 60°C) showed better total carotenoid content of a carrot beverage stored at 4°C for 28 days than the combination of HPH and heat treatment at 90°C for 5 min (Liu et al., 2019b). Rosehip nectar treated at 125 MPa resulted in higher total carotenoid content than that treated at 75 MPa. In addition, the total carotenoid content increased with increasing HPH passes from 1 to 3 passes. Lycopene is the main carotenoid in rosehip fruit (Saricaoglu et al., 2019). The effect of HPH treatment of carrot juice at 20, 60, 100, 150, and 180 MPa on the total carotenoid content of carrot juice was investigated by Liu et al. (2019a). The total carotenoid content was unaffected by the HPH treatment. HPH treatment at 180 MPa with moderate inlet temperature of 60°C showed a better preservation of carotenoids in carrot beverage than HPH treatment combined with heat treatment at 90°C for 5 min. During storage of the carrot beverage, the half-life, $t_{1/2}$ of carotenoids (24.60–40.29 day) by HPH treatment was higher than the value of 19.21 day by heat treatment. This signified the negative effect of heat treatment on carotenoid retention during storage (Liu et al., 2019c). Overall, carotenoids remain quite stable during HPH treatment.

CONCLUSIONS

HPH is a promising technology that could open up new opportunities to preserve phytochemicals of liquid food products. Studies on using HPH have shown relative improvement in recovery of carotenoids and phenolic compounds from plant matrix. The impact of HPH treatment on the stability of phytochemicals is depended on the type of phytochemical. Generally, the stability of carotenoids in various food matrices upon HPH treatment is maintained. The stability of polyphenols upon HPH treatment is dependent on the HPH pressure that was applied and number of HPH passes. Conversely, the stability of vitamin C is affected by HPH treatment. The HPH pressure applied, number of HPH cycles or passes and inlet temperature of a HPH system are key factors that will have an impact on the extractability and stability of phytochemicals. In addition, the HPH valve type or design and/or scale of HPH may also be the factors that can have an impact on the extractability and stability of phytochemicals. More studies to investigate these factors should be carried out. Nevertheless, HPH has a great potential to be used as treatment to improve the extractability and maintaining the stability of phytochemicals. HPH can also be used together with milder thermal processing. More research on the impact of HPH on the nutritional quality of food products should also be investigated in the future.

AUTHOR CONTRIBUTIONS

SY: original draft preparation and writing. CS: review. WC: conceptualization, writing, review, and editing. All authors contributed to the article and approved the submitted version.

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Bioactive Pigments of *Monascus purpureus* Attributed to Antioxidant, HMG-CoA Reductase Inhibition and Anti-atherogenic Functions

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Monascus purpureus is known to produce pigment molecules. The pigments were extracted from *M. purpureus* fermented rice. *In-vitro* antioxidant effects of pigments were observed and presumed to alleviate oxidative stress related atherosclerosis effect in rats fed with high fat diet (HFD) for 14 weeks. The formation of lipid peroxide due to the oxidation of serum lipid was higher in rats fed with HFD. While, the feeding of fermented rice (groups III-V) significantly lowered the formation of lipid peroxide (27.1–51.7%) in serum of rats, indicated antioxidative effect of pigments. In addition, feeding of fermented rice lowered serum cholesterol and triacylglycerol by 44.82 and 45.30%, respectively. Whereas, LDL-cholesterol levels were decreased by 70.12% and HDL-cholesterol increased by 34.58%. The atherogenic indices (LDL/HDL and TC/HDL) were reduced by 77.80 and 61.05%, respectively, in rats fed with fermented rice. These data confirmed the anti-atherosclerotic effect of pigments. Further liver enzyme, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity was significantly inhibited up to 54%. The identification of statins, sterols and fatty acids in fermented rice revealed the HMG-CoA reductase inhibitory activity. This was confirmed by synthesis of lower levels of cholesterol and triacylglycerol in liver of rats fed with fermented rice. Accordingly antioxidant, inhibition of HMG-CoA reductase, anti-atherogenic functions of *M. purpureus* fermented rice is attributed to the collective effect of bioactive metabolites.

Keywords: antioxidant, HMG-CoA reductase, Monascus, pigment, anti-atherogenic

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INTRODUCTION

Red mold rice (RMR) is a traditional food additive of the Chinese for thousands of years. In many Asian countries, *Monascus* fermented rice has been consumed as food and folk medicine. *Monascus* spp. are well-known for various bioactive metabolites, viz., pigments (red pigments: monascorubramine and rubropunctamine, orange pigments: monascorubrin and rubropunctatin, yellow pigments: ankaflavin and monascin), isoflavones, polyketides, lipids, dimerumic acid an antioxidant (Aniya et al., 2000), γ-Aminobutyric Acid (GABA), an antihypertensive metabolite (Feng et al., 2012), metabolites with antibacterial, antitumor, and immunosuppressive properties (Martinkova et al., 1999) and, structural analogs of lovastatin (pravastatin, compactin, monacolin J). Monacolins, in particular monacolin K (MK), inhibitor of 3-hydroxy-3-methylglutaryl coenzyme

A (HMG-CoA) reductase is marketed as lovastatin drug (Hachem et al., 2020). Apart from food color, *Monascus* pigments extended its use in biological activities, like anti-inflammatory, anticancer and antihyperlipidemic activities (Lin et al., 2011).

Monacolin K, a new hypocholesterolemic agent was first isolated from *M. ruber* by Endo (1979a), that competes with HMG-CoA reductase due to its structure similarity with HMG-CoA (3-Hydroxy 3-Methylglutaryl Coenzyme A). Thus, inhibits the cholesterol synthesis by prohibiting the formation of mevalonic acid an essential intermediate product for the cholesterol biosynthesis.

Dihydromonacolin-MV and dehydromonacolin MV2 isolated from *M. purpureus* and its hyper pigment mutant have shown biological activities (Dhale et al., 2007a,b). Dihydromonacolin-L, a potent inhibitor of cholesterol biosynthesis was isolated from the *M. ruber* (Endo et al., 1985). Our earlier toxicological studies of RMR did not show any adverse effect in rats (Mohan-Kumari et al., 2009).

In recent years, a high fat and cholesterol diet has been implicated as one of the risk factors for cardiovascular diseases (CVD) related problems in developed and developing countries. Atherosclerosisis is the accumulation of cholesterol-rich plaques in arteries, a key contributor to CVD, prevents sufficient blood flow to the heart. Atherosclerosis greatly progress due to the elevation of blood lipids from circulating lipoproteins and inflammation (Hansson, 2005). The atherogenic index (AI) is expressed for cholesterol level in the blood can be a risk factor for heart disease and atherosclerosis. Atherogenic risks also include oxidative stress, inflammation, obesity, diabetes, endothelial dysfunction, shear stress, homocysteine, infection, and genetic factors. Therefore, lowering the cholesterol levels in patients with cardiovascular diseases has become an important concern. Even though the diet and exercise is successful in certain patients for the management of hyperlipidemia, pharmacological intervention is required to effectively lower cholesterol. Cholesterol lowering agents that inhibit HMG-CoA reductase (EC 1.1.1.34) are prominent among the drugs of choice for treating hyperlipidemia. The available hypolipidemic drugs lowers circulating cholesterol levels either by prevention of cholesterol absorption, or increasing hepatic uptake of low density lipoproteins (LDL) or inhibit cholesterol synthesis. The HMG-CoA reductase inhibitors significantly reduce cholesterol biosynthesis in animal and human subjects owing to the structural similarity of HMG-CoA (substrate of the enzyme) and statins. Lovastatin produced by *Aspergillus terreus, M. purpureus*, and other fungi (Endo, 1979b) is a well-known inhibitor of HMG-CoA reductase and widely used as a hypocholesterolemic drug in humans (Alberts et al., 1980).

During the *M. purpureus* fermentation process multitude of secondary metabolites, pigments, monacolins, phytosterols, isoflavonoids, fatty acids, and others are produced. This study is aimed to investigate these metabolites effect which attributes antioxidant effect of RMR on total cholesterol (TC), triacylglycerol (TG), lipoprotein cholesterol (low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), serum lipid, hepatic lipid, and HMG-CoA reductase enzyme in hypercholesterolemic albino rats.

MATERIALS AND METHODS

Chemicals

Cholesterol and bile acid mixtures were purchased from Sigma chemical Co. (St. Louis, MO, USA). Mineral mix, vitamin mix and cellulose were obtained from Sisco Research Laboratories, Mumbai, India. Casein was purchased from Nimesh Corporation, Mumbai. The rice used for cultivating *M. purpureus* was purchased locally. Kits used were as follows: Total cholesterol (TC) assay kit (Product No. 11403002, Kerala, India), triacylglycerol (TG) assay kit (Product No. 11410102, Kerala, India), were purchased from Agappe Diagnostics Ltd. Kerala, India. All the solvents used were of analytical grade.

Microorganism and Red Mold Rice Preparation

M. purpureus MTCC 410 obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India was maintained on potato dextrose agar (PDA, Hi Media, India) slants at 4–8°C. It was sub cultured every 30 days.

Seed culture was prepared by inoculating a loopful of *M. purpureus* MTCC 410 to 100 ml basal medium (pH 6.0) containing (dextrose (100 g), peptone (10 g), KNO_3(2 g), $NH_4H_2PO_4$ (2 g), $MgSO_4.7\ H_2O$ (0.5 g), $CaCl_2$ (0.1 g) in 1L distilled water (Su et al., 2003). After incubation at 30°C for 48 h at 110 rpm, 5% seed medium (volume/weight) was used for solid state fermentation.

The traditional RMR was prepared by inoculating 500 g of rice (sterilized/cooked) with 5% (v/w) seed culture of *M. purpureus* (Su et al., 2003) under solid state fermentation and inoculated rice was incubated at 30°C [Adolf Khuner Therm-Lab, Birfelden (Basel), Switzerland] for 14 days in a slanting position with intermittent shaking. After fermentation, RMR was dried at 45–50° C for 24 h and then powdered. The powdered RMR was used for the experiments.

M. purpureus Bioactive Molecules

Pigments

The pigments were extracted with 95% ethanol at 30°C for 60 min on rotary shaker (150 rpm). After filtration, the pigments were quantified by measuring the optical density (OD) with appropriate dilution. The OD was measured at 375, 475, and 500 nm for yellow, orange, and red pigments, respectively. Pigment yield was calculated as OD Units using

TABLE 1 | Polyketide pigment of RMR methanol extract.

Strain	OD Units/g dry cell mass			
	Red (500 nm)	Orange (475 nm)	Yellow (375 nm)	Total pigments
MTCC 410	202.8 ± 12.56	183.4 ± 9.86	120.3 ± 2.23	506.5

Values are mean ± standard deviation (SD, n = 3).

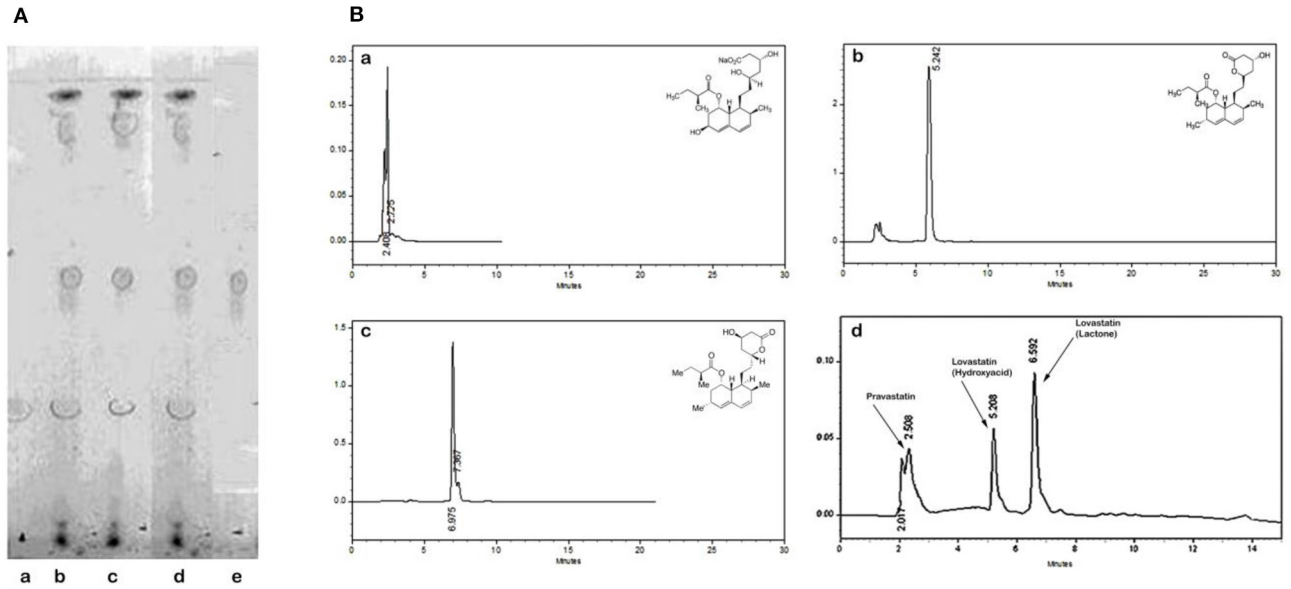

FIGURE 1 | Comparison of chromatograms of pravastatin (a), lovastatin hydroxyacid form (b), lovastatin lactone form (c), and RMR extract (d). **(A)** TLC. (a) Standard lovastatin, (b-d) RMR, e-Standard pravastatin. **(B)** HPLC. (a) Elution profile of standard pravastatin; (b) Elution profile of standard hydroxyl acid form of lovastatin; (c) Elution profile of standard lactone form of lovastatin; (d) Elution profile of statins from RMR extract.

following formula.

$$\text{OD Units} = \frac{\text{OD X Total vol of extraction X Dilution}}{\text{Red mould rice (g)}}$$

Statins

RMR powder added to 75% ethanol (1:10, w/v) was kept at 30°C for 1 h on rotary shaker [Adolf KhunerTherm-Lab, Birfelden (Basel), Switzerland] at 180 rpm. After centrifuging for 10 min at 10,000 rpm, samples were filtered through a 0.45 μm millex-LH (Millipore Corp., Bedford, MA 01730) filter before injection. The identification of statins were carried out by HPLC equipped with linear isocratic system, using Shimadzu Liquid Chromatograph LC-10A (Shimadzu, Japan) fitted with water's ODS (5 μm, 25 cm × 4.6 mm i.d.) column with a UV detector (237 nm) eluted with acetonitrile:water (72:28, v/v) at a elution of 1.0 ml/min maintained column temperature at 30°C with the injection volume of 20 μl. Statins were identified by comparing with the retention time of standards.

Fatty Acids

Total lipid extraction from dry biomass and analysis of fatty acids was carried out according to the method (Somashekar et al., 2003). The fatty acids were identified with the retention time of authentic fatty acid standards from Sigma. Fatty acids were identified by converting the fatty acids to fatty acid methyl esters (FAMEs) as described earlier (Kate, 1964) with slight modification. FAMEs were separated by Shimadzu GC-15A equipped with a DEGS packed column with isothermal temperature with flame ionization detector. The injector temperature was set at 220°C, detector temperature was maintained at 230°C and column temperature at 180°C. N_2 was used as carrier gas at a flow rate of 40 ml min^{-1}.

Phytosterol

About 10–20 mg of extracted lipid was subjected to saponification by methanolic KOH (6%, w/v) and refluxed for 2 h at 80°C in boiling water bath, cooled and diluted with 1 vol. of water. Total sterol was repeatedly extracted 3 times with hexane. The total extract was pooled together and evaporated to dryness. The final residue was dissolved in 1 ml chloroform and estimated by Liberman-Burchard method (Syed Mubbasher et al., 2003). Briefly to 200 μl sample, 800 μl chloroform and 2 ml of the Liberman-Burchard reagent (5 ml of H_2SO_4 dissolved in 10 ml acetic anhydride) was added in a graduated test tube. The final volume was made up to 7 ml with chloroform and incubated in dark for 15 min. The absorbance of resultant solution was determined spectrophotometrically at 640 nm (Shimadzu 160 UV A). Sitosterol was used as standard for comparison.

Antioxidant Activity

Antioxidant activity of *M. purpureus* extract was determined by estimating DPPH radical scavenging activity, inhibition of ascorbate autoxidation, lipid peroxidation, and reducing activity. All the experiments were carried out in triplicates by maintaining appropriate blanks and controls. Butylated hydroxyl anisole was used standard as for comparison.

The DPPH scavenging activity (Blois, 1958) of was measured accordingly (Moon and Terao, 1998). The inhibition of ascorbate autoxidation was determined according to Mishra and Kovachich (1984). Lipid peroxidation inhibitory activity was measured according to Kulkarni et al. (2004). The reducing power

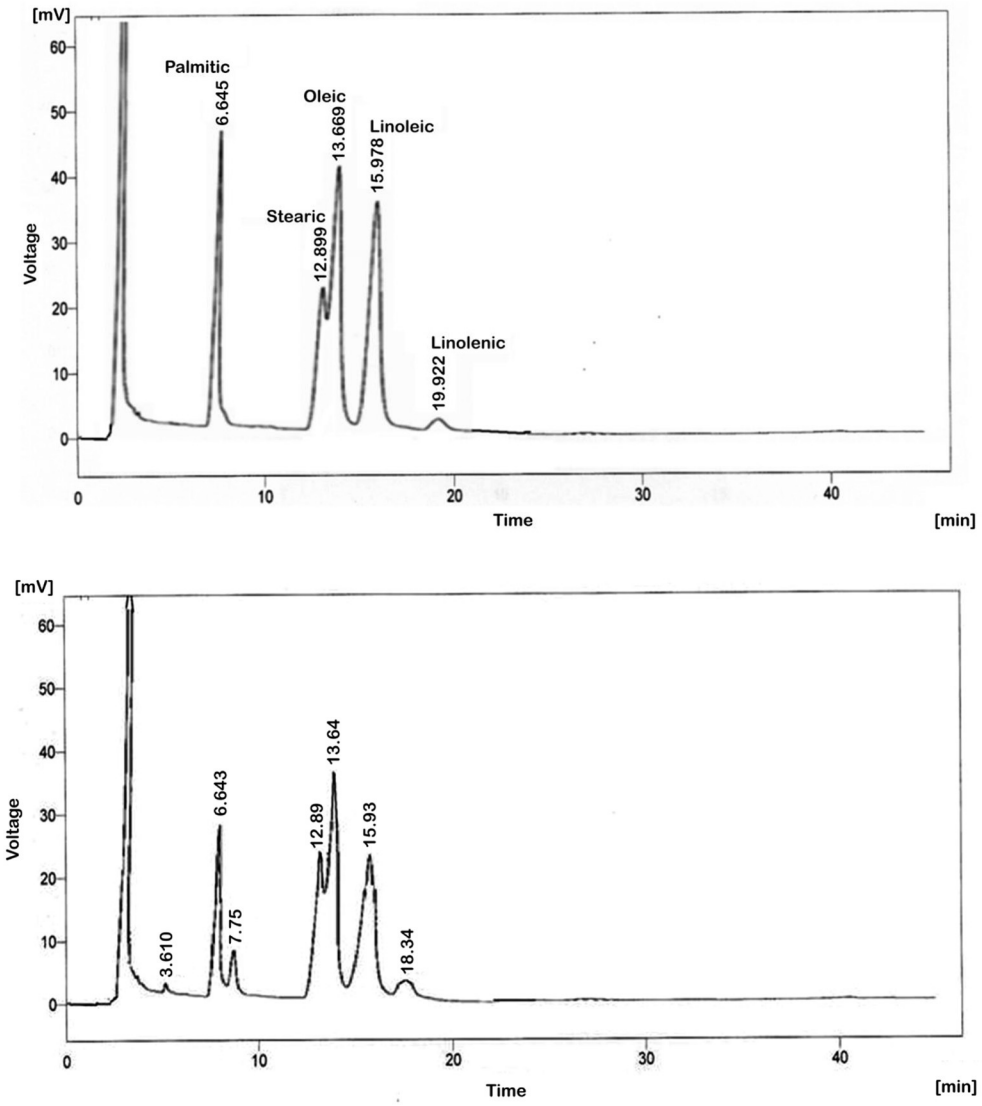

FIGURE 2 | GC profiles of standard fatty acids **(A)**, and the fatty acids of *M. purpureus* RMR.

was determined according to Oyaizu (1986). Percentage of antioxidant activity was determined using the standard formula.

