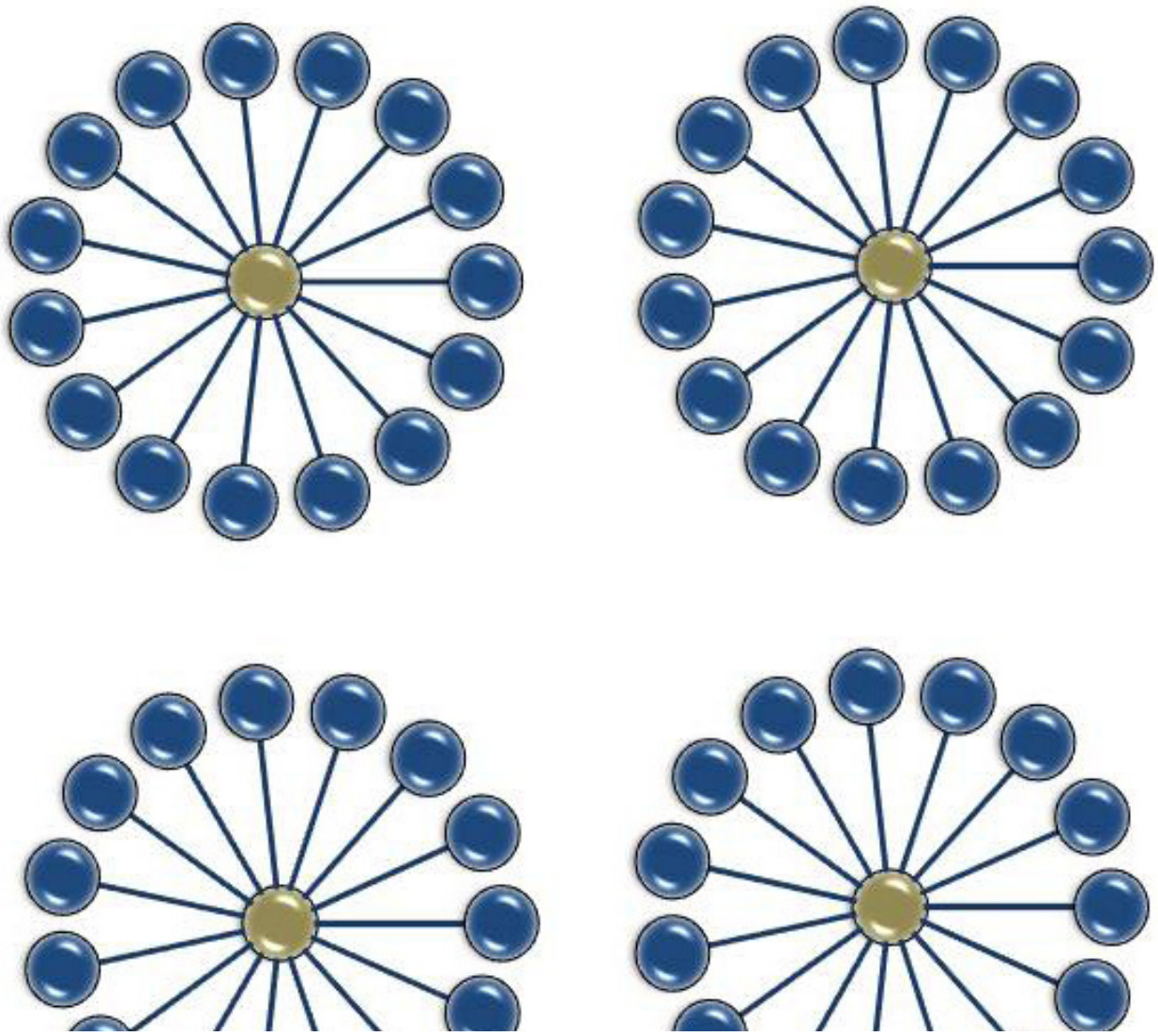
MICROBIOTECHNOLOGY BASED SURFACTANTS AND THEIR APPLICATIONS

EDITED BY: Pattanathu K.S.M. Rahman
PUBLISHED IN: Frontiers in Microbiology



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ISSN 1664-8714
ISBN 978-2-88919-752-1
DOI 10.3389/978-2-88919-752-1