IN VIVO HYPOLIPIDEMIC ACTION OF RMR

Experimental Animals

Sixty four male albino rats (CFT-Wistar strain) weighing 150–180 g were used as experimental animals. The rats were bred in animal house facility at Central Food Technological Research Institute, Mysore, India. The experimental protocol was approved by the Institute animal ethical committee. Animals were grouped by randomized design on a body weight basis and were kept (12 h light/dark cycles, RH 60% and at 25 ± 2°C) in stainless steel cages for 14 week. Four animals in control group were sacrificed immediately to analyze cholesterol and triacylglycerol levels in serum and liver and established a base line lipid profile data.

Treatment Groups and Dosages

American Institute of Nutrition (AIN)-76 diet formulation was followed for the preparation of experimental diets with slight modification. Animals were divided into six groups of 10 animals each. The control group rats (I group) were fed a normal diet of AIN-76 formulation. Group II were fed with high fat diet (HFD, cholesterol). Group III to V rats were fed with HFD supplemented with 8, 12, and 16% of RMR powder, while Group VI rats were fed with HFD supplemented with lovastatin (0.08 g/kg). Appropriate proportion of cholesterol, bile salts, RMR and standard lovastatin were supplemented to the control diet at the expense of corn starch. Cholesterol (1.0%) and bile acids (0.15%) were added to the diet to induce hyperlipidemia in rats. Diets

TABLE 2 | Antioxidant activty of RMR methanol extract.

	Phenolics mg/100 g	DPPH scavenging (IC_{50})	Inhibition of ascorbate autoxidation (%)	Reducing (equivalent cysteine, μM)	Lipid peroxidation (IC_{50})
Methanol extract	141.32 ± 0.01	100.78 ± 2.66	21.10 ± 0.95	1.35 ± 0.05	47.02 ± 1.91
BHA	–	18.96 ± 1.28	9.76 ± 1.45	0.65 ± 0.11	38.02 ± 1.53

Values are mean ± standard deviation (SD, n = 3).

TABLE 3 | Food intake of male rats fed dietary *M. purpureus* RMR for 14 weeks.

Concentration of RMR mixed with diet (w/w)	Average food Intake (g/rat/day)							
	0th week	2nd week	4th week	6th week	8th week	10th week	12th week	14th week
Control	12.67 ± 1.43	13.50 ± 1.42	14.89 ± 2.20	13.93 ± 1.44	9.62 ± 0.84	14.31 ± 2.35	15.42 ± 0.69	14.37 ± 0.97
HCD	13.20 ± 1.30	13.74 ± 1.37	14.82 ± 1.73	14.41 ± 1.76	8.78 ± 1.22	14.28 ± 0.84	15.79 ± 0.79	13.98 ± 1.06
HCD + 8% RMR	13.11 ± 1.71	13.68 ± 1.63	14.75 ± 1.43	14.08 ± 1.94	9.87 ± 0.70	15.06 ± 0.54	15.86 ± 1.11	13.82 ± 0.33
HCD + 12% RMR	11.58 ± 2.31	13.41 ± 1.46	14.24 ± 1.65	14.24 ± 1.84	9.81 ± 0.30	14.95 ± 2.32	16.13 ± 0.55	14.08 ± 1.41
HCD + 16% RMR	13.36 ± 1.10	13.25 ± 1.62	14.25 ± 1.45	14.36 ± 1.94	9.86 ± 0.61	15.13 ± 0.81	15.90 ± 0.79	14.50 ± 0.42
HCD + 80 mg Lovastatin	12.61 ± 1.28	13.38 ± 1.36	14.70 ± 2.15	14.32 ± 2.07	9.41 ± 0.60	15.19 ± 1.39	16.34 ± 0.71	15.84 ± 0.58

Values are mean ± SEM of six animals. No significant difference between control and M. purpureus red mold rice fed groups ($p < 0.05$).

were prepared every week and stored at 4°C. Throughout the study (14 weeks), the animals had free access to food and water *ad libitum*. The animals were observed thoroughly for the onset of any signs of toxicity. Daily food intake and gain in weekly body weight gained was monitored. At the end of the experimental period (7th and 14th week), overnight fasted rats were sacrificed. Blood samples were collected by cardiac puncture and serum separated by centrifugation at 6,000 *g*. Organs like liver, heart, brain, kidney, adipose, lungs, spleen and testis were excised, blotted, weighed and stored at −20°C till they were processed. A portion of the liver was stored in 10% neutral formalin and processed for histological examination.

Serum Cholesterol and Triacylglycerol Analysis

Serum cholesterol and triacylglycerol were measured using commercial enzymatic kits, respectively. HDL cholesterol was estimated after precipitation of LDL with heparin-$MnCl_2$ reagent (Warnick and Albers, 1978).

Liver Cholesterol and Triacylglycerol Analysis

Triacylglycerol in liver was estimated by the method of Fletcher (1968). Total lipids from liver after extraction was estimated by the method of Folch et al. (1957). Total cholesterol content of liver was estimated by the method of Searcy and Bergquist (1960).

3-Hydroxy-3-Methylglutaryl-CoA Reductase Activity

Preparation of microsomes and HMG CoA reductase activity was assayed according to the method of Hulcher and Oleson (1973). The reaction mixture was containing 0.5–1.0 mg of microsomal protein, 150 nmoles of HMG-CoA, 2 μmoles of NADPH and 0.8 ml of 0.1 M triethanolamine in 0.02 M EDTA buffer (pH 7.4) without dithiothreitol. The final volume was made up to1 ml and kept for incubation at 37°C for 30 min (Hulcher and Oleson, 1973).

Statistical Analysis

The statistical analyses of experimental data e were subjected to one-way analysis of variance (ANOVA) using Duncan's test. Statistical significance was considered when $P < 0.05$. Statistical analysis were performed by software SPSS, release 9.0 (SPSS, Inc.).

RESULTS

Identification of Bioactive Molecules

Major bioactive molecules were identified using various techniques like HPLC, GCMS and UV visible spectroscopy.

Pigments

Production of more red pigment (202.8 OD units) was observed compared to orange (183.4 OD units) and yellow pigments (120.3 OD units). These results indicated the production of three major colored pigment like yellow, orange and red (**Table 1**).

Statins

Qualitative and Quantitative Analysis by TLC and HPLC

Statins (lovastatin and pravastatin) from *M. purpureus* RMR, extracted with ethanol, were identified after TLC and HPLC analysis. For TLC, pravastatin and lovastatin standards obtained from sigma were used. After performing TLC, Rf values were determined in relation to the standards. Standards showed an Rf

TABLE 4 | Body weight gain of male rats fed with *M. purpureus* RMR for 14 weeks.

Concentration of RMR mixed with diet (w/w)	Average body weight gained (g)							
	0th week	2nd week	4th week	6th week	8th week	10th week	12th week	14th week
Control	177.40 ± 2.12^{f}	219.9 ± 10.05^{c}	267.4 ± 21.56^{c}	297.3 ± 26.70^{c}	315.4 ± 22.7^{a}	318.2 ± 35.38^{a}	332.8 ± 44.05^{a}	339.0 ± 63.79^{a}
HCD	169.00 ± 1.05^{d}	218.8 ± 8.84^{c}	266.2 ± 16.86^{c}	298.1 ± 20.21^{c}	313.8 ± 20.36^{a}	324.8 ± 15.55^{a}	342.6 ± 17.04^{a}	355.6 ± 20.07^{a}
HCD+8% RMR	164.80 ± 1.40^{c}	204.3 ± 15.71^{b}	247.4 ± 18.65^{b}	279.4 ± 19.48^{bc}	316.8 ± 26.45^{a}	326.4 ± 27.35^{a}	343.6 ± 23.68^{a}	349.2 ± 21.92^{a}
HCD+12% RMR	161.00 ± 1.05^{b}	184.7 ± 8.55^{a}	223.6 ± 17.63^{a}	245.8 ± 20.48^{a}	316.8 ± 26.45^{a}	299.8 ± 27.55^{a}	315.4 ± 35.42^{a}	331.8 ± 19.59^{a}
HCD+16% RMR	155.70 ± 1.42^{a}	185.1 ± 10.07^{a}	226.8 ± 14.97^{a}	244.9 ± 14.56^{a}	286.4 ± 12.97^{a}	298.2 ± 11.43^{a}	321.8 ± 12.76^{a}	328.4 ± 11.59^{a}
HCD+80 mg Lovastatin	172.50 ± 0.71^{e}	200.8 ± 7.24^{b}	246.8 ± 13.55^{b}	267.4 ± 21.36^{b}	326.8 ± 40.34^{a}	321.2 ± 17.09^{a}	338.2 ± 17.71^{a}	330.8 ± 8.04^{a}

Values are mean ± SD of six animals. Mean values within each column with different superscripts are significantly different Duncan's test at $p < 0.05$.

TABLE 5 | Effect of feeding *M. purpureus* RMR for 7 weeks on organ weight of male rats.

Concentration of RMR mixed with diet (w/w)	Relative organ weight (g/100 g body weight)							
	Liver	Lungs	Kidney	Heart	Testis	Adrenal	Brain	Spleen
Control	3.08 ± 0.16^{a}	0.41 ± 0.10^{ab}	0.73 ± 0.07^{ab}	0.27 ± 0.03^{a}	0.90 ± 0.14^{a}	0.03 ± 0.01^{a}	0.62 ± 0.06^{a}	0.26 ± 0.03^{b}
HCD	4.30 ± 0.23^{b}	0.40 ± 0.06^{a}	0.71 ± 0.02^{a}	0.28 ± 0.01^{ab}	0.84 ± 0.04^{a}	0.02 ± 0.01^{a}	0.60 ± 0.15^{b}	0.21 ± 0.04^{a}
HCD + 8% RMR	3.76 ± 0.25^{a}	0.43 ± 0.04^{ab}	0.73 ± 0.05^{bc}	0.31 ± 0.02^{bc}	0.83 ± 0.15^{a}	0.02 ± 0.01^{a}	0.59 ± 0.59^{c}	0.26 ± 0.05^{b}
HCD +12% RMR	3.55 ± 0.39^{a}	0.47 ± 0.07^{ab}	0.75 ± 0.06^{ab}	0.32 ± 0.04^{c}	1.18 ± 0.20^{b}	0.03 ± 0.01^{a}	0.60 ± 0.12^{c}	0.23 ± 0.05^{ab}
HCD +16% RMR	3.42 ± 0.30^{ab}	0.49 ± 0.08^{b}	0.74 ± 0.06^{bc}	0.34 ± 0.02^{c}	1.14 ± 0.05^{b}	0.03 ± 0.00^{a}	0.68 ± 0.68^{c}	0.24 ± 0.05^{ab}
HCD + 80 mg Lovastatin	3.49 ± 0.25^{a}	0.47 ± 0.10^{b}	0.73 ± 0.06^{c}	0.30 ± 0.02^{c}	0.98 ± 0.20^{b}	0.02 ± 0.00^{a}	0.63 ± 0.10^{c}	0.20 ± 0.02^{ab}

Values are mean ± SD of six animals. Mean values within each column with different superscripts are significantly different Duncan's test at $p < 0.05$.

values of 0.52 and 0.39 for pravastatin and lovastatin, respectively. Spots appearing at this Rf, with the ethanol extract of *M. purpureus* identified (**Figure 1A**) the presence of two statins. Hence for conformation, HPLC was carried out.

Further the HPLC chromatograms confirmed the presence of lovastatin (hydroxyacid and lactone form) and pravastatin in RMR extract. The retention time (*Rt*) was 5.22 and 6.97 min for hydroxyacid and lactone form of lovastatin, respectively. The pravastatin showed elution time at 2.40 min when compared with standard (**Figure 1B**). HPLC data shows, the lactone form was the major form of lovastatin.

These results indicated the biosynthesis of lovasatin (hydroxyacid and lactone form) and pravastatin in RMR.

Fatty Acids

Unsaturated fatty acids such as oleic, linoleic, and linolenic acids also help to reduce serum lipids (Ma et al., 2000). In order to quantitate useful fatty acids in *M. purpureus* RMR, the profile were estimated by GC. Each of the fatty acid was identified using the retention time with reference to that of the standard. Palmitic, stearic, oleic, linoleic and linolenic acids were identified as the major fatty acids in *M. purpureus* RMR (**Figure 2**). RMR had higher concentrations of unsaturated fatty acids (linoleic and oleic acids) than saturated fatty acids (palmitic and stearic acid). Production of linolenic acid (0.2 ± 0.66%) concentration was very less compared to other fatty acids like oleic acid (27.90 ± 0.90%) and Linoleic acid (24.60 ± 0.66%).

Antioxidant Activity

A compound can exert its antioxidant activity by scavenging radicals, decomposing peroxides, or chelating metal ions. The antioxidant activity of crude methanol extract was carried out by *in vitro* assays for DPPH radical scavenging, inhibition of ascorbate autoxidation, reducing activity, and lipid peroxidation (**Table 2**). All the experiments were carried out in triplicates by maintaining appropriate blanks and controls.

The phenolic content of methanol extract of RMR was 141.32 ± 0.01 μg ml^{-1} and expressed as mg gallic acid equivalent/100 g dry weight. The IC_{50} value to scavenge DPPH radical for methanol extract, and BHA were found to be 100.78, 50.01 and 18.96 μg ml^{-1}, respectively (**Table 2**).

Inhibition of ascorbate autoxidation and reducing activity by crude methanol extract of RMR was found to be more compared to BHA. The BHA showed lipid peroxidation inhibition activity of 38.02 ± 1.53 μg/ml (**Table 2**).

IN VIVO HYPOLIPIDEMIC ACTION OF RMR

Effect of RMR on Food Intake and Body Weight Gain in Rats

The normal, high fat and RMR supplemented diets were prepared. Cholesterol and bile acids were added to diet at 1.0 and 0.15% respectively to induce hypercholesterolemia in rats. Effects of RMR supplementation to HCD on food intake and body weight gain were presented in **Tables 3**, **4**. No significant differences in average food intake were observed between control

TABLE 6 | Effect of feeding *M. purpureus* RMR for 14 weeks on relative organ weight of male rats.

Concentration of RMR mixed with diet (w/w)	Relative organ weight (g/100 g body weight)							
	Liver	Lungs	Kidney	Heart	Testis	Adrenal	Brain	Spleen
Control	3.35 ± 0.36^{a}	0.40 ± 0.07^{a}	0.69 ± 0.16^{a}	0.35 ± 0.06^{a}	1.01 ± 0.18^{a}	0.02 ± 0.00^{b}	0.54 ± 0.11^{a}	0.25 ± 0.05^{a}
HCD	5.12 ± 0.45^{c}	0.43 ± 0.04^{a}	0.67 ± 0.06^{a}	0.32 ± 0.02^{a}	0.92 ± 0.05^{a}	0.02 ± 0.01^{a}	0.56 ± 0.07^{a}	0.22 ± 0.02^{a}
HCD+8% RMR	4.64 ± 0.59^{b}	0.38 ± 0.09^{a}	0.62 ± 0.04^{a}	0.22 ± 0.09^{a}	0.86 ± 0.18^{a}	0.03 ± 0.01^{a}	0.49 ± 0.10^{a}	0.27 ± 0.02^{a}
HCD+12% RMR	4.42 ± 0.09^{b}	0.55 ± 0.12^{a}	0.68 ± 0.09^{a}	0.33 ± 0.08^{a}	1.16 ± 0.04^{a}	0.03 ± 0.01^{a}	0.68 ± 0.11^{a}	0.26 ± 0.07^{a}
HCD+16% RMR	4.34 ± 0.51^{b}	0.61 ± 0.15^{a}	0.67 ± 0.10^{a}	0.35 ± 0.04^{a}	1.13 ± 0.23^{a}	0.03 ± 0.00^{ab}	0.69 ± 0.05^{a}	0.29 ± 0.06^{a}
HCD + 80 mg Lovastatin	4.37 ± 0.25^{b}	0.53 ± 0.07^{a}	0.68 ± 0.02^{a}	0.37 ± 0.10^{a}	1.11 ± 0.15^{a}	0.03 ± 0.01^{a}	0.67 ± 0.10^{a}	0.27 ± 0.04^{a}

Values are mean ± SD of six animals. Mean values within each column (liver) with different superscripts are significantly different Duncan's test at $p < 0.05$.

TABLE 7 | Effect of RMR on serum/Liver cholesterol and triglycerides levels in HCD fed male rats.

	Serum				Liver			
	7th week		14th week		7th week		14th week	
Conc. of RMR in diet (w/w)	cholesterol (mg dl^{-1})	Triglyceride (mg dl^{-1})	Cholesterol (mg dl^{-1})	Triglyceride (mg dl^{-1})	Cholesterol (mg dl^{-1})	Triglyceride (mg dl^{-1})	Cholesterol (mg dl^{-1})	Triglyceride (mg dl^{-1})
Control	66.24 ± 4.52^{a}	94.00 ± 25.36^{ab}	70.90 ± 13.80^{bc}	99.07 ± 11.43^{a}	96.29 ± 16.40^{a}	75.24 ± 8.57^{a}	62.96 ± 18.35^{a}	76.37 ± 5.24^{a}
HCD	150.02 ± 17.29^{e}	145.54 ± 38.43^{c}	165.81 ± 13.56^{e}	151.53 ± 13.02^{d}	189.93 ± 8.53^{d}	148.79 ± 58.92^{b}	152.19 ± 26.34^{d}	125.38 ± 14.93^{b}
HCD + 8% RMR	126.96 ± 6.37^{d}	110.25 ± 11.41^{b}	121.90 ± 6.48^{d}	113.77 ± 12.09^{b}	163.76 ± 14.10^{c}	107.05 ± 44.53^{ab}	114.67 ± 5.40^{c}	115.97 ± 18.78^{b}
HCD + 12% RMR	96.97 ± 9.78^{c}	94.29 ± 13.80^{ab}	105.20 ± 5.38^{cd}	103.07 ± 4.76^{bc}	133.20 ± 22.56^{b}	96.78 ± 42.44^{a}	96.72 ± 22.32^{bc}	95.05 ± 19.50^{a}
HCD + 16% RMR	82.78 ± 7.62^{a}	79.60 ± 11.27^{a}	83.91 ± 24.37^{ab}	86.35 ± 6.11^{ab}	97.89 ± 9.70^{ab}	86.17 ± 15.57^{a}	83.04 ± 22.68^{ab}	80.88 ± 10.99^{a}
HCD + 80 mg lovastatin	80.25 ± 5.42^{ab}	76.56 ± 5.25^{a}	77.85 ± 2.69^{a}	84.70 ± 1.90^{a}	92.50 ± 11.76^{a}	85.12 ± 12.17^{a}	79.75 ± 14.23^{ab}	79.80 ± 8.06^{a}

Control, normal diet (without cholesterol); HCD, High Cholesterol Diet (containing 1% cholesterol and 0.15% bile salts); HCD + 8 % RMR; HCD + 12 % RMR, HCD + 16 %; HCD + 80 mg Lovastatin Data are presented as mean ± SD (n = 6). Mean values within each column with different superscripts are significantly different ($p < 0.05$).

and RMR fed groups. RMR fed groups results with lower average body weight gain compared to control group at initial periods of feeding (1–7 weeks), however, no significant change in body weight gain was observed during 8–14 weeks of feeding. There were no marked differences in mean relative organ weights of various vital organs except in liver of HCD and RMR fed groups compared to that of control groups. Changes in liver weight are ascribed to high fat diet. The average weight of the liver of HCD fed rats increased by 14.7% over that control diet and addition of RMR reduced liver weights ranges from 10.46 to 8.27 g in rats fed diets containing 8, 12 and 16 % RMR, respectively (**Tables 5**, **6**). The results indicate that the daily food intake and gain in body weight of rats were normal and did not differ among various experimental groups.

Effect of RMR on Serum and Liver TC and TG Levels

Change in serum and liver TC and TG levels of rats fed HCD and HCD supplemented with different concentration of RMR were given in **Table 7**. High cholesterol diet fed group showed 2.26 and 1.97 fold increase in serum and liver cholesterol levels. Similarly high cholesterol diet induced 1.54 and 1.97 fold increase in serum and liver triacylglycerols, respectively. RMR supplementation in high cholesterol diet resulted in a dose dependent decrease in serum and liver cholesterol and triacylglycerol levels compared to high cholesterol fed group at 7 and 14 weeks. The cholesterol and triacylglycerol levels in serum were significantly decreased by 44.82 and 45.30%, respectively, in rats fed with 16% RMR for 7 weeks. Similarly, 48.45 and 42.08% decrease in liver cholesterol and triacylglycerols were observed in 16% RMR fed group for 7 weeks compared to high cholesterol fed group. However, at the end of 14 week, the decrease observed in serum and liver cholesterol and triacylglycerol levels in RMR fed groups were not relatively higher than 7 weeks feeding of RMR.

Effect of RMR on Serum and Liver HDL and LDL-C Levels

The effects of various concentration of RMR on serum HDL- and LDL-C levels are presented in **Table 8**. High cholesterol diet induced 3.62 fold increases in LDL-C level and 4.12 fold in LDL/HDL ratio compared to control animals. LDL-C level was increased by 5.14 fold in high cholesterol diet group. Supplementation of 16% RMR in the diet for 14 weeks significantly decreased LDL-C by 3.37 fold compared to high cholesterol control group.

TABLE 8 | Effect of RMR on serum HDL and LDL levels of HCD fed male rats.

Conc. of RMR in diet (w/w)	7th week				14th week			
	HDL-C (mg dl^{-1})	LDL-C (mg dl^{-1})	LDL-C/ HDL-C	TC/HDL-C	HDL-C (mg dl^{-1})	LDL-C (mg dl^{-1})	LDL-C/ HDL-C	TC/HDL-C
Control	33.30 ± 8.26^{a}	32.94 ± 2.77^{b}	0.99 ± 0.34^{b}	2.55	46.96 ± 3.71^{ab}	28.48 ± 2.10^{a}	0.61 ± 0.57^{b}	2.02
HCD	29.27 ± 8.33^{ab}	119.29 ± 11.07^{a}	4.08 ± 1.33^{a}	6.06	40.20 ± 2.71^{a}	146.61 ± 5.05^{c}	3.65 ± 1.86^{a}	5.40
HCD + 8% RMR	33.48 ± 4.07^{c}	92.48 ± 5.25^{b}	2.76 ± 1.29^{b}	4.42	48.51 ± 2.85^{ab}	92.30 ± 1.90^{b}	1.90 ± 0.67^{b}	3.37
HCD + 12% RMR	37.50 ± 3.88^{bc}	58.37 ± 5.18^{a}	1.56 ± 1.34^{a}	3.05	51.12 ± 1.93^{ab}	66.08 ± 1.51^{b}	1.29 ± 0.78^{a}	2.69
HCD + 16% RMR	42.92 ± 0.71^{a}	39.59 ± 1.19^{b}	0.92 ± 0.54^{bc}	2.29	54.43 ± 1.84^{b}	43.48 ± 1.45^{ab}	0.80 ± 0.79^{b}	2.11
HCD + 80 mg lovastatin	42.28 ± 2.35^{a}	38.12 ± 2.14^{a}	0.90 ± 0.91^{c}	2.26	55.28 ± 0.43^{b}	44.77 ± 0.89^{ab}	0.81 ± 2.08^{b}	2.11

Control, normal diet (without cholesterol); HCD, High Cholesterol Diet (containing 1% cholesterol and 0.15% bile salts); HCD + 8 % RMR; HCD + 12% RMR, HCD + 16%; HCD + 80 mg Lovastatin Data are presented as mean ± SD (n = 6). Mean values within each column with different superscripts are significantly different ($p < 0.05$). LDL-C low-density lipoprotein cholesterol, HDL-C high-density lipoprotein cholesterol.

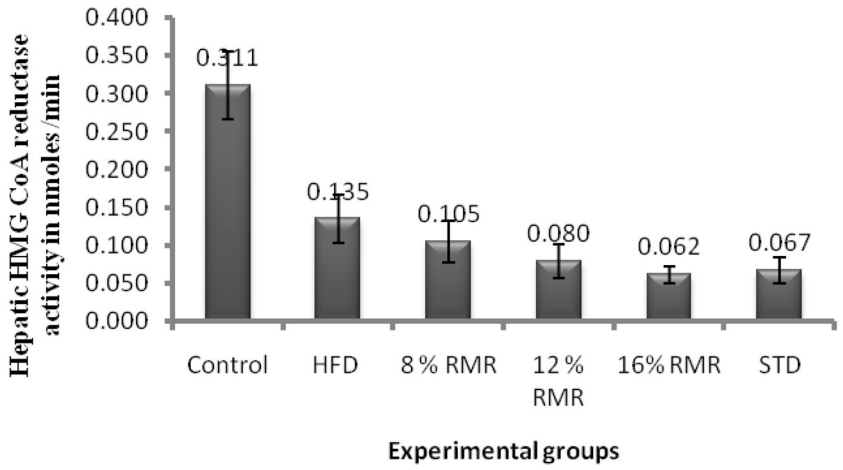

FIGURE 3 | Hepatic 3-hydroxy-3-methylglutaryl (HMG-CoA) reductase activity in rats fed with control and experimental diets for 14 weeks. Eight, twelve, and sixteen percentage fermented rice contains 4, 6, and 8 mg/g of lovastatin. Mean values within each group are significantly different Duncan's test at $p < 0.05$.

3-Hydroxy-3-methylglutaryl-CoA Reductase Activity

3-Hydroxy-3-methylglutaryl coenzyme A reductase is generally considered to catalyze the rate-limiting reaction in cholesterol biosynthesis. High cholesterol feeding decreased HMG-CoA reductase activity by 56.45 % in control rats (**Figure 3**). RMR supplementation in high cholesterol diet resulted in a dose dependent inhibition of HMG-CoA reductase activity in liver compared to high cholesterol HCD fed group at 14 weeks. RMR feeding reduced HMG-CoA reductase activity by 22–54% compared HCD fed rats.

No significant damage in liver tissue of RMR groups was observed in microscopic examination compared to control group and HCD group (**Figure 4**). Liver sections of group III, IV, V supplemented with RMR showed relatively moderate cholesterol infiltration compared to that of group II fed with HCD. Group VI fed with lovastatin showed fatty infiltration similar to that of RMR fed groups.

DISCUSSION

M. purpureus MTCC 410 RMR produces polyketides, statins (hydroxyl and lactone form of lovastatin and pravastatin), sterols and significant amount of saturated (palmitic and stearic) and unsaturated fatty acids (oleic, linoleic, and linolenic) by solid-state fermentation. Production of higher concentration of metabolites in solid-state fermentation can be attributed to better hyphal adhesion and invasion into the microstructure of the rice grain (Johns and Stuart, 1991). The hyphal mode of fungal growth is also tolerant to low water activity and high osmotic pressure conditions. Thus, the conditions apparently made the fungus competitive for efficient bioconversion of solid substrates to produce these products of interest in higher concentration.

Moreover, plant phytosterols, saponins, fungal metabolites such as mevinolin, monacolin K are being investigated for their antihyperlipidemia and antiatherosclerotic properties (Kroon et al., 1982; Harwood et al., 1993; Ikeda and Sugano, 1998). Monacolin K, the secondary metabolite produced during fermentation of rice by *Monascus* spp. has been shown to

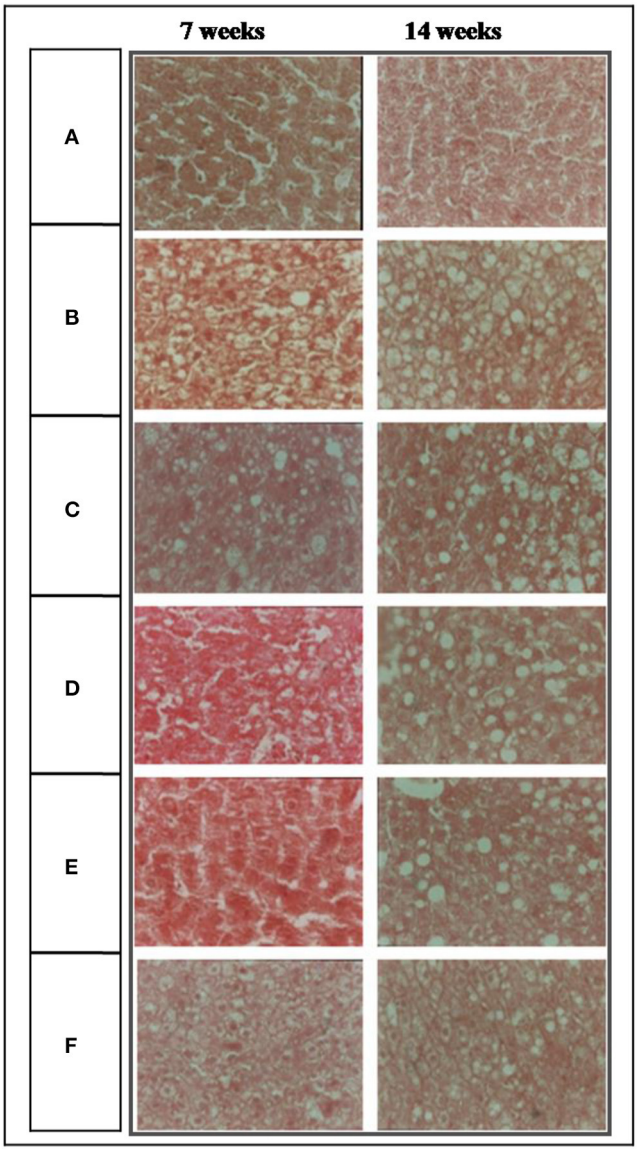

FIGURE 4 | Photomicrographs (400X) of liver of experimental rats. **(A)** Normal diet, **(B)** high fat diet (HFD), **(C)** (HFD +8% RMR), **(D)** (HFD +12% RMR), **(E)** HFD +16% RMR, and **(F)** HFD +0.08 mg lovastatin.

reduce cholesterol by inhibiting HMG-CoA reductase enzyme in liver. But the safety of RMR as a dietary supplement to reduce high cholesterol was a concern due to the presence of high concentrations of a mycotoxin- citrinin in different commercial *Monascus* products.

Hypercholesterolemia is considered as the major risk factors for atherosclerosis and cardiovascular diseases. Management of hyperlipidemia with healthy less atherogenic diet, less stressful life style, and physical activity is successful for certain patients, but a majority of patients need therapeutical intervention to effectively lower hyperlipidemia. Statins that inhibit HMG CoA reductase are widely used drugs to control hypercholesterolemia (Alberts, 1990; Blankenhorn et al., 1993).

In this study, supplementation of RMR of *M. purpureus* MTCC 410 to rats at a dose of 16% for 7 weeks showed, 44.82 and 48.45% reduction in serum and liver cholesterol levels; 45.30 and 42.08% reduction in serum and liver triacylglycerol, respectively, compared to HCD fed group. LDL-C levels decreased by 66.81% while HDL-C increased by 46.63% in serum of 16% RMR fed rats for 7 weeks. RMR feeding to hypercholesterolemia rats resulted in 22–54% inhibition of HMG-CoA reductase activity in liver. Thus, RMR is very effective in reducing cholesterol and triacylglycerol and LDL-C levels in hyperlipidemia rats. The combination of dietary sterols with statin drugs was also suggested for lowering cholesterol more effectively than statin alone (Plat and Mensink, 2001). The bioactive molecules sterols and statins identified in extract were attributed to the lipid lowering activity. The RMR been developed as a health food in human dietary supplements due to its functional properties like lowering cholesterol level and blood pressure. Further, RMR reduced liver weight and fatty infiltration, suggesting inhibition of lipids/fats deposition in liver. These findings are in concurrence with reported decrease in liver weight, lipid deposition in liver and atheroma in aorta of rabbits fed with *Monascus purpureus* (Li et al., 1998). While the clinical trials with *Monascus* spp. as dietary supplement in human subjects showed an effective reduction in serum cholesterol and triacylglycerol with mild side effects (Wang et al., 1997).

Functional food product marketed in the United States as Cholestin^{TM} by Pharmanex Inc., Simi Valley, CA (Wang et al., 1997). It is reported to contain many nutritional components including phytosterols, saponin, sapogenin, unsaturated fatty acids, isoflavones, trace elements zinc, selenium, and HMG-CoA reductase inhibitor (Wang et al., 1997). The exact mechanism by which RMR reduces serum cholesterol and triacylglycerols is not completely understood, because many *Monascus* species could synthesize small quantity ($<$500 mg Kg^{-1}) of monocolin K and only monacolin K cannot be accounted for hypolipidemic effect of *Monascus* (Chang et al., 2002; Casas-López et al., 2003). Therefore, *Monascus* species which can produce high monacolin K are being developed to apply as functional food. It is believed that, atherosclerotic index (ratio of non-HDL-cholesterol to HDL-cholesterol) is important risk factor for atherosclerosis. Our data clearly demonstrate that RMR significantly decreased the ratio, even in the lower dose group. The inhibition of HMG-CoA reductase by other forms of monacolins like, monacolin J, L, M, X, and their hydroxy acid form, as well as dehydromonacolin (MV2 and K), dihydromonacolin (MV and L), compactin, 3α-hydroxy-3,5-dihydromonacolin L are also accounted for the reduced cholesterol synthesis and anti-atherogenic effect of RMR (Li et al., 2004; Dhale et al., 2007a,b). Another risk factor for atherosclerosis is lower level of HDL-cholesterol. Whereas, feeding of RMR increased serum HDL-C, even in the lower dose group to increase HDL-C significantly. The HCD diet induced an increase in plasma and liver peroxides (malnoldehyde, lipid hydroperoxide and conjugated dienes) which may indicate elevated oxidative stress in the body. Monacolins, known as statins, isolated from *Monascus* spp. were found to protect body organs against oxidation of LDL and inhibit HMG-CoA reductase (Istvan and Deisenhofer, 2001). The statins play important role in atherosclerosis as

antioxidant by preventing the oxidation of LDL during oxidative stress. Cholesterol diet induces oxidative stress by conceding antioxidant molecules and antioxidant enzymes. The statins and supplementation of RMR containing statin, pigment and other secondary metabolites effectively reduced the oxidative stress by alleviating antioxidant molecules and enzymes in the liver of hypercholesterolemic rats (Mohan-Kumari et al., 2011a,b). The coronary heart disease (CHD) do not always have elevated levels of LDL but, the normal LDL levels with high levels of stress are more prone to CHD (Rosenson, 2004; Mohan-Kumari et al., 2011a). The increased oxidation of serum lipid and LDL-cholesterol in serum and its entry into artery leads to the formation of atherosclerotic lesion. The formation of atherosclerotic plaque in macrophages due to foam cells is prone to damage (Osterud and Bjorklid, 2003; Rosenson, 2004).

The feeding of RMR reduced such oxidative stress by modulating the antioxidant enzymes and molecules (Mohan-Kumari et al., 2011b). The data suggested RMR can lower or slowing down oxidative stress related atherosclerosis pathological process. However, the authentic comparison of the secondary metabolites of RMR is in their effects on severity of cholesterol-induced atherosclerosis. The cholesterol lowering and anti-atherogenic effect of RMR in this study is comparable to therapeutic drug, lovastatin. The monacolins also increase the activity of LDL-receptors and reduce total cholesterol and LDL levels in the body (Pitman et al., 1998). Thus, the significant inhibition of HMG Co-A reductase and anti-atherogenic effect of RMR observed in this study can be attributed to synergistic effect of monacolins (statins) which are suppressing cholesterol synthesis.

Commercial RMR products of *Monascus* contain high levels of citrinin, a mycotoxin affecting liver and kidney. Hence, the safety of RMR is of big concern to use it as a food supplement. Our earlier work, acute and sub-acute toxicological studies on RMR of *M. purpureus* MTCC 410 did not show any adverse effect in rats (Mohan-Kumari et al., 2009) and produced low levels of 1.427 mg kg^{-1} of citrinin. RMR supplementation in HCD did not induce any histopathological alterations in liver but moderately reduced fatty infiltration in liver compared to HCD fed group.

CONCLUSIONS

In conclusion, our data suggest that *M. purpureus* RMR can be considered as a potent anti-atherogenic food supplement with no adverse effects and possess health benefits related to regulation of blood lipids by inhibiting the HMG CoA reductase. The RMR significantly decreased LDL-C level in serum. The HDL value and HDL-C to LDL-C ratio were increased in rats fed on RMR.

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.

ETHICS STATEMENT

The animal study was reviewed and approved by Institute Animal Ethics Committee, Central Food Technological Research Institute (CSIR), Mysore.

AUTHOR CONTRIBUTIONS

HPMK conceptualized, executed the experiments, data analysis, and wrote the paper. KAN and GV designed the experiment and reviewed the paper. KN carried out histopathology. All the authors read and approved the final manuscript.

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REVIEW
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Agro-Industrial Residues: Eco-Friendly and Inexpensive Substrates for Microbial Pigments Production

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Many commodities are abundantly produced around the world, including soybean, corn, rice sugarcane, cassava, coffee, fruits, and many others. These productions are responsible for the generation of enormous amounts of daily residues, such as cassava and sugarcane bagasses, rice husk, and coffee peel. These residues are rich sources for renewable energy and can be used as substrates for industrial interest products. Microorganisms are useful biofactories, capable of producing important primary and secondary metabolites, including alcohol, enzymes, antibiotics, pigments, and many other molecules. The production of pigments was reported in bacteria, filamentous fungi, yeasts, and algae. These natural microbial pigments are very promising because synthetic colorants present a long history of allergies and toxicity. In addition, many natural pigments present other biological activities, such as antioxidant and antimicrobial activities, that are interesting for industrial applications. The use of inexpensive substrates for the production of these metabolites is very attractive, considering that agro-industrial residues are generated in high amounts and usually are a problem to the industry. Therefore, in this article we review the production of microbial pigments using agro-industrial residues during the current decade (2010–2020), considering both submerged and solid state fermentations, wild-type and genetically modified microorganisms, laboratorial to large-scale bioprocesses, and other possible biological activities related to these pigments.

Keywords: microbial pigments, biopigments, agro-industrial residues, biological activities, antimicrobial activity, antioxidant activity

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INTRODUCTION

Color has major importance in human activities. From traffic signals to arts and clothing, color has multiple cultural meanings. It is also central in interpreting edible items regarding their ingestion suitability, that is, if some food or beverage is fresh, ripe, safe, nutritional, or rotten (for instance, red vs. gray beef, green vs. yellow banana) (Sen et al., 2019).

In this sense, molecules capable of bestowing color to products (food products, in particular) are especially interesting from a technological standpoint. Such molecules are generally called pigments and comprise a variety of chemical structures able to absorb light in the visible range (400–700 nm wavelengths). By chemical definitions, soluble colored substances are called colorants, while insoluble colored substances are called pigments. Nevertheless, under a biological perspective, the colored substances are called pigments, irrespective of their solubility (De Carvalho et al., 2014).