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MICROBIOTECHNOLOGY BASED SURFACTANTS AND THEIR APPLICATIONS

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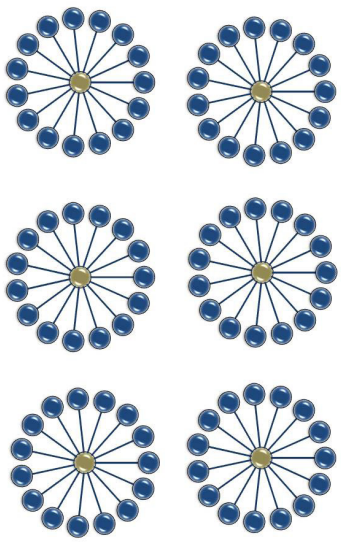

Structure of micelles produced by biosurfactant. Image by Dr Pattanathu K.S.M. Rahman

Biosurfactants are structurally diverse group of bioactive molecules produced by a variety of microorganisms. They are secondary metabolites that accumulate at interfaces, reduce surface tension and form micellar aggregates. This research topic describes few novel microbial strains with a focus on increasing our understanding of genetics, physiology, regulation of biosurfactant production and their commercial potentials. A major stumbling block in the commercialization of biosurfactants is their high cost of production. Many factors play a significant role in making the process cost-effective and the most important one being the use of low-cost substrates such as agricultural residues for the production of biosurfactants. With the stringent government regulations coming into effect in favor of production and usage of the bio-based surfactants, many new companies aim to commercialize technologies used for the production of biosurfactants and to bring down costs.

This Research Topic covers a compilation of original research articles, reviews and research commentary submitted by researchers enthusiastically working in the field of biosurfactants and highlights recent advances in our knowledge of the biosurfactants and understanding of the biochemical and molecular mechanisms involved in their production, scale-up and industrial applications. Apart from their diverse applications in the field of bioremediation, enhanced oil recovery, cosmetic, food and medical industries, biosurfactants can also boast off their unique eco-friendly nature to attract consumers and give the chemical surfactants a tough competition in the global market.

This biosurfactant focused research topic aims to summarize the current achievements and explore the direction of development for the future generation of biosurfactants and bioemulsifiers. Some of the biosurfactant optimization processes presented are well-structured and already have a well-established research community. We wish to stimulate on-going discussions at the level of the biosurfactant production including common challenges in the process development, novel organisms and new feedstock and technologies for maximum benefit, key features of next generation biosurfactants and bioemulsifiers. We have compiled the research outputs of international leaders in the filed of biosurfactant particularly on the development of a state-of-the-art and highly-efficient process platform.

Citation: Rahman, P. K. S. M., ed. (2016). Microbiotechnology Based Surfactants and Their Applications. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-752-1

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frontiers in Microbiology

EDITORIAL
published: 01 December 2015
doi: 10.3389/fmicb.2015.01344

Editorial: Microbiotechnology Based Surfactants and Their Applications

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Keywords: biosurfactants, bioemulsifiers, actinobacteria, enzymes, market research

This editorial is an annotation on the exciting research topic "Microbiotechnology based surfactants and their applications" that covers a compilation of original research articles, reviews and mini-reviews submitted by researchers enthusiastically working in the field of biosurfactants.

Biosurfactants, which for a long time have been confused with bioemulsifiers, derived their name from biologically produced surfactants. The term "Surfactants" was, however coined by Antara products in 1950—which covered all products having surface activity, including wetting agents, emulsifiers, dispersants, detergents, and foaming agents. The terms biosurfactants and bioemulsifiers have been used interchangeably for a long time until a demarcation has been suggested by several researchers including (Uzoigwe et al., 2015). They emphasized that although biosurfactants and bioemulsifiers are both amphiphilic in nature and produced by variety of microbes, there are marked differences between them in terms of their physico-chemical properties and physiological roles. Authors strongly presented their opinion that bioemulsifiers are not biosurfactants as only biosurfactants have the surfactant effect of reducing surface tension, although both can emulsify solutions. Debating on the topic of emulsification, another study by Das et al. (2014) from China, showed that emulsification potential and also the antimicrobial activity of rhamnolipid biosurfactants produced by crude oil extracted *Pseudomonas* sp. IMP67 is effected by the ratio of monorhamnolipid (MRL) and dirhamnolipid (DRL) congeners. The MRL and DRL congeners were analyzed by thin layer chromatography and rhamnose quantification. Rhamnolipids from *Pseudomonas* sp. IMP67 also reduced the minimum inhibitory concentrations (MICs) of some antibiotics signifying the synergistic role of these rhamnolipids with antibiotics.