Synthetic colorants have been facing market resistance starting from 1960s due to multiple reasons, including allergenicity, toxicity, teratogenicity, and carcinogenicity problems (Sen et al., 2019). In addition to that, multiple synthetic colorants depend on precursors that are petroleum-based, a non-renewable resource (Kumar et al., 2015). These restrictions, combined with an increased advertising desire to label products (majorly food) as natural, ecological, biological, or eco-friendly have sparked the study of biopigments (Babitha, 2009). Biological pigments are considered safe if they are non-toxic, non-allergenic, non-carcinogenic, and biodegradable (Sen et al., 2019).

Among biological pigment sources, microbes like bacteria, yeasts, molds, and algae are being targeted as ideal resources to be tapped. Microbial pigments are forefront in colorant development due to their independence from weather conditions, considerable assortment of shades, fast growth, and substrate-dependent cost effectiveness, characteristics considered to be superior in comparison to plant and animal-sourced pigments (Lopes et al., 2013; Panesar et al., 2015). Such advantages, combined with shifts in consumer preferences, have been responsible for a steep rise in the biopigment market, with natural colors expected to grow 7% annually as a category (De Carvalho et al., 2014; Sen et al., 2019).

Microbial pigments are especially interesting when one considers that the major cost regarding their production is related to microbes growth media. Thus, as a rule of thumb, low-cost medium translates into low-cost pigment (Panesar et al., 2015; Sen et al., 2019). Among such low-cost media, agro-industrial residues are being regarded as ideal substrates for microbial pigment production, serving as sources of carbon, nitrogen, and minerals (Lopes et al., 2013; Panesar et al., 2015; Sen et al., 2019).

The agro-industrial residues are defined as many different wastes from the food and agriculture industry. These residues include multiple plant-based materials, such as straws, stems, stalks, leaves, husks, shells, peels, lint, seeds, pulps, stubbles, bagasse, spent coffee grounds, brewer's spent grains, and some animal byproducts, including feathers and whey (Madeira et al., 2017; Venil et al., 2020a). The efficient transformation of these residues has become a central environmental issue in recent years, with great attention being given to energy generation (e.g., biodiesel production) (Vandamme, 2009). However, new materials, chemicals and valuable products in general are being obtained from agro-industrial waste, including pigments (Madeira et al., 2017). These added-value products are obtained from cheaper materials that otherwise would cause environmental damage, either directly (phenolic or via other toxic compounds) or indirectly (by changing nutritional aspects of the ecosystem, normally causing eutrophication).

Microbial pigments are already used for food and textile coloring, with proposed uses in candles, soaps, ballpoint, salmon, yogurt, and highlighter pens (Venil et al., 2013; Sen et al., 2019). The applications of microbial-derived pigments, however, are not restricted to coloring. Some of these compounds have additional properties, such as antioxidant, antiparasitic, antimicrobial, and anticancer (Venil et al., 2013, 2020a; Sen et al., 2019).

In this article, we review the current literature on microbial pigment production using agro-industrial residues as substrates, encompassing the last decade (2010–2020). Exceptions were made for cornerstone articles and chapters that were considered of major interest for the reader but were outside the selected timeframe.

PIGMENT-PRODUCING MICROORGANISMS

Microorganisms used in the production of biopigments include bacteria, yeasts, molds, and algae. The term "microbial pigments" is somewhat vague and can include some species that would not be considered *de facto* microorganisms (such as some filamentous fungi). In general, microbial pigment defines any non-plant, non-animal sourced biological pigment.

The microorganisms targeted for pigment production must satisfy a series of criteria: they should be non-pathogenic, non-toxic, able to use a wide range of carbon and nitrogen sources, able to give reasonable color yield, be tolerant to high salt concentration, be tolerant to variable temperatures and pH. They must also produce pigments that are easy to extract (Babitha, 2009; Kumar et al., 2015; Panesar et al., 2015; Venil et al., 2020a).

Biopigments can be classified based on chemical structure, with the main representatives being canthaxanthin, astaxanthin, prodigiosin, phycocyanin, violacein, riboflavin, β-carotene, melanin, and lycopene (Malik et al., 2012; Sen et al., 2019). All of them are currently used in the food industry (Sen et al., 2019). A variety of microbial pigments for which molecular identities were determined are shown in **Table 1**, along with synthesizing organism and pigment color. The molecular structure for some of the listed pigments is shown in **Figure 1**. For an updated list of macroscopic fungal pigments, please refer to Lagashetti et al. (2019).

AGRO-INDUSTRIAL RESIDUES USED FOR THE PRODUCTION OF MICROBIAL PIGMENTS

There is a need to explore novel strains of microorganisms and appropriate strategies for commercial production of microbial pigments (Nigam and Luke, 2016). Since synthetic culture media are usually expensive, the use of agro-industrial waste would be a profitable mean of reducing the production cost (Panesar et al., 2015). The Food and Agriculture Organization (FAO), in 2013, reported that 250 million tons of agro-industrial wastes are generated globally per year, during processing of different plant

TABLE 1 | Microbial pigments and their source organisms [based on data from Malik et al. (2012), Panesar et al. (2015), Lagashetti et al. (2019), and Sen et al. (2019)].

Pigment (class)	Color	Microorganisms
Ankaflavin	Orange	*Monascus purpureus*
Anthraquinone	Red	*Pacilomyces farinosus, Penicillium oxalicum,*
Arpink Red	Dark red	*Penicillium oxalicum*
Astaxanthin	Pink red	*Agrobacterium aurantiacum*, *Haematococcus pluvialis*, *Paracoccus carotinifaciens*, *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*)
Atrovenetin	Yellow	*Penicillium melinii*
Azaphilones	Red	*Penicillium purpurogenum*, *Talaromyces atroroseus*
Canthaxanthin	Red	*Bradyrhizobium* sp., *Brevibacterium* sp., *Dietzia maris*, *Haloferax alexandrinus*, *Lactobacillus pluvialis*, *Monascus roseus*
Carotenoids	Red	*Blakeslea trispora*, *Dunaliella salina*, *Fusarium sporotrichioides*, *Mucor circinelloides*, *Neurospora crassa*, *Phycomyces blakesleeanus*, *Rhodotorula rubra*
Cycloprodigiosin	Red	*Pseudoalteromonas denitrificans*
Granadaene	Orange red	*Streptococcus agalactiae*
Indigoidine	Blue	*Corynebacterium insidiosum*
Lutein	Yellow	*Chlorella* spp.
Lycopene	Red	*Blakslea trispora*, *Fusarium sporotrichioides*
Melanin	Dark brown, Black	*Bacillus thuringiensis* H-14, *Cryptococcus* sp., *Saccharomyces neoformans* var. *nigricans*, *Streptomyces virginiae*, *Yarrowia lipolytica*
Monascin	Yellow	*Monascus* sp.
Monascorubramine	Red	*Monascus* sp.
Naphthoquinone	Brownish yellow	*Fusarium* sp.
Naphtoquinone	Dark red	*Cordyceps unilateralis*
Phycocyanin	Blue	*Arthrospira* sp. (formerly *Spirulina* sp.), *Pseudomonas* spp.,
Phycoerythrin	Red	*Porphyridium cruentum*
Physcion	Yellow	*Aspergillus ruber*
Prodigiosin	Red	*Alteromonas rubra*, *Pseudoalteromonas rubra*, *Rugamonas rubra*, *Serratia* sp., *Streptomyces* sp., *Streptoverticillium rubrireticuli*, *Vibrio gaogenes*
Prodoginine	Red	*Streptoverticillium rubrireticuli*
Pyocyanin	Blue green	*Pseudomonas aeruginosa*
Riboflavin	Yellow	*Ashbya gossypi*, *Bacillus subtilis*
Rubrolone	Red	*Streptomyces echinoruber*
Rubropunctatin	Orange	*Monascus* sp.
Staphyloxanthin	Golden yellow	*Staphylococcus aureus*
Torularhodin	Orange red	*Rhodotorula glutinis*
Violacein	Violet	*Chromobacter violaceum*, *Janthinobacterium lividum*, *Pseudoalteromonas* spp.
Xanthomonadin	Yellow	*Xanthomonas oryzae*
Zeaxanthin	Yellow	*Flavobacterium* sp., *Paracoccus zeaxanthinifaciens*, *Sphingobacterium multivorum*, *Staphylococcus aureus*

crops, mainly cereals, starchy roots, fruits, and other vegetables (Heredia-Guerrero et al., 2017).

Natural raw materials and by-products generated by industry are widely used as culture medium due to their low cost, once the components of the medium can represent from 38 to 73% of the total production cost (Panesar et al., 2015). Agro-industrial residues are untreated and underutilized, while being rich in nutrient components, such as carbohydrates, proteins, fibers, minerals, and vitamins. The utilization of this waste not only eliminates the disposal problems, but also the environment pollution (toxicity to aquatic life, pollution of surface and ground waters, altered soil quality, phyto-toxicity, odorous, and colored natural waters) and negative impact on human and animal health (Panesar et al., 2015; Zihare et al., 2018; Nayak and Bhushan, 2019). The environmental concern is related to the content of these wastes, since most of them include phenolic compounds that have toxic potential, in addition these wastes exhibit high value of biological oxygen demand, chemical oxygen demand, and other suspended solids that can be considered pollutant to the environment (Sadh et al., 2018; Venil et al., 2020a). Furthermore, plant cell walls found in the agro-industrial residues are composed of lignocellulose, a recalcitrant component, consisting of cellulose, hemicellulose and lignin, that can also be an environment pollutant (Sánchez, 2009). Recovery of high value-added components from the waste and their re-utilization as food additives or therapeutics are another interesting aspect to valorize these agro-industrial residues, we will not focus on

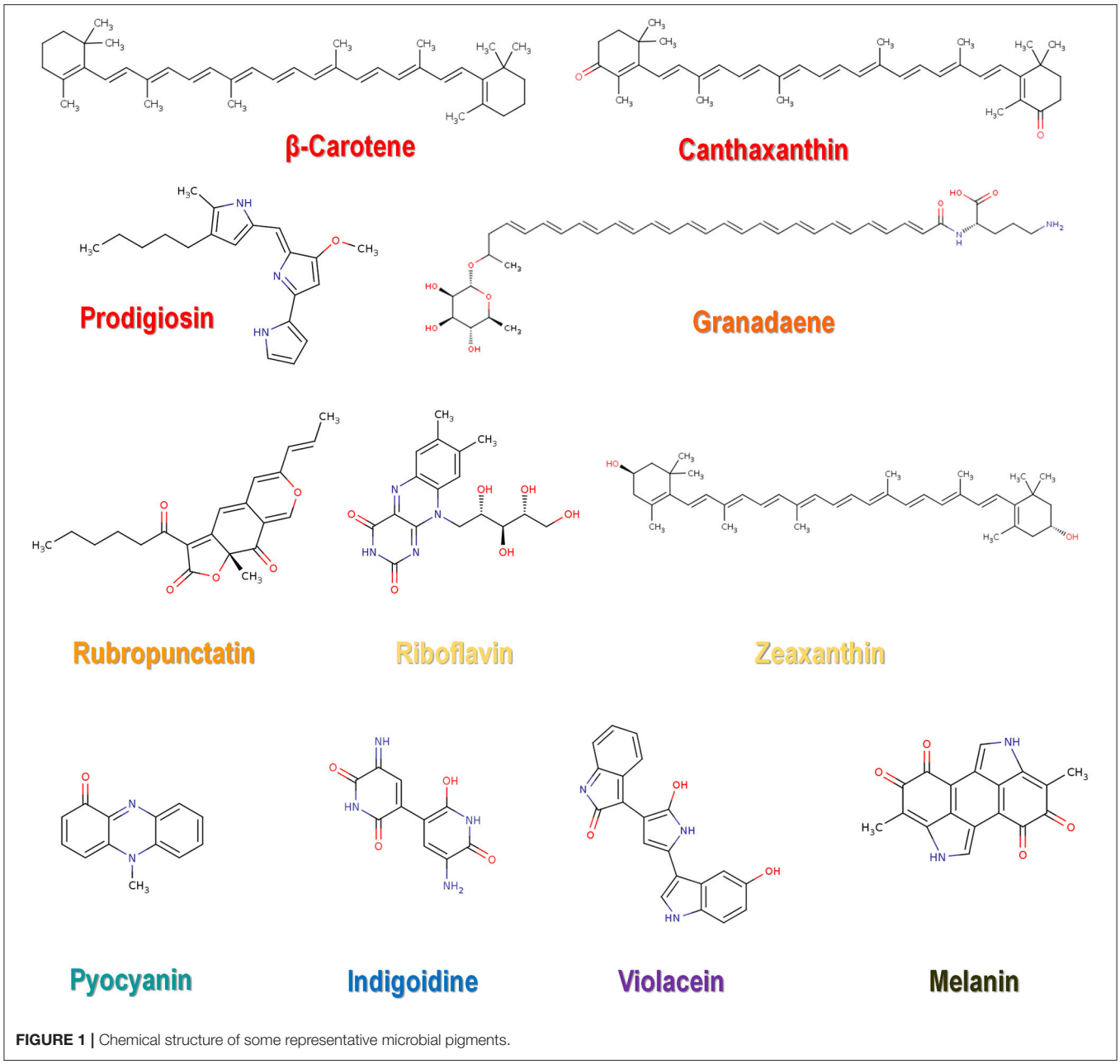

FIGURE 1 | Chemical structure of some representative microbial pigments.

this approach in the present review. For more information about this strategy, please refer to Nayak and Bhushan (2019). A representative scheme of the different steps for microbial pigment production using agro-industrial waste is proposed in **Figure 2**.

In this work we reviewed the literature from 2010 to 2020 and selected studies with the production of microbial pigments using agro-industrial residues (**Table 2**). We restricted our selection to focus on the microorganisms that can produce pigments in an inexpensive manner using agro-industrial residues that are alternatives to decrease the costs of production. During this decade, there were several works using waste as substrate, the sole substrate or supplemented with nutrients. The microorganisms more commonly used were fungi, both molds and yeasts. It is important to highlight some genera such as *Monascus* and *Rhodotorula* that were the most prevalent in these studies, especially *Monascus purpureus*, *Rhodotorula glutinis*, and *R. mucilaginosa*. Furthermore, carotenoids and *Monascus*-polyketides were thus the pigments commonly produced by these microorganisms.

Yeasts are unicellular and have high growth rates, which favor these microorganisms in the production of biopigments (Bhosale and Gadre, 2001). Yeasts are pigment producers

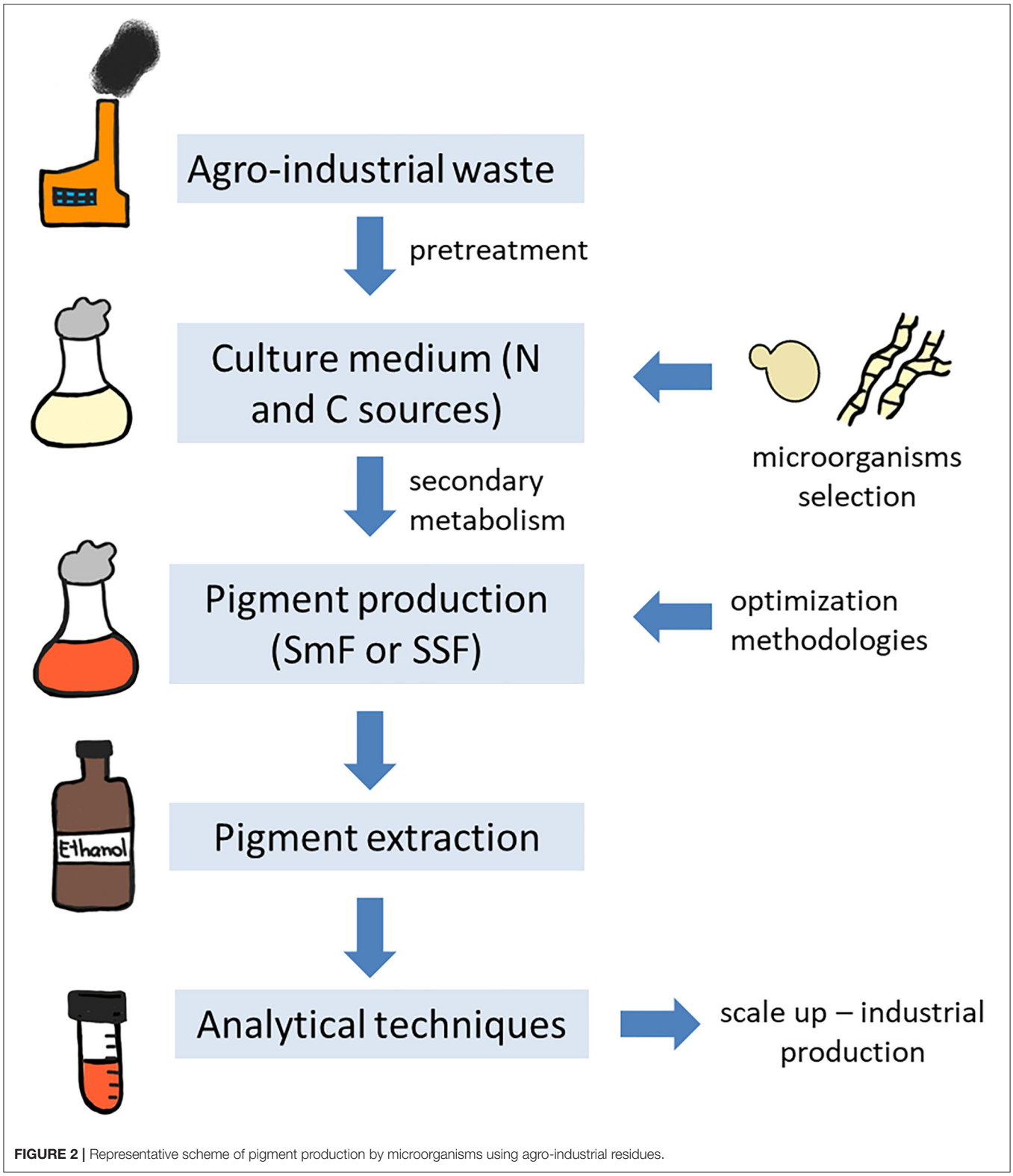

FIGURE 2 | Representative scheme of pigment production by microorganisms using agro-industrial residues.

considered as safer in comparison to filamentous fungi, due to the concerns about mycotoxin production by some of the latter. For instance, *Monascus* spp. is well-known for the production of citrinin, a nephrotoxic and hepatotoxic mycotoxin (Blanc et al., 1995). However, some genetic studies were published, currently modifying genes, eliminating non-essential genes of filamentous

TABLE 2 | Microbial pigments produced with agro-industrial waste and fermentation conditions (2010–2020).

Agro-industrial residues	Microorganisms	Type	Pigment	Fermentation	Scale	Additional information	References
Petiole oil palm fronds	*Monascus purpureus* FTC 5357	Filamentous fungus	Red pigments	SSF	Laboratory	Peptone to supplement, 30°C, 8 days, 75% moisture, yield: 207 AU/g.d	Daud et al., 2020
High test molasses/sweet whey/corn steep liquor	*Erwinia uredovora* DSMZ 30080 and *Rhodotorula glutinis* number 32	Bacterium and yeast	Carotenoids	SmF	Laboratory (100 ml)	150 rpm, 4 days, 30°C, dark improved carotenoid production, yield: 1.46 ± 0.02 mg/L	Galal and Ahmed, 2020
Peanut seed oil	*Serratia marcescens* 11E	Bacterium	Prodigiosin	SmF	Laboratory (100 ml)	28°C, 150 rpm, 36 h, yield: 2 g/L	Hernández-Velasco et al., 2020
Paper mill sludge/sugarcane bagasse	*Planococcus* sp. TRC1	Bacterium	β-carotene	SSF	Laboratory (100 g)	Supplemented with minimal salt media and yeast extract, 30°C, 120 h, mixing every 12 h, 80% moisture, yields: 38.54 ± 1.4 mg/g and 47.13 ± 1.9 mg/g	Majumdar et al., 2020
Onion peels/mung bean husk	*Rhodotorula mucilaginosa* MTCC-1403	Yeast	β-carotene, phytoene, torulene and Torularhodin	SmF	3 L bioreactor stirred tank	pH 6.1, 25.8°C, 119.6 rpm, 84 h, 1.0 vvm, yield: 719.69 μg/g	Sharma and Ghoshal, 2020
Maltose syrup	*Monascus ruber* CCT 3802	Filamentous fungus	Orange, yellow and red pigments	SmF and SSF	Laboratory (petri dishes and 120 ml)	pH influenced the pigment production, 30°C, 120 rpm, 192 h	De Oliveira et al., 2019
Fruits and vegetable waste	*Blakeslea trispora* MTCC884	Filamentous fungus	Carotenoids	SSF	Laboratory	28°C, 4 days, 200 rpm, yield: 0.127 mg/mL	Kaur et al., 2019
Bengal gram husk	*Talaromyces purpureogenus* CFRM02	fungus	Red pigments	SmF	Laboratory (100 ml)	pH 5.5, 12 h day and night, 30°C, 10 days, 110 rpm, yield: 0.565 ± 0.05 AU/mL	Pandit et al., 2019
Oil palm frond	*Monascus purpureus* FTC 5356	fungus	Red pigments	SSF	Laboratory	50% moisture, pH 6, 2% peptone, 100% petiole, and 10^8 spores/mL, 8 days, 30°C, yield: 2.93 AU/g	Said and Hamid, 2019
Cassava wastewater	*Rhodotorula glutinis* CTT 2182	Yeast	Carotenoids	SmF	Laboratory (150 ml)	30°C, 200 rpm, 120 h, darkness, yield: 0.98 mg/L	Santos Ribeiro et al., 2019
Mesquite pods/corn steep liquor	*Xanthophyllomyces dendrorhous* ATCC 24202	Yeast	Carotenoids	SmF	Laboratory (25 ml)	Mesquite pods extract (2%), corn steep liquor (0.3%) and yeast extract (0.3%), pH 5.5, 120 h, 200 rpm, 20°C, yield: 293.41 ± 31.12 μg/g	Villegas-Méndez et al., 2019
Sugarcane juice	*Rhodotorula rubra* l02	Yeast	Carotenoids	SmF	Laboratory (250 ml)	1.9% reducing sugar, 2% sucrose-and maltose-based media, Mg^{2+}(0.16 % and 0.196 %), 30°C, 200 rpm and 1,600 lm of fluorescent lighting for 72 h, yield: 30.39 mg/g	Bonadio et al., 2018
Corn cob	*Monascus purpureus* ATCC 16436	Filamentous fungus	Orange and red pigments	SSF	Laboratory	24 g corn cobs, 2.17 M glycerol, pH 4.5, 30°C, 10 days, 150 rpm, 12 × 10^{11} spores/mL, yield: 133.77 UA/mL and 108.02 UA/mL	Embaby et al., 2018
Sugarcane bagasse hydrolysate	*Monascus ruber* Tieghem IOC 2225	Filamentous fungus	Red pigment	SmF	Laboratory (50 ml)	30°C, 150 rpm, 12 days, supplementation with glucose and cellobiose, darkness, yield: 18.71 AU	Hilares et al., 2018

(Continued)

TABLE 2 | Continued

Agro-industrial residues	Microorganisms	Type	Pigment	Fermentation	Scale	Additional information	References
Waste orange peels	*Monascus purpureus* ATCC 16365 and *Penicillium purpurogenum* CBS 113139	Filamentous fungi	Yellow, orange and red pigments	SSF, Semi-SSF and SmF	Laboratory	5 g orange peels, 65% moisture, 25°C and 30°C, yields: 9 AU/g, 0.95 AU/mL, 0.58 AU/mL	Kantifedaki et al., 2018
Coffee husk/pulp extract	*Rhodotorula mucilaginosa* CCMA 0156	Yeast	Carotenoids	SmF	Laboratory (300 ml)	8.36% coffee husk extract, 0.636% glucose, 0.368% peptone and 0.5% tween 80, 10^7 cell/mL, 28°C, 160 rpm, 5 days, dark, yields: 16.36 ± 0073 mg/L and 21.35 ± 0067 mg/L	Moreira et al., 2018
Crude glycerol/Corn maceration water/rice parboiling water	*Sporidiobolus salmonicolor* CBS 2636	Yeast	Carotenoids	SmF	2 L bioreactor Stirred tank (semi-continuous process)	8% crude glycerol, 8% corn maceration water, and 2% rice parboiling water, 25°C, pH 4.0, 180 rpm, 1.5 vvm, 50% working volume, yields: 34.8 g/L/h and 41.4 g/L/h	Colet et al., 2017
Cassava bagasse	*Rhodotorula mucilaginosa*	Yeast	Carotenoids	SmF	Laboratory (100 ml)	2% cassava bagasse, pH 6.0, 25°C, 4 days, yield: 12.0–12.5 mg/L.	Manimala and Murugesan, 2017
Wastes of potato chips manufacturing	*Monascus purpureus* Went NRRL 1992	fungus	Red, orange and yellow pigments	SSF	Laboratory (10 g)	67% moisture, pH 6.5, 1.5 mm particle size and 2% ammonium sulfate, 140 × 10^3 spores/10 g dry substrate, 15 days, 30°C, darkness, yields: 126.5, 204.7, and 322.9 AU/g	Abdel-Raheam et al., 2016
Liquid pineapple waste	*Chryseobacterium artocarpi* CECT 8497	Bacterium	Yellowish-orange pigment	SmF	50 L- Bioreactor stirred tank	20% liquid pineapple waste, 12.5 % L-tryptophan 1.25 % KH_2PO_4, 30°C, 200 rpm, a. r. 10 L/min, pH 7.0, yield: 152 mg/L	Aruldass et al., 2016
Olive pomace	*Xanthophyllomyces dendrorhous* ATCC24202 and *Sporidiobolus salmonicolor* ATCC24259	Yeasts	Astaxanthin	SSF	Laboratory (100 g)	15°C, pH 4.5, 90% moisture, 10^6 cells/mL, 12 days, yield: 220.24717.47 mg/gdp	Eryilmaz et al., 2016
Bakery waste	*Monascus purpureus* ATCC 16365	Filamentous fungus	Orange, yellow and red pigments	SSF and SmF	Laboratory	5 g bread waste, moisture 60%, 30°C, 100 mL, 250 rpm (SmF), yield: 24 AU/g glucose	Haque et al., 2016
Glycerol and soy peptone bagasses	*Serratia marcescens* Xd-1	Bacterium	Prodigiosin	SSF	Laboratory (2 g)	0.17% glycerol bagasse, 033% soy peptone bagasse, moisture 83.5%, 1 mm particles of bagasse, 28°C, 48 h with mixing every 12 h, yield: 40.86 g/kg	Xia et al., 2016
Liquid pineapple waste	*Chromobacterium violaceum UTM5*	Bacterium	Violacein and deoxyviolacein	SmF	50 L -Bioreactor stirred tank	10% pineapple waste, 24 h, 30°C, 200 rpm, a.r. 10 L/min, pH 7.0, yield: 16256 ± 440 mg/L	Aruldass et al., 2015
Sugarcane bagasse hydrolysate	*Dietzia maris* NIT-D	Bacterium	Trans-canthaxanthin (carotenoid)	SmF	Laboratory (50 ml)	1.5% of total reducing sugars, 2% peptone, 0.5% yeast extract, 0.25% NaCl and 1 mg/100 mL of glutamic acid, pH 5.5, 25°C, 5 days, 120 rpm, 2% inoculum	Goswami et al., 2015

(Continued)

TABLE 2 | Continued

Agro-industrial residues	Microorganisms	Type	Pigment	Fermentation	Scale	Additional information	References
Raw glycerol/corn steep liquor/sugarcane molasses	*Sporodiobolus pararoseus* CCT 7689	Yeast	Carotenoids	SmF	Laboratory (250 ml)	3% raw glycerol and 5.29% corn steep liquor or 4% sugar cane molasses, 0.65% corn steep liquor, 25°C, 180 rpm, 1×10^7 cell/mL 168 h darkness, yield: 520.94 μg/L	Machado and de Medeiros Burkert, 2015
Carob pulp syrup/sugarcane molasses	*Rhodosporidium toruloides NCYC 921*	Yeast	Carotenoids	SmF	Laboratory (200 ml)	7.5% sugarcane molasses, 10% carob pulp syrup, pH 5.5, 30°C, 150 rpm, yield: 9.79 μg/L/h	Freitas et al., 2014
Slaughterhouse wastewater	*Phormidium autumnale*	Microalgae	Carotenoids	SmF	Bubble column bioreactor (2 L)	26°C, pH 7.6, C/N ratio of 30, 1 vvm, darkness, residence time of 168 h, yield: 107,902.5 kg/year	Rodrigues et al., 2014
Raw glycerol/corn steep liquor/parboiled rice water	*Sporidiobolus pararoseus*	Yeast	Carotenoids	SmF	Laboratory (100 ml)	4% glycerol, 4% corn steep water, 2% parboiled rice water, 25°C, pH 4.0, 180 rpm, yield: 843 μg/L	Valduga et al., 2014
Raw glycerol	*Monascus ruber* CCT 3802	Filamentous fungus	Yellow, orange and red pigments	SmF	Bioreactor stirred tank (4 L)	1% glucose, 1% glycerin, 0.5% glycine, 0.5% K_2HPO_4, 0.5% KH_2PO_4 and micronutrients, 30°C, 350 rpm, 1 vvm, pH 6.5, yield: 8.28 UA	Bühler et al., 2013
Grape waste/cheese whey/soybean meal/feather meal/soy protein/rice husk	*Penicillium chrysogenum* IFL1 and IFL2, *Fusarium graminearum* IFL3, *Monascus purpureus* NRRL 1992, *P. vasconiae* IFL4	Filamentous fungi	Yellow, orange and red pigments	SmF	Laboratory (50 ml)	1% of each waste, pH 6.5, 7 days, 30°C, 125 rpm, 10^6 spores/mL	Lopes et al., 2013
Brewery wastewater	*Rhodotorula glutinis* ATCC 15125	Yeast	Carotenoids	SmF	Laboratory (500 ml)	115 rpm, 25°C, 168 h, yields: 0.6 and 1.2 mg/L	Schneider et al., 2013
corn cob waste stream cellulose	*Penicillium resticulosum*	Filamentous fungus	Red pigments	SmF	Laboratory (50 ml)	12 days, 25°C, 60–70%, relative humidity, dark, yield: 497.03 ± 55.13 mg/L	Sopandi et al., 2013
Vegetable cabbage waste	*Pseudomonas* sp.	Bacterium	Melanin	SmF	Laboratory (50 ml)	25°C, 200 rpm, 48–72 h, 2.79 mg/mL	Tarangini and Mishra, 2013
Sugarcane bagasse	*Chromobacterium violaceum*	Bacterium	Violacein	SmF	Laboratory (50 ml)	3 g sugarcane bagasse, 10% L-tryptophan, 200 rpm, 30°C, 24 h, yield: 0.82 g/L	Ahmad et al., 2012
Corn meal	*Monascus purpureus* CMU001	Filamentous fungus	Red pigments	SSF	6 × 10-in. plastic bags	5 g of waste, salt solution, 8% glucose, 30°C for 14 days, 1×10^6 spores/mL, yield: 129.63 U/gds	Nimnoi and Lumyong, 2011
Waste chicken feathers	*Rhodotorula glutinis* MT-5	Yeast	Carotenoids	SmF	Laboratory (100 mL)	0.8% peptone of chicken feather, 4% glucose, 0.4% yeast extract, pH 6.0, 30°C, 200 rpm, yield: 6.47 mg/g	Taskin et al., 2011
Corn cob powder	*Monascus purpureus* KACC 42430	Filamentous fungus	Yellow and red pigments	SSF	Laboratory (5 g)	60% moisture, 30°C, 4 mL of spores/gram of dry substrate, 7 days, yield: 25.42 OD Units/gram	Velmurugan et al., 2011
Rice bran	*Rhodotorula glutinis*	Yeast	β-carotene	SSF	Laboratory (5 g)	pH 5, 70% moisture,C:N ratio 4, yield: 2.12 mg/kg rice bran	Roadjanakamolson and Suntornsuk, 2010

SSF, Solid State Fermentation; SmF, Submerged Fermentation; vvm, volume of air per volume of liquid per minute; a.r., aeration rate; C:N ratio, carbon:nitrogen ratio.

fungi in order to increase the production of pigment and decrease mycotoxin secretion (Lagashetti et al., 2019). In the case of *Monascus*, several techniques were performed to decrease the production of citrinin: changes in the nitrogen composition of the medium, the dissolved oxygen or the pH, as well as genetic alterations of the strains (Sen et al., 2019).

In the reviewed studies, some investigated the production of citrinin by *Monascus* strains. *M. purpureus* Went NRRL 1992 did not produce citrinin using potato waste in a solid-state fermentation. The mycotoxin detection was performed by thin layer chromatography (Abdel-Raheam et al., 2016). *M. purpureus* NRRL 1992 produced citrinin in potato dextrose agar, with mycotoxin production being evaluated by ESI-MS/MS (electrospray ionization *tandem* mass spectrometry). Nevertheless, the authors did not evaluate its production in the agro-industrial residues (Lopes et al., 2013). *M. purpureus* ATCC 16365 also produced citrinin in orange processing waste, however the authors did not demonstrate the methodology used in this study to detect citrinin (Kantifedaki et al., 2018). The detection of mycotoxin production by filamentous fungi is very important, considering the industrial production of pigments and it is a fundamental step to guarantee the safety of the final product.

Microorganisms from these studies were wild-type, and many of them were purchased from collection cultures or isolates. This fact is surprising due to the great advances regarding mutation techniques and heterologous expression with the purpose of obtaining strain improvement. Strain development is important because pigments produced by wild type strains are usually too low in quantity and take longer fermentation periods, making the process uneconomical (Sen et al., 2019). Due to the recent application of molecular techniques to improve pigment production, more studies will be probably published in a near future with recombinant strains grown on agro-industrial residues.

Regarding the wastes, pre-treatment is important for promoting breakdown of these residues, mainly formed by cellulose, hemicellulose and lignin. This breakdown will increase the availability of the nutrients from the substrate to the microorganisms. Various pre-treatment methods such as physical, chemical, biological (enzymatic), and combined are available (nee'Nigam et al., 2009). In the selected studies, the agro-industrial wastes were pre-treated (treated prior to use). Some examples of physical pre-treatment deal with paper mill sludge and sugarcane bagasse that are milled and sieved to achieve an uniform size of particles (Majumdar et al., 2020), as well as onion peels and mung bean husk (Sharma and Ghoshal, 2020), petiole oil palm fronds (Daud et al., 2020) and fruits and vegetable wastes (Kaur et al., 2019). Chemical treatments of the waste were performed in some studies. Bengal gram husk was pre-treated with hydrochloride acid, promoting an acid hydrolysis to improve the availability of the substrate (Pandit et al., 2019). Sugarcane bagasse was pre-treated under alkaline condition with sodium hydroxide and afterwards hydrolyzed with a commercial cellulase complex (Hilares et al., 2018). In this case there was the combination of a biological treatment. Bakery waste was also hydrolyzed with enzymes produced by *Aspergillus awamori* and *Aspergillus oryzae* before its use (Haque et al., 2016). In the majority of the studies, the agro-industrial wastes are used as carbon, nitrogen, and micronutrient sources by microorganisms. However, in some cases, the residues can be added as an inert support for the fermentation process, such as the bagasse used in the prodigiosin production by *Serratia marcescens* (Xia et al., 2016).

Brazil is one of the biggest agricultural commodity producers, since it is considered as the world's biggest producer of sugar, coffee, orange juice, and soybeans (Da Silva Vilar et al., 2019). In this review, 11 studies were performed in Brazil, due to the great availability of such residues in this country. Wastes used in these works were maltose syrup, cassava wastewater, sugarcane (juice, molasses, and hydrolyzed bagasse), solid coffee waste, crude glycerol, rice parboiling water, slaughterhouse wastewater, corn steep liquor (or corn maceration water), grape waste, cheese whey, soybean meal, feather meal, soy protein, and rice husk) (**Table 2**). In order to evaluate each residue as carbon and/or nitrogen source, **Table 3** brings an average composition of the agro-industrial residues regarding carbon and nitrogen composition, according either to the works cited in this review or, in some cases to the composition obtained from other works, when this information was not available. It is important to highlight that this composition is variable, depending on the source of the waste; however, an average composition can be useful for planning new projects in this area.

MODES OF FERMENTATION AND SCALE OF PRODUCTION

Traditional methods of microorganism isolation, culture and products extraction are now substituted by novel biotechnological techniques and strategies, via the advent of genetic engineering and fermentation technologies (Nigam and Luke, 2016). Different types of fermentation are used to produce pigments depending on the chosen strain and the type of pigment that will be extracted. Production of pigments by fermentation has a great number of advantages, including abundance of raw materials, absence of seasonal variation, cheaper production, easier extraction, perfectible yields, and procurement of biodegradable pigments (Venil et al., 2013; Charalampia et al., 2017). In addition, pigments produced by microorganisms through fermentation present higher stability to heat, to light exposure and pH variations, and are highly soluble in water (when compared to plant pigments) (Nigam and Luke, 2016). Some types of pigments are only produced by microorganisms (Dufossé, 2006) and the possibily of using industrially important species, such as *Escherichia coli* and *Saccharomyces cerevisiae*, to express these pigments heterologously are excellent alternatives (Venil et al., 2013).

Microorganisms produce pigments mainly by two types of fermentations processes: solid state fermentation (SSF) and submerged fermentation (SmF). In the SSF technique, the substrates are used by the microorganisms very slowly, and then the same substrate can be used for longer periods. This technique provides controlled release of nutrients during the process (Subramaniyam and Vimala, 2012). This type of

TABLE 3 | Average carbon and nitrogen composition of the agro-industrial residues.

Agro-industrial residue	Carbon-composition	Nitrogen-composition	References
Bakery waste	Starch (37.42 ± 0.032 g)	Proteins (14.72 ± 0.55 g) and other nitrogen sources (2.58 ± 0.97 g)	Haque et al., 2016
Carob pulp syrup	Sugar (75 g L^{-1} reducing sugar)	ni	Freitas et al., 2014
Carrot peel	Carbohydrates (2.98 ± 0.75 g)	Proteins (9.70 ± 0.25 g)	Chantaro et al., 2008
Cassava wastewater	Carbohydrates (58.11 ± 2.13 g)	Total nitrogen (1.94 ± 0.08 g)	Santos Ribeiro et al., 2019
Cheese whey	Lactose (77%)	Proteins (13%)	Lopes et al., 2013
Coffee pulp and husk	Carbohydrates (17.10–13.38%)	Proteins (24.21–30.50%)	Moreira et al., 2018
Corn cob	Cellulose (32.3–45.6 %), hemicellulose (39.8 %)	ni	Embaby et al., 2018
Corn steep liquor	Sugar (0.1–11.0% glucose)	Total nitrogen (2.7–4.5%)	Liggett and Koffler, 1948
Feather meal	ni	Protein (83.7%)	Lopes et al., 2013
Grape waste	Carbohydrates (21%)	Proteins (10%)	Lopes et al., 2013
High test molasses	Sugar (74–79%)	Low amounts (0.1%)	Murphy, 1984
Liquid pineapple waste	Sugars (reducing sugar, glucose, sucrose, fructose)	ni	Aruldass et al., 2016
Maltose syrup	Maltose and glucose (70.0 g L^{-1} maltose and 2.23 g L^{-1} glucose)	ni	De Oliveira et al., 2019
Mesquite pods	Sucrose, glucose, fructose	ni	Villegas-Méndez et al., 2019
Mung bean husk	Sugar (327.78 ± 2.08 mg/g)	Proteins (262.23 ± 3.59 mg/g)	Sharma and Ghoshal, 2020
Olive pomace	Fatty acids (59.03% and 63.81%)	Proteins (2.43 ± 0.00%) to (3.87 ± 0.17%) and other nitrogen sources (0.39 ± 0.0%) to (0.62 ± 0.02%)	Wedyan et al., 2017
Onion peels	Sugar (851.33 ± 4.62 mg/g)	Proteins 165.10 ± 5.13 mg/g)	Ifesan, 2017; Sharma and Ghoshal, 2020
Orange peel	Cellulose (71.2 g/kg), hemicellulose (128 g/kg)	Crude protein (57.2 g/kg)	Ahmadi et al., 2015
Papel mill sludge	Cellulose (62%), hemicellulose (15%) and lignin (20%)	ni	Majumdar et al., 2020
Parboiled rice water	Present	Present	Valduga et al., 2014
Peanut seed oil	Fatty acids (80% of these fatty acids are either oleic acid or linoleic acid)	ni	Rachaputi and Wright, 2015
Petiole oil palm fronds	Cellulose (44%), hemicellulose (27.3%) and lignin (10.1%)	ni	Ikubar et al., 2018
Rice bran	ni	Protein (114.2 g/kg)	Roadjanakamolson and Suntornsuk, 2010
Rice husk	Cellulose (31.12%), hemicellulose (22.48%) and lignin (22.34%)	ni	Kumar et al., 2010
Slaughterhouse wastewater	ni	Total nitrogen (128.5 ± 12.1 mg/L)	Rodrigues et al., 2014
Soybean meal	Carbohydrates (35%)	Proteins (50%)	Lopes et al., 2013
Soybean protein	Carbohydrates (12%)	Proteins (79%)	Lopes et al., 2013
Sugarcane bagasse	Cellulose (32–34%), hemicellulose (19–24%) and lignin (25–32%)	ni	Haghdan et al., 2016
Sugarcane juice	Sugar (19 g reducing sugar)	ni	Bonadio et al., 2018
Sweet whey	Lactose (4.5%)	Proteins (0.8%)	Morr, 1989
Wastes of potato chips	Starch (61.49%)	Low amounts of protein	Korish and El-Sanat, 2007

ni, not informed.

fermentation is suitable to filamentous fungi and microorganisms that require low moisture content for their growth. Their growth as SSF mimic (Joshi and Attri, 2006). This technique is very effective in waste valorization due to the microorganism conversion of waste into value-added products with low power supply and high yield. Arikan and co-authors observed higher production of a yellow pigment by *Aspergillus carbonarius* using pomegranate pulp through SSF than SmF (Arikan et al., 2020). According to Carvalho et al. (2005), the majority of *Monascus* researchers observed pigment production varying from hundreds of absorbance units per mL culture medium, in SmF; in contrast thousands of absorbance units/g dry substrate was observed

in SSF (Carvalho et al., 2005). In some cases, however, the higher production using SSF requires longer times of culture in comparison with SmF. The low energy to perform a SSF is due to the simplified bioreactors used in this type of fermentation, mainly when we compare some types of SmF bioreactors, such as stirred tank, that requires high energy consumption to maintain constant agitation during the process. In addition, it is considered an easy process, with minimal pretreatment of the waste being necessary (sometimes not needed at all) and generating less wastewater (Wang and Yang, 2007; Arun et al., 2020). Some of the common substrates used in SSF are wheat bran, rice and rice straw, fruit and vegetable waste, paper pulp, bagasse, and coconut coir (Panesar et al., 2015).

On the other hand, in SmF the substrates are consumed very rapidly, then it is possible to replace or supplement the culture media (fed batch or continuous culture strategies are necessary in some cases), or to use fast-growing microorganisms during the process (Subramaniyam and Vimala, 2012). It is important to highlight that in some cases supplementation of the medium in SSF is also needed, because of the poor nature of the substrate or low availability of the nutrients in the beginning of the bioprocess (substrates that are not pretreated, for example). This type of fermentation is commonly used for bacteria, due to their necessity of higher moisture in the fermentation process (Subramaniyam and Vimala, 2012). Some common substrates used in SmF are soluble sugars, molasses, fruit and vegetable juices, and sewage/wastewater (Panesar et al., 2015). Based on the data of this review (**Table 2**), the majority of the studies use SmF to produce pigments instead of using SSF. Solid-state fermentation systems could present some advantages because of the potential with natural substrates. Nonetheless, pigment production is performed commercially almost entirely in SmF (Sánchez-Muñoz et al., 2020). The main obstacles to SSF are the low amenability of the process to regulation, the heterogeneous fermentation conditions and the low reproducibility of the results. Moreover, some difficulties to scale-up may also appear, as well as often unfeasible determination of biomass or complicated product purification by downstream processes (Hölker and Lenz, 2005).

Agro-industrial residues decrease the costs of the fermentation process. Maximization of the pigment production while decreasing the production costs has been the goal of current techniques applied to produce microbial pigments at large scale. Medium optimization is especially important to maximize the production. Optimizing the medium includes controlling the conditions of the fermentation, such as temperature, pH, aeration, agitation, and media components. Process optimization techniques have used statistical experimental designs and response surface analysis with limited use of artificial intelligence (like genetic algorithms). Response surface methodology (RSM) is an effective approach for the process optimization in pigment production (Gharibzahedi et al., 2012; Sen et al., 2019).

Some studies reported in this review used statistical methodologies to optimize the production of pigments, with RSM being by far the most used. Sharma and Goshal optimized pH, temperature, and agitation conditions in the production of carotenoids by *R. mucilaginosa* (Sharma and Ghoshal, 2020). Embaby and co-authors used a three-step optimization, including RSM, to study the concentration of glycerol and inoculum size in the production of orange and red pigments by *M. purpureus* using corn cobs (Embaby et al., 2018). Aruldass and co-authors used RSM to optimize the production of yellowish-orange pigments by *Chryseobacterium artocarpi*. The authors used three independent variables: concentrations of liquid pineapple waste, L-tryptophan, and KH_2PO_4 (Aruldass et al., 2016). The production of astaxanthin by *Xanthophyllomyces dendrorhous* in olive pomace was optimized with RSM, testing temperature, moisture content and pH condition (Eryilmaz et al., 2016). Prodigiosin production by *S. marcescens* was optimized by RSM with three variables: glycerol bagasse, soybean peptone bagasse, and initial moisture content (Xia et al., 2016). Raw glycerol, steep liquor, and sugarcane molasses concentrations were tested in the optimization experiments of carotenoids production by *Sporidiobolus pararoseus*, as well as glycerol, corn steep liquor and parboiled rice water concentrations (Valduga et al., 2014; Machado and de Medeiros Burkert, 2015). Moisture, pH, and carbon-to-nitrogen ratio were evaluated in the carotenoids production by *R. glutinis* using rice bran (Roadjanakamolson and Suntornsuk, 2010). The Taguchi methodology was applied to optimize the production of carotenoids by *Xanthophyllomyces dendrorhous*, using four variables: concentrations of mesquite pods extract, corn steep liquor, yeast extract, and malt extract (Villegas-Méndez et al., 2019).

Another approach used in some studies is the optimization of one factor at a time (OVAT). For instance, the authors evaluated pH, temperature, and light on the carotenoids production by *Planococcus* sp. (Majumdar et al., 2020). Optimization of various factors such as pH, growth temperature, incubation time and the addition of nitrogen components was evaluated on the production of β-carotene by *Blakeslea trispora* (Kaur et al., 2019). Five operational factors such as the initial moisture content of oil palm frond, initial pH, supplementation of nitrogen source, the percentage of petiole to leaflet, and inoculum size were investigated on the red pigments production by *M. purpureus* (Said and Hamid, 2019). In the conventional approach OVAT, the conditions are optimized by changing one factor at a time while maintaining the other variables constant. This is a simple approach to implement and helps to choose the main variables. However, this methodology is time-consuming, because several experiments are necessary to study all the relevant conditions and also ignores the combined interaction(s) among the variables (Vishwanatha et al., 2010).

Variables that were most frequently evaluated in the optimization studies were pH, substrate concentration, and initial moisture. The pH is particularly important in the pigments production. pH of the medium plays an important role in activating key enzymes involved in pigment production and excretion by *M. pupureus* CCT3802, according to Orozco and Kilikian (2008). In addition, pH influences the color of the pigments. According to De Oliveira and co-authors, pH values close to neutrality lead to the formation of red pigments by *Monascus* sp. (De Oliveira et al., 2019). On the other hand, medium pH around 3 results in the production of

yellow pigments by *Monascus anka* (Shi et al., 2015). Substrate concentration is considered as an important variable to study, in particular the nitrogen source is a key regulation factor. It has been consistently shown that selective nitrogen sources largely influence the composition of *Monascus* pigments and this variable correlates with pH of the medium (Shi et al., 2015), highlighting the importance of the statistical methodologies to study the interactions among variables. Moisture is also a significant variable in SSF systems. Adequate water content of the substrate facilitates the oxygen transport, promoting microorganism growth. Nevertheless, excessive water content may lead to the reduction of oxygen transfer and diffusion because of substrate agglomeration, lowering the porosity of the substrate and increasing the risk of contamination, since an advantage of SSF is to select microorganisms that grow under low water content (Said and Hamid, 2019). Indeed, some filamentous fungi and yeasts are capable to grow at a water activity (a_w) of 0.61, the lowest a_w value for growth of microorganisms (Grant, 2004).

Large scale production with high yields and low costs of production are the biggest challenges faced by industry. Recent developments in molecular biology could be crucial. The genes responsible for the synthesis of several pigments have been cloned and recombinant DNA technology would be an alternative to overproduction of pigments. With the advances in the gene technology, the scientists intend to create cell factories for the production of pigments using the heterologous expression of biosynthetic pathways from already known or novel pigment producers (Malpartida and Hopwood, 1984; Pfeifer and Khosla, 2001; Venil et al., 2013). Martínez and co-authors reviewed the recombinant production of melanin by *Escherichia coli*, *Pseudomonas putida*, and *Streptomyces kathirae* (Martínez et al., 2019). In another work, the authors deleted the 15-kb citrinin biosynthetic gene cluster in *M. purpureus* industrial strain KL-001, using CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas system, obtaining a mutant strain that did not produce the mycotoxin and also increased red pigment production in 2–5% (Liu et al., 2020). From a technical point of view, the vast majority of the studies reported in the present review, were at a laboratory scale. Nevertheless, certain pigments are in a more advanced stage of scaling up. Some studies used SmF bioreactors, such as stirred tank and bubble column (Bühler et al., 2013; Rodrigues et al., 2014; Aruldass et al., 2015, 2016; Colet et al., 2017; Sharma and Ghoshal, 2020). These more advanced studies are interesting, since they show the high potentialities of scaling up microbial pigments targeting a future industrial application.

Industrial scale synthesis of microbial pigments is mainly limited by the high costs of production, the co-production of toxins in some of these processes, and the resistance of the final product to extreme processes conditions, such as high temperature and extreme pHs (conditions that are found in industrial processes) (Narsing Rao et al., 2017). Some microbial pigments are produced industrially, such as β-carotene (*Blakeslea trispora* and *Dunaliella salina*) and lycopene (*Blakeslea trispora)*, *Monascus*-derived pigments in Natural Red^{TM} (*Penicillium oxalicum*), riboflavin *(Aspergillus gossypii*), phycocianin (*Spirulina platensis*), and astaxanthin (*Paracoccus carotinifasciens* and *Haematococcus pluvialis*) (Dufosse et al., 2014). The estimation of the universal food colorant market is anticipated to achieve 3.75 billion USD by 2022 (Venil et al., 2020b). Besides, the carotenoids (astaxanthin, betacarotene, canthaxanthin, lutein, lycopene, zeaxanthin) market in 2019/2020 is supposed to reach $1.5–1.8 billion with annual growth rate of 3.9% (Barredo et al., 2017; Venil et al., 2020a). The global demand for natural pigments, including microbial pigments is high and tends to grow exponentially in the next years.

OTHER BIOLOGICAL ACTIVITIES OF MICROBIAL PIGMENTS PRODUCED WITH AGRO-INDUSTRIAL WASTE

Microbial pigments not only add color, but they also have interesting biological properties such as antioxidant, antimicrobial, anticancer, immunoregulation, anti-inflammatory, antiproliferative, and immunosuppressive activities (among others) (Kirti et al., 2014; Manimala and Murugesan, 2014; Kumar et al., 2015; Sen et al., 2019; Muthusamy et al., 2020). Because of these pharmacological properties, there are many more advantages of using natural pigments over synthetic colorants (Venil et al., 2013).

The most common biological activities were antibacterial and antioxidant in the reviewed studies (**Table 2**). The antimicrobial activity of pigments can be a strategy to improve the source microbe ability of competing with other microorganisms (De Carvalho et al., 2014). The violet crude extract produced by *Chromobacterium violaceum* using liquid pineapple waste contains the pigments violacein and deoxyviolacein. This extract has antibacterial activity against *Staphylococcus aureus* ATCC 29213 and methicillin-resistant *S. aureus* (MRSA) (Aruldass et al., 2015). The extracted β-carotene produced by *Planococcus* sp. TRC1 using paper mill sludge and sugarcane bagasse was active against *Bacillus subtilis*, *Salmonella enterica*, *Escherichia coli*, and *Proteus vulgaris*. In addition, this pigment had antioxidant activity (Majumdar et al., 2020). Red pigment produced by *Talaromyces purpureogenus* using Bengal gram husk was active against *B. cereus*, *B. subtilis*, *S. aureus*, *Micrococcus luteus*, *E. coli*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, and *Salmonella typhimurium*. The extracted pigment presented antioxidant activity and was non-toxic to the crustacean *Artemia franciscana* (Pandit et al., 2019). Carotenoids produced by *R. mucilaginosa* using solid coffee waste exhibited antioxidant and antimicrobial activities against pathogenic bacteria: *Salmonella cholerasus*, *E. coli*, *S. aureus*, and *L. monocytogenes*, as well as against toxigenic fungi like *Aspergillus flavus*, *A. parasiticus*, *A. carbonarius,* and *A. ochraceus* (Moreira et al., 2018). β-carotene produced by *Blakeslea trispora* using fruit and vegetable waste, astaxanthin produced by *Xanthophyllomyces dendrorhous* in olive pomace, and melanin produced by *Pseudomonas* sp. using cabbage waste, also presented antioxidant activity (Tarangini and Mishra, 2013; Eryilmaz et al., 2016; Kaur et al., 2019).

CONCLUSIONS AND CHALLENGES

The use of agro-industrial residues as substrate for microbial pigment synthesis is a green, sustainable way of solving a pollution problem while cutting costs in the production of added value assets. Besides reducing the carbon footprint, microbial pigments also satisfy a growing demand for natural colorants. The majority of these pigments are also vegan, circumventing the need for animal-based colors (dairy and poultry-waste based microbe production would not be vegan, however).

Considering the advantages of using microorganisms as pigment factories, there is a growing need for biodiversity sampling, in search for new molecular entities (including greater color variety). Not only that, but genetic engineering and synthetic biology approaches are expected to provide strains with increased productivity and tolerance to cultivation conditions. Brazil is an interesting place to bioprospect new microorganisms, due to its great natural biodiversity and its agriculture-based economy, providing a large variety of agro-industrial waste to be studied.

Besides the use of a strain that produces high yields of pigments in an inexpensive medium, the current and future challenges in this area are related to the safety of the final products, due to the mycotoxin co-produced by some of the microorganisms. Technologies to produce microorganisms with these characteristic are available and in some cases, they are not expensive, such as the new genome- editing methodology CRISPR. Another important approach is the stability of the natural pigments that are in some cases a problem for the final product. More studies about different modes of production are needed in order to find new pigments with high stability to be applied in different industries. Lastly, the use of agro-industrial residues needs to be more implemented in industries not only to decrease the costs of the production but also to execute a circular economy, that is an eco-friendly approach, extremely necessary in the current days.

The road is still long and largely unpaved for microbe-based biopigments, and as their use expands, so will the development of associated technologies with proportional cost reduction. In summary, the microbial pigment production using agro-industrial residues is a rare win-win scenario, in which "One man's trash is another man's treasure."

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Realm of Microbial Pigments in the Food Color Market

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Colors are added to food items to make them more attractive and appealing. Food colorants therefore, have an impressive market due to the requirements of food industries. A variety of synthetic coloring agents approved as food additives are available and being used in different types of food prepared or manufactured worldwide. However, there is a growing concern that the use of synthetic colors may exert a negative impact on human health and environment in the long run. The natural pigments obtained from animals, plants, and microorganisms are a promising alternative to synthetic food colorants. Compared to animal and plant sources, microorganisms offer many advantages such as no seasonal impact on the quality and quantity of the pigment, ease of handling and genetic manipulation, amenability to large scale production with little or no impact on biodiversity etc. Among the microorganisms algae, fungi and bacteria are being used to produce pigments as food colorants. This review describes the types of microbial food pigments in use, their benefits, production strategies, and associated challenges.

Keywords: microbial pigment, food, fermentation, bio-colorant, market

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INTRODUCTION

The world cannot be imagined without colors and this is equally true for the food that we eat. Some food materials such as fruits, vegetables etc. have striking natural shades and hues and therefore, do not require any further coloration. However, for many food items addition of food colorants is an integral part of their recipe before final packaging or serving. Food colorants enhance the visual appeal and grant unique identity to the food so that it could look more attractive and seem enjoyable to eat. Many times, food color is also associated with the flavor, safety and nutritional value (Sigurdson et al., 2017). The market of food colorants was estimated to be USD 3.88 billion in 2018 and it is estimated to reach USD 5.12 billion by 2023 with a compound annual growth rate (CAGR) of 5.7% [Food Colors Market by Type (Natural, Synthetic, Nature-Identical), Application (Beverages, Processed Food, Bakery & Confectionery Products, Oils & Fats, Dairy Products, Meat, Poultry, Seafood), Form, Solubility, and Region—Global Forecast to 2023[1]]. Food coloring has been known to be in practice as early as 1,500 BC (Burrows, 2009). Earlier, all the colorants used were of natural origin such as saffron, paprika, turmeric, various flowers etc. (Burrows, 2009). In the midst of nineteenth century, synthetic colors were started to be produced and owing to their low production cost, high tinctorial strength, and chemical stability they became popular as food colorants (Sigurdson et al., 2017). However, in later years several health issues were realized with the

[1] https://www.marketsandmarkets.com/Market-Reports/food-colors-market-36725323.html

use of many potentially hazardous synthetic chemicals as food colorants which led to the banning of various such food color additives e.g., Quinoline Yellow, Yellow 2G, Ponceau SX, Brilliant Black B etc.[2]. At present, although strict regulations are in practice in different countries toward approval of a synthetic colorant for intended use as food additive, people with the growing awareness about personal health and environment are now more inclined toward their substitute obtained from natural sources. However, there is still a substantial share of synthetic colors in the market of food colorants. Mono- and di-azo dyes are the most commonly used synthetic food colorants approved by FDA and EU. The other approved food grade colorants include Triarylmethane derivatives, xanthenes dyes, quinophthalones, and indigoid compounds (Corradini, 2018).

Natural pigments, the colored compounds synthesized by plants, animal, microorganisms or derived from mineral ores are a promising alternative to the synthetic food colorants (Corradini, 2018). Titanium dioxide (E171), calcium carbonate (E170), iron oxides (E172) are some examples of mineral pigments approved as food colorants by FDA[3]. Although for similar shades the cost of synthetic colors is on lower side for most cases in comparison to natural colors but the mass production of natural colors may fill this gap. Unlike synthetic colorants, they have nutritional values and associated with cytotoxic, antioxidant, antimicrobial, antimalarial, anticancer, antitumor, and antifouling activities (Ramesh et al., 2019) (**Figure 1**). Not only the natural colorants/pigments but their identical compounds made by chemical processes are also exempted from the certification process before use as food additive (Sen et al., 2019). Although plants are a major source of natural pigments, pigments obtained from microbial sources offer special advantages. Compared to plants, microbial pigments are more stable, cost-effective, uninfluenced by seasonal variations, amenable to yield improvement, and smoothly extractable (Nigam and Luke, 2016). Also, the excessive use of microbial culture for pigment production is not likely to harm the biodiversity and environment. Currently, a variety of different food color additives produced through fermentative processes are available in the market. Monascus pigments, Astaxanthin from *Xanthophyllomyces dendrorhous*, Arpink Red from *Penicillium oxalicum*, Riboflavin from *Ashbya gossypii*, and carotene from *Blakeslea trisporatrispora* (Venil et al., 2013) are the examples of some microbial food grade pigments.

Pigment production is one of the strategies of bacteria to escape from adverse effect of UV radiations. The photo-protective pigments help bacteria to cope up with prolonged exposure to UV radiation (Wynn-Williams et al., 2002). Some of these pigments are also well-known for their ability to provide protection against oxidative damage which helps in stimulation of immune response and cancer inhibition (Krinsky and Johnson, 2005). Symbiotic pigmented bacteria are known to protect their host from other pathogens (Egan et al., 2002). Fungi also produce pigments as a protection strategy against abiotic stresses like UV radiation and desiccation (Issac, 1994). Endophytic fungi have been reported to protect the host plant from insects or other microorganisms by producing the pigment Anthraquinones (Gessler et al., 2013). In microalgae, pigments are known to play light harvesting, photo protective and structural roles and they are also involved in carbon and energy storage (Mulders et al., 2014). Microbial pigments are thus more than simple coloring compounds because of the associated biological activities that can be of potential human benefit in case of their use as food additives. This article comprises the up to date details about the types, advantages and challenges related to the production and use of microbial food pigments.

[2] http://importedfoods.afdo.org/food-color-additives-banned-in-the-usa.html
[3] http://ifc-solutions.com/food-coloring/mineral-pigments

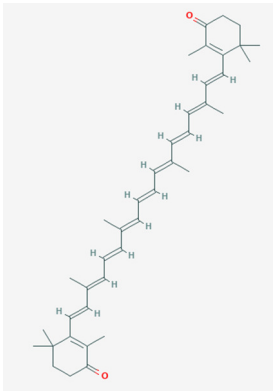
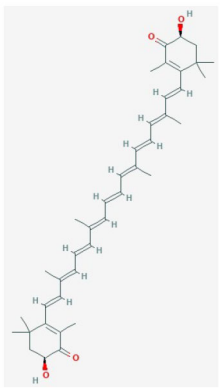
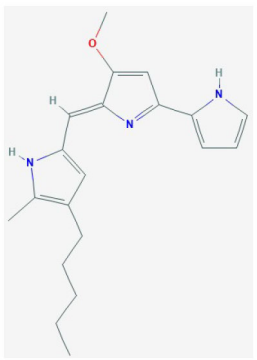
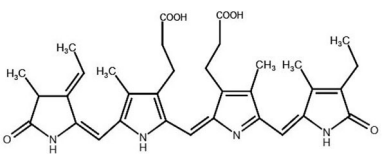
TYPES OF MICROBIAL PIGMENTS AS FOOD COLORANTS

Among microorganisms, fungi, bacteria, and microalgae are well-known to produce a range of natural pigmented substances having marked variation in chemical compositions, function, stability and solubility. These naturally occurring pigments are reflection of the secondary metabolites with great commercial value in food & dairy, cosmetics, pharmaceutical, textile, and dyeing industry (Narsing Rao et al., 2017). They belong to distinct categories based on their chemical composition, functional activities and natural occurrence such as derivatives of flavonoids, pyrroles, carotenoids, etc. Riboflavin, Beta-carotene, Canthaxanthin, Prodigiosin, Phycocyanin, Melanin, Violacein, Astaxanthin, and Lycopene are the major pigments reported from microbial sources having application as food colorants (Sen et al., 2019). The important natural food pigments reported to be produced by microorganisms and their advantages are discussed below and their details are also summarized in **Tables 1**, **2**.

1. Riboflavin, also known as B2 vitamin, is a water soluble pigment of yellow color having applications as a dietary supplement and food additive in dairy products, sauces, baby foods, fruit, and energy juices. It helps body break the dietary polymeric compounds such as carbohydrates, proteins, and fats to generate energy and use oxygen. Microorganisms such as *Candida guilliermundii, Debaryomyces subglobosus, Eremothecium ashbyii, Ashbya gossypi*, *Clostridium acetobutylicum* have been reported to produce it (Unagul et al., 2005; Hong et al., 2008; Nigam and Luke, 2016; Dufossé, 2018).

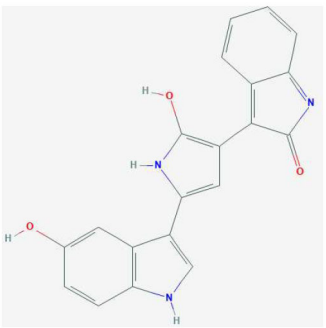
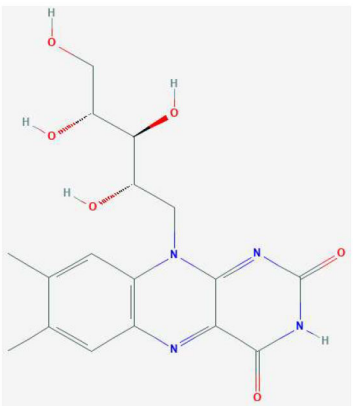
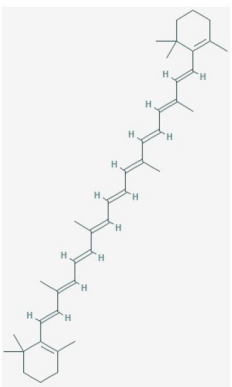
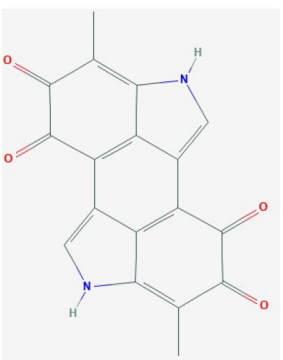
2. Beta-carotene, a red-orange water insoluble organic pigment, is a very good source of vitamin A for human body that boosts immunity, prevents aging and helps in night vision issues (Eroglu et al., 2012). Several microorganisms such as *Dunaliella salina, Blakeslea trispora, Mucor circinelloides, Phycomyces blakesleeanus, Rhodotorula glutinis, Rhodotorula gracilis, Rhodotorula rubra* are known to produce it (Ruegg, 1984; Nigam and Luke, 2016; Sigurdson et al., 2017; Dufossé, 2018). Some natural colorants belonging to carotenoid family obtained from *Haematococcus pluvialis* and *Phaffia rhodozyma* are extensively used as food additives for animals and fish as well as in pharmaceuticals and aquaculture fields (Stafsnes et al., 2010).

TABLE 1 | Structural details of important microbial pigments used as food colorants.

Pigment	Molecular formula	Chemical structure	References
Canthaxanthin	$C_{40}H_{52}O_2$		National Center for Biotechnology Information. PubChem Compound Summary for CID 5281227, Canthaxanthin[1].
Astaxanthin	$C_{40}H_{52}O_4$		National Center for Biotechnology Information. PubChem Compound Summary for CID 5281224, Astaxanthin[2].
Prodigiosin	$C_{20}H_{25}N_3O$		National Center for Biotechnology Information. PubChem Compound Summary for CID 135455579[3].
Phycocyanin	$C_{33}H_{38}N_4O_6$		Ramesh et al., 2019

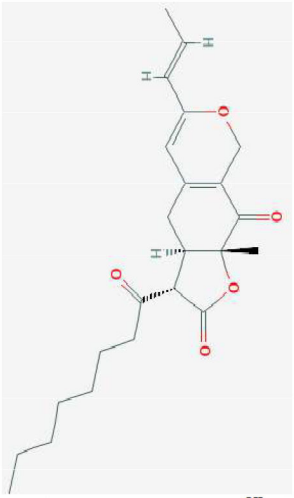
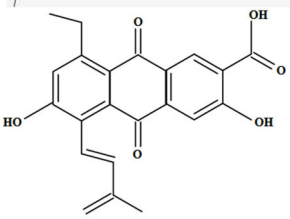
(Continued)

TABLE 1 | Continued

Pigment	Molecular formula	Chemical structure	References
Violacein	$C_{20}H_{13}N_3O_3$		National Center for Biotechnology Information. PubChem Compound Summary for CID 11053, Violacein[4].
Riboflavin	$C_{17}H_{20}N_4O_6$		National Center for Biotechnology Information. PubChem Compound Summary for CID 493570, Riboflavin[5].
Beta-carotene	$C_{40}H_{56}$		National Center for Biotechnology Information. PubChem Compound Summary for CID 5280489, beta-Carotene[6].
Melanin	$C_{18}H_{10}N_2O_4$		National Center for Biotechnology Information. PubChem Compound Summary for CID 6325610, Melanin[7].

(Continued)

TABLE 1 | Continued

Pigment	Molecular formula	Chemical structure	References
Lycopene	$C_{40}H_{56}$		National Center for Biotechnology Information. PubChem Compound Summary for CID 446925, Lycopene[8].
Ankaflavin	$C_{23}H_{30}O_5$		National Center for Biotechnology Information. PubChem Compound Summary for CID 15294091, Ankaflavin[9].
Arprink Red	$C_{22}H_{18}O_6$		Ramesh et al., 2019

[1]*https://pubchem.ncbi.nlm.nih.gov/compound/Canthaxanthin (accessed December 21, 2020).*
[2]*https://pubchem.ncbi.nlm.nih.gov/compound/Astaxanthin (accessed December 21, 2020).*
[3]*https://pubchem.ncbi.nlm.nih.gov/compound/135455579 (accessed December 21, 2020).*
[4]*https://pubchem.ncbi.nlm.nih.gov/compound/Violacein (accessed December 21, 2020).*
[5]*https://pubchem.ncbi.nlm.nih.gov/compound/Riboflavin (accessed December 21, 2020).*
[6]*https://pubchem.ncbi.nlm.nih.gov/compound/Melanin (accessed December 21, 2020).*
[7]*https://pubchem.ncbi.nlm.nih.gov/compound/beta-Carotene (accessed December 21, 2020).*
[8]*https://pubchem.ncbi.nlm.nih.gov/compound/Lycopene (accessed December 21, 2020).*
[9]*https://pubchem.ncbi.nlm.nih.gov/compound/Ankaflavin (accessed December 21, 2020).*

3. Canthaxanthin is orange to dark pink colored, keto-carotenoid and lipid soluble pigment. There are reports of its production by Bacteriochlorophyll containing microbes such as *Bradyrhizobium* sp. and *Halobacterium* sp. (Jaswir et al., 2011; Surai, 2012; Chuyen and Eun, 2017). The production of canthaxanthin from microalgae like *Nannochloropsis gaditana* (Millao and Uquiche, 2016) and *Chlorella zofingiensis* (Li et al., 2006) has also been reported. Canthaxanthins are effective antioxidants and inhibit the oxidation of lipids in liposomes (Woodall et al., 1997).

TABLE 2 | Microbial sources, bioactivity and applications of natural pigments.

Pigment	Source organism	Bioactivities known	Applications	References
Canthaxanthin	• *Bradyrhizobium Sepp*, • *Halobacterium* sp.	• Antioxidant • Anticancer	Food colorant, salmon food and poultry feed	Jaswir et al., 2011; Surai, 2012; Chuyen and Eun, 2017; Hamidi et al., 2017
Astaxanthin	• *Halobacterium salinarium,* • *Agrobacterium aurantiacum* • *Paracoccus carotinifaciens* • Yeast • Microalgae	• Antioxidant • Photoprotector • Anti-inflammatory • Antimicrobial	Animal and fish food and food colorants	Dufossé, 2009, 2016; Guedes et al., 2011; Asker, 2017; Pogorzelska et al., 2018
Prodigiosin	• *Serratia marcescens* • *Pseudoalteromonas rubra*	• Anticancer • Antimetastatic activity • Immunosuppressant • Antimalarial	Coloring agents in yogurt, milk, and carbonated drinks	Nagpal et al., 2011; Namazkar and Ahmad, 2013; Kamble and Hiwarale, 2012
Phycocyanin	• *Aphanizomenonflos-aquae* • *Spirulina* sp. • *Pseudomonas* spp.	• Cytotoxicity • Apoptosis • Anti-alzhelmeric Activity • Antioxidant	Sweets and ice cream	Barsanti et al., 2008; Eriksen, 2008; Cuellar-Bermudez et al., 2015;
Violacein	• *Chromobacterium violaceum* • *Janthinobacterium lividum* • *Pseudoalteromonas tunicata* • *Pseudoalteromonas* spp.	• Anticancer • Antioxidant • Antifungal • Antiviral • Anti-tuberculosis • Ant parasitic • Antiprotozoal • Anti-HIV • Anti-malarial	Used in food, cosmetic, and textile industries	Matz et al., 2004; Konzen et al., 2006; Durán et al., 2012; Dufossé, 2018
Riboflavin	• *Candida guilliermundii*, • *Debaryomyces subglobosus,* • *Eremothecium ashbyii*, • *Ashbya gossypii*, • *Clostridium acetobutylicum*	• Anticancer • Antioxidant • Protection against cardiovascular diseases • In vision	In food industry	Powers, 2003; Unagul et al., 2005; Hong et al., 2008; Dufossé, 2018
Beta-carotene	• *Dunaliella salina*, • *Blakeslea trispora*, • *Mucor circinelloides*, • *Phycomycesblakes leeanus*, • *Rhodotorula glutinis*, • *Rhodotorula gracilis*, • *Rhodotorula rubra*	• Anticancer • Antioxidant • Suppression of cholesterol synthesis	Food colorant, Vitamin A source	Ruegg, 1984; Terao, 1989; Kot et al., 2016 Sigurdson et al., 2017
Melanin	• *Saccharomyces*, • *Neoforman*	• Antioxidant • Antibiofilm • Antimicrobial • Anti-HIV	Eye glasses, Cosmetic creams and food items	Vinarov et al., 2003; Valla et al., 2004; Surwase et al., 2013
Lycopene	• *Lycopersicon esculentum*, • *Fusarium* sp. • *Sporotrichioides* • *Blakesleatrispora*	• Antioxidant • Anticancer	Meat colorant	Di Mascio et al., 1989; Giovannucci et al., 2002
Arpink red	• *Penicillium oxalicum*	……..	Food colorant	Kumar et al., 2015
Monascus pigments (ankaflavine, monascine)	• *M. pilosus,* • *M.purpureus* • *M. rubera* • *M. froridanus*	• Antimicrobial • Anticancer • Anti-obesity activities	Food colorant	Joshi et al., 2003; Feng et al., 2016

4. Prodigiosin, a red colored multipurpose pigment, is reported to be produced by *Serratia marcescens, Vibrio psychoerythrus, Rugamonas rubra, Streptoverticillium rubrireticuli,* and other eubacteria (Nagpal et al., 2011). It is used in yogurt, milk and carbonated drinks (Namazkar and Ahmad, 2013). Prodigiosin has been shown to have insecticidal, antifungal, antibacterial, anticancer, and anti-malarial activities (Kavitha et al., 2010; Kamble and Hiwarale, 2012).

5. Phycocyanin is a blue colored photosynthetic pigment produced by blue-green algae that contain chlorophyll A (Sen et al., 2019). It is water soluble and an accessory pigment to chlorophyll. It is found in *Aphanizomenon flos-aquae* and *Spirulina* sp. (Barsanti et al., 2008; Cuellar-Bermudez et al., 2015). It is used in sweets, ice creams and also as a dietary supplement rich in proteins. Pyocyanin also acts as bio-control agent that have anti-bacterial,

anti-fungal and anti-alzhelmeric activity (Jayaseelan et al., 2014).

6. Melanin is a natural pigment which is known to be produced by a wide variety of microorganisms such as *Colletotrichum lagenarium, Aspergillus fumigates Vibrio cholerae, Shewanella colwelliana, Alteromonas nigrifaciens* (Soliev et al., 2011). This pigment is also present in animals and plants. Besides several other uses such as in eye glasses, cosmetic creams, pharmaceuticals, they are also added in food items (Sen et al., 2019). The pigment is also reported to be associated with anti-HIV activity (Surwase et al., 2013).

7. Violacein is a versatile purple colored pigment that possess numerous biological activities. Various bacteria like *Chromobacterium violaceum, Pseudoalteromonas, Collimonas, Janthinobacterium, Microbulbifer* are known to produce this pigment (Choi et al., 2015). It is highly demanded at large scale in cosmetics, food, medicine and textiles (Dufossé, 2018). The pigment is associated with several useful bioactivity including antibacterial, anticancer, antiviral, enzyme modulation, antiulcerogenic, and anti-leishmanial (Soliev et al., 2011).

8. Astaxanthin is a lipid soluble orange-red pigment present in yeast, microalgae, marine organisms, and in feather of some birds (Sen et al., 2019). It is also reported to be produced by various bacteria such as *Halobacterium salinarium, Agrobacterium aurantiacum, Paracoccus carotinifaciens* (Guedes et al., 2011; Asker, 2017; Zuluaga et al., 2017; Pogorzelska et al., 2018). It is associated with anti-aging and memory improving activities and used as coloring agent in animal and fish foods (Capelli and Cysewski, 2013).

9. Lycopene is an approved meat coloring agent in several countries. It is a water insoluble biopigment belonging to carotene. It is present in tomato and other red fruits and vegetables (Di Mascio et al., 1989; Giovannucci et al., 2002) and can also be chemically synthesized. However, microbial production of lycopene is comparatively more economical and sustainable and has been produced in microbial hosts like *Blakeslea trispora, E. coli*, and yeasts by genetic engineering methods (Chen et al., 2016).

10. Arprink red is a red colored extracellular metabolite of the anthraquinone class produced by *Penicillium oxalicum*. It is also suggested to have anticancer effects when used as food supplements (Sardaryan, 2002). It is used as food colorant in various food products in different amounts as recommended by Codex Alimentarius Commission (Kumar et al., 2015).

11. *Monascus* pigments are a group of fungal secondary metabolites called azaphilones produced by filamentous fungi belonging to the genus *Monascus* of Ascomycetes group (Chung et al., 2008). They are red (monascorubramine and rubropunctamine), yellow (ankaflavin and monascin) and orange (rubropunctatin and monascorubrine) colored pigments (Vendruscolo et al., 2013). These pigments are extracted from various species of this fungi i.e., *M. pilosus, M. purpureus, M. ruberand, M. froridanus,* etc. and being used as food colorants for many years in red wines, yogurt, sausages, hams, and meats (Dufossé et al., 2005). They are also known to exhibit antimicrobial, anticancer, anti-obesity, and antioxidant activities (Vendruscolo et al., 2013).

FERMENTATION CONDITIONS FOR MICROBIAL PIGMENT PRODUCTION

Microbial pigments intended to be used as food additive or colorants are being commercially obtained from bacteria, fungi, and algae (Nigam and Luke, 2016). For industrial production of pigments the desired microorganism should possess properties like acceptability of wide range of carbon and nitrogen sources and tolerance of process pH and temperature (Kirti et al., 2014; Kumar et al., 2015). In addition, the yield should be sufficiently high enough to make it a cost-effective affair. The microorganisms capable to produce promising pigments can be isolated and screened through bioprospecting programs in different environment. Alternatively, the known pigment producing microorganisms can be subjected to strain improvement techniques for the desired yield and properties. A combination of the aforesaid two approaches can also be applied. Various factors mainly the type of fermentation, media components (carbon, nitrogen sources, and minerals), pH, temperature, time of incubation, moisture content and aeration rate (**Figure 2**) affect the growth and yield of pigments through microbial fermentation. Solid state and submerged fermentation approaches are used for the production of microbial pigments (Tuli et al., 2015). Solid state fermentation offers the advantages of higher yield and productivity as well as the direct applicability of the fermented product as a colorant without isolating the product (Babitha, 2009). SSF is particularly suitable for the growth of fungi and the use of this technique also leads to savings in wastewater and yield of the metabolites (Tuli et al., 2015). The optimum value of various factors affecting the fermentation conditions vary with the microorganism used for pigment production. For instance, optimum temperature range for pigment production by *Monascus* spp. is 25–28°C whereas *Pseudomonas sp.* prefers the temperature of 35–36°C (Kumar et al., 2015). The maximum growth and production of carotenoid from *Sarcina* sp after an incubation period of 72 h was reported by Joshi et al. (2011). The same incubation period has been found as the optimum for pigment production by *Rhodototrula* and *Micrococcus* (Attri and Joshi, 2005; Joshi and Attri, 2006). On the other hand, an incubation period of 48 hr has been reported as optimum for *Chromobacter* for pigment production by Attri and Joshi (2006).

The pH can affect the pigment production and its shade. *Monascus* sp. produces pigments optimally at pH between 5.5 and 6.5 whereas *Rhodotorula* does it as pH 4.0–4.5. Lycopene formation occurs at neutral to slightly alkaline pH whereas β-carotene formation occurs at acidic pH (Joshi et al., 2003). Carbon sources have impact on the microbial growth and shade of the microbial pigment. Depending on the species used monosaccharide or their polymers can be the optimum carbon source choice for pigment production (Joshi et al., 2003). A range of inorganic and organic nitrogenous compounds may be preferred by different microbes for maximum pigment

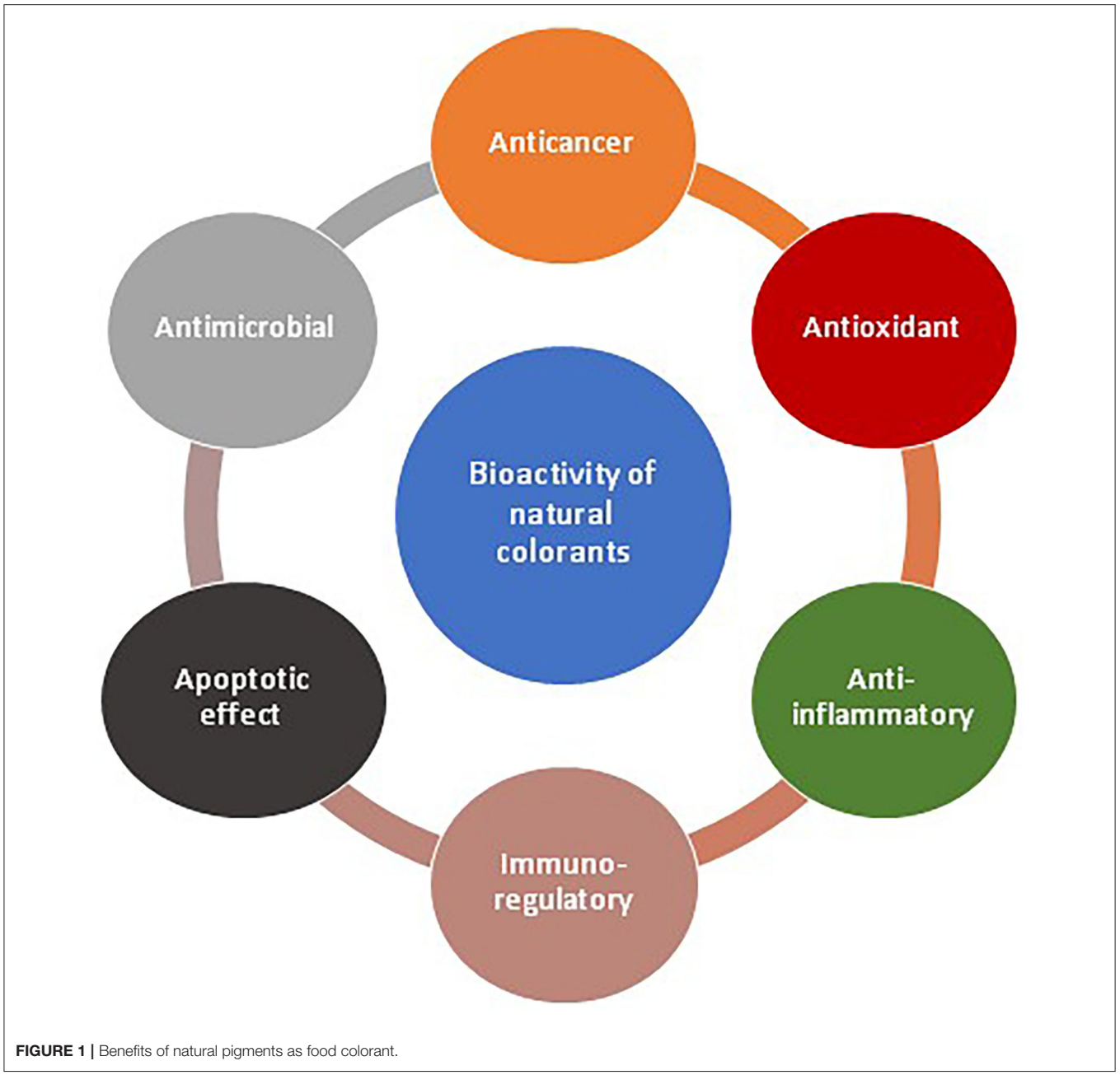


FIGURE 1 | Benefits of natural pigments as food colorant.

production. In addition, various minerals have also been documented to affect microbial pigment production (Joshi et al., 2003).

Although synthetic media can be used for the microbial production of pigments but use of agrochemical waste is suggested to be much better in terms of reducing the overall production cost (Panesar et al., 2015). Hamano and Kilikian (2006) demonstrated the use of corn steep liquor favorable for the production of pigment by *Monascus ruber*. A waste stream cellulose culture medium was utilized and optimized for pigment production by *Penicillum* sp. (Sopandi et al., 2013). Tinoi et al. (2005) produced carotenoid pigment by culturing *Rhodotorula glutinis* on hydrolyzed mung bean waste flour. Whey and soya protein have also been successfully used as raw material for the production of microbial pigments (Kaur et al., 2008; Panesar et al., 2015). Various food and vegetable waste products have also been utilized for microbial pigment production (Nigam and Luke, 2016).

TECHNICAL ADVANCEMENT TOWARD MICROBIAL PIGMENT PRODUCTION

Numerous technical advancements have been reported in recent years toward successful production of microbial pigments intended for various industrial applications. The progress in

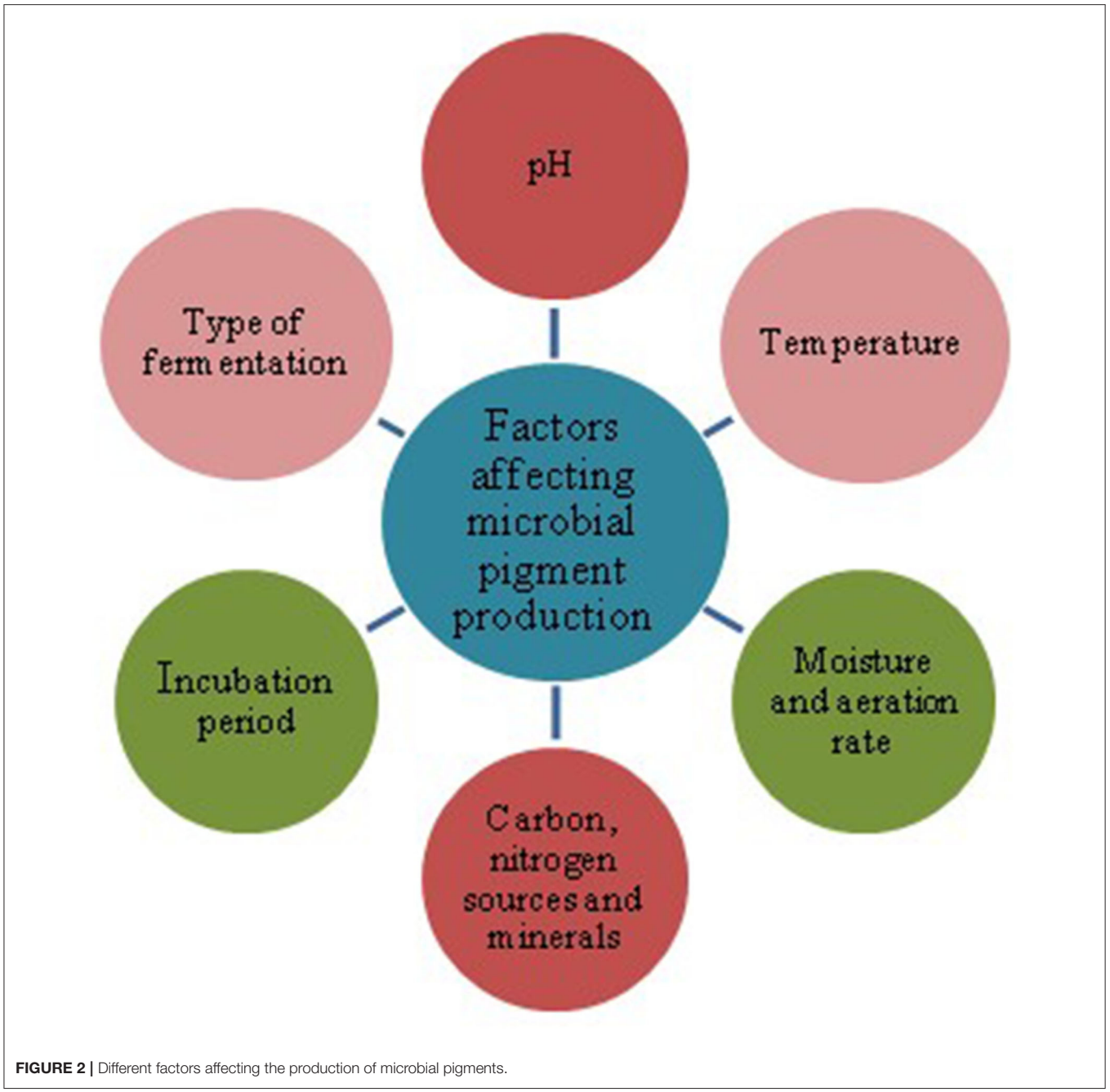


FIGURE 2 | Different factors affecting the production of microbial pigments.

various aspects related to fermentative production of pigments is discussed below.

Strain Improvement

Microbial pigments of desired characteristics with increased yield can be obtained through the use of established strain improvement techniques. Often a wild microbial strain is associated with limited production of pigment which negatively affects the economy of the process. The routinely used method of random mutagenesis and selection has resulted in the increase of pigment yield. Exposure to UV light and other mutagens such as 1-methyl-3-nitro-1-nitrosoguanidine, Ethyl methyl sulfonate is known to cause several fold increase in microbial pigment production (Nigam and Luke, 2016). Fakorede et al. (2019) have reported 5 fold increase in pigment production by *Serratia marcescens* (GBB151) after mutagenic treatment with Ethidium bromide. Techniques of genetic engineering have also been successfully employed for enhancing the microbial pigment yield and alter its molecular structure and color (Sen et al., 2019). Although information on the complete blueprint of

the biochemical synthetic pathways and the intermediates is generally a prerequisite for all such genetic manipulations targeting the rate limiting step for enhancing production. Bartel et al. (1990) and McDaniel et al. (1993) have reported such genetic alteration for blue pigment Actinorhodin, produced by *Streptomyces coelicolor*. Increased production of Zeaxanthin and other pigments by genetic engineering of *Synechocystis* sp. Strain PCC 6803 has been reported by Lagarde et al. (2000). There are reports of the development of cell factories using heterologous expression for the production of microbial pigments (Nielsen and Nielsen, 2017; Sankari et al., 2018). Technique of transposon mutagenesis applied on *Pseudomonas fluorescens* revealed 8 genes involved in blue pigment production and antioxidant protection (Andreani et al., 2019).

Optimization of Fermentation Conditions and Downstream Processing

The optimization of fermentation conditions and development of economic downstream processing can lead to the cost-effective production of microbial pigments. Media optimization includes variation in fermentation conditions like temperature, pH, incubation time, nutritional sources, aeration, and agitation rate etc. for selection of conditions that provide best yield. The technique of Response surface methodology (RSM) has many advantages over classical methods used for media optimization. Fewer experiments are required to derive an optimum combination of all the variable factors under investigation. Optimization of fermentation conditions thus require less time and efforts leading to reduction in the overall cost. Hamidi et al. (2017) determined the optimum values of temperature, pH and saline concentration and the effect of light on total carotenoid production by *Halorubrum* sp. TBZ126 using response surface methodology. Optimization of culture medium for yellow pigments production with *Monascus anka* mutant using response surface methodology has been reported by Zhou et al. (2009). Artificial neural network (ANN) is another technique that can be used to study the impact of fermentation conditions as well as their optimization for microbial pigment production. Singh et al. (2015) have investigated the application of Artificial Neural Network (ANN) in modeling a Liquid State Fermentation (LSF) for red pigment production by *Monascus purpureus* MTCC 369 and reported that ANN model can be used to predict the effects of fermentation parameters on red pigment production with a high correlation.

The conventional method of organic solvent extraction of pigments from fermentation broth is a complicated and time-consuming process with disadvantages of high cost, low yield and possible solvent leftover in the purified product as contaminants (Sen et al., 2019). Sen et al. (2019) have also mentioned that use of non-ionic resin due to their high loading ability is particularly suitable for large scale recovery of pigments and the technique also offer the advantage of direct absorption of compounds form the culture broth, thereby reducing the overall cost. Wang et al. (2004) have described the use of a non-ionic adsorbent (X-5) resin in the presence of Tween 80 for direct recovery of prodigiosin from the culture broth of *S. marcescens*. A novel approach of perstraction for recovery of intracellular pigments through submerged fermentation of *Talaromyces* spp. in a surfactant rich media has been described by Morales-Oyervides et al. (2017).

Pigment Stabilization

Stability against light, pH, temperature, UV radiation, and food matrices is an important issue with regard to the suitability of a microbial pigment for industrial applications and strategies like microencapsulation, nanoemulsion, and nanoformulations have been suggested for this purpose (Sen et al., 2019). In encapsulation solids, liquids, or gaseous materials are packaged in matrices (encapsulants) which sustain and release their contents under specific conditions (de Boer et al., 2019). The technique offer multiple advantages such as protection against light, moisture, or heat and also increase the brightness of the natural colorants and enhance their stability for many industrial applications (de Boer et al., 2019). The commonly used techniques for encapsulating colorants are spray-drying, electrospraying and anti-solvent precipitation (de Boer et al., 2019). A number of reports are available on the microencapsulation of microbial pigments and their enhanced applicabilities. Lycopin and carotenoids have been reported to be encapsulated by the methods of spray drying and lyophilization, respectively (Rocha et al., 2012; Nogueira et al., 2017). The strategy of nano-encapsulation or nano-emulsions which employs the droplet sizes of 100 nm or less with water, soil and emulsifier can be used for encapsulating microbial pigments which offers further advantages of stronger kinetic stability and resistance to chemical and physical changes because of larger surface area per unit (Gupta et al., 2016; Sen et al., 2019). There are various studies about the positive impact of nanoemulsion on stability of β carotene (Yi et al., 2014; Sen et al., 2019). Although various nanostructures are known to confer stability to carotenoid pigments, encapsulated polymeric nanocapsules are most utilized due to its stability during storage, high efficiency to encapsulate and to control the release of the carotenoid (Dos Santos et al., 2018). Bazana et al. (2015) have explored some nanoencapsulation techniques such as emulsification, coacervation, inclusion complexation and nanoprecipitation for lycopene. Other strategies for enhancing pigment stability that has been worked upon are like additions of copigment compounds, such as polymers, phenolic compounds, and metals as well as the exclusion of O_2 during processing (Cortez et al., 2017). Various patents have been filed for novel methods of pigment stabilization such as the hard candy coating for various colors (Cortez et al., 2017). The technique of genetic engineering has also been applied for enhancing the natural pigment stability. A novel method of gene-encoded acyltransferase of aromatic acyl groups has been filed for patent by Tanaka et al. (2011).

Analysis and Detection of Microbial Pigments

Various analytical techniques developed over the years are in use to detect and analyze microbial pigments. TLC, UV–VIS spectrophotometry, FTIR, NMR, HPLC are the techniques in

routine use for identification and characterization of microbial pigments. A handheld Raman spectrometer, working on the principle of excitation laser, has been employed for the detection of microbial pigments in various environments (Jehlicka and Oren, 2013; Kumar et al., 2015). Mass spectrometry coupled with electrospray ionization can be used for classifying the pigments producing fungi (Smedsgaard and Frisvad, 1996).

MAJOR CHALLENGES ASSOCIATED WITH THE USE OF MICROBIAL PIGMENTS AS FOOD COLORS

Both extracellular and intracellular production of pigments is known in microorganisms. Although commercial production of food pigments from microorganism offers special advantages, the species under use must be amenable to culture with a fast growth rate and productivity in limited space and time (Ramesh et al., 2019). In addition, it must be non-toxigenic, non-pathogenic, and able to grow on a wider range of cheaper raw materials with stability under harsh physical and chemical process conditions (Ramesh et al., 2019). Due to various factors natural colors are more expensive as compared to their synthetic counterparts. In confectionary items biopigments can be 20 times more expensive as synthetic pigments (Sigurdson et al., 2017). Microbial pigments may also be associated with the tendency to react with the other food components and may generate unwanted odors and flavors (Sen et al., 2019). Extraction and purification of microbial pigments from fermentation broth is a time consuming, low yielding and costly affair (Nigam and Luke, 2016) and use of organic solvent may itself overcome the idea of obtaining natural pigments (Hicketier and Buchholz, 2002).

The use of synthetic media for microbial production can overprice the production cost although cheap agro-industrial residues such as coconut residue, soybean meal, corn syrup, starch, cheese whey, rice water, jackfruit seed extract, mustard waste, sugar beet molasses, etc. are promising alternative media substitutes for pigment production (Venil et al., 2014). However, the availability of such byproducts throughout the year at many places may be difficult. The another issue with natural pigments is their stability and sensitivity toward light, pH, UV, temperature, oxygen, heat, and other environmental conditions that may lead to color loss and a reduced shelf life (Sen et al., 2019). Various encapsulation strategies as well as genetic engineering methods have been developed to address this issue. In future the development of novel techniques like the combination and evaluation of new pigment stabilizing material will further enhance their prospects to be used as value-added natural food pigments (Cortez et al., 2017). Since all food additives are under very strict legislation and approval mechanism, it is of paramount importance that microbial pigment production and its purification process must not allow any unwanted toxic or allergic metabolite in the final product (Gao et al., 2003).

CONCLUSION

Given the growing public perception and concern over the use of safe food ingradients, the industrial demand of natural pigments is expected to increase in future by many folds. Microbial pigments are attractive alternative to synthetic food colorants not only because of their natural origin but also due to their several proven health benefits. Although a plethora of microorganisms have been reported to produce food grade pigments at laboratory level, large scale production and purification of the products from many of them is still a challenge. More studies are required with respect to media and fermentation condition optimization for sufficient production and easy recovery of microbial pigments. In addition, classical strain improvement methods as well as the advance techniques of genetic or metabolic engineering can be used for sustainable production of microbial pigments of high use. The strain improvement methods can also be preceded by bioprospecting programs to screen and identify novel pigment producing microbial strains from different environments in adherence to the Nagoya protocol and other applicable state rules. Exploration of traditional fermentative food in isolated or tribal region can also lead to the identification of promising pigment producing isolates. Although only non-pathogenic microbes are acceptable for food grade pigments, co-production of toxic or undesirable compound by so called "safe" organism is also possible and therefore, appropriate cost effective purification strategies are to be devised.

AUTHOR CONTRIBUTIONS

BR, MB, and BP prepared the first draft of the manuscript. GJ critically evaluated the same and prepared the final version with the help of all of them and MA. All authors contributed to the article and approved the submitted version.

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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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