If there is one major stumbling block in the flourishing of the business of biosurfactants it is their high cost of production. There are many factors that can play a significant role in order to bring down the expenses and make the process cost-effective. One such factor is the usage of low-cost substrates for the production of biosurfactants. Second to this could be the exploration of new strains or strains and classes which has been less-explored for biosurfactant production. An extensive review by Kugler and co-authors precisely talks about the class *Actinobacteria* and suggest a lack of structural information on a large proportion of Actinobacterial surfactants. Authors claim that the sheer magnitude of Actinobacterial surfactants that still remains undetermined is evident from this comprehensive review (Kügler et al., 2015). A better understanding of the diversity of the Actinobacterial surfactants would allow to further explore their potential for various novel biotechnological applications just as in case of lipopeptide biosurfactants produced by many microorganisms including *Bacillus* species. Lipopeptides, a series of chemical structural analogs of many different families, are one of the five major classes of biosurfactants known. Among the different families identified, 26 families covering about 90 lipopeptide compounds have been reported in last two decades (Liu et al., 2015).

Not only the less-researched strains and classes but a significant leap is required investigating the carbon sources that would work best for high biosurfactant production. Addressing this area are the original research articles by Antoniou et al. (2015), Gudiña et al. (2015) and Ismail et al. (2014), and a review by Banat et al. (2014).

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *16 September 2015*
Accepted: *16 November 2015*
Published: *01 December 2015*

Citation:
Rahman PKSM and Sekhon Randhawa KK (2015) Editorial: Microbiotechnology Based Surfactants and Their Applications. Front. Microbiol. 6:1344. doi: 10.3389/fmicb.2015.01344

Eleftheria Antoniou and co-researchers from Greece, investigated the biosurfactant production yield of marine hydrocarbon degraders isolated from Elefsina Bay (Eastern Mediterranean Sea) in presence of heavy oil fraction of crude oil as substrate. Their data particularly emphasized on *Paracoccus marcusii* to be an optimal choice for various bioremediation applications. They reported that the isolated pure strains were found to have higher specific production yields (50 ± 20 mg/l) than the complex microbial marine community-consortia (20 mg/l) (Antoniou et al., 2015). Crude oil was the best energy source for these marine hydrocarbon degraders whereas corn steep liquor (CSL) turned out to be an ideal substrate for *Bacillus subtilis* #573 (Gudiña et al., 2015). Authors reported a yield of 1.3 g/l surfactin using 10% CSL in the medium, which increased to as high as 4.8 g/l when supplemented with the optimum concentration of three metals (iron, manganese, and magnesium) simultaneously. Wael Ismail and his team on the other hand came out with another interesting finding that the expression levels of the *rhl*ABC genes in *Pseudomonas* sp. strain AK6U greatly varies depending on the sulfur source. They showed that a biosurfactant yield of 1.3 g/l was obtained in presence of dibenzothiophene (DBT) as a carbon source which was higher than obtained in presence of DBT-sulfone (0.5 g/l) and the inorganic sulfate (0.44 g/l) (Ismail et al., 2014). To bring together these types of "carbon-source" based studies for "low-cost" biosurfactant production technologies Ibrahim M. Banat and co-authors wrote an intensive review where they discussed how and why despite so many developments on biosurfactants their commercialization remain difficult, costly and to a large extent irregular and what role does the low-cost renewable raw substrates and fermentation technology play in reducing the overall production cost.

Some other interesting studies that focus on rhamnolipids and their applications are also included under this special research topic. Madsen et al. (2015), compared the impact of anionic biosurfactant rhamnolipid and the synthetic surfactant SDS on the structure and stability of three different commercially used enzymes—the cellulase Carezyme®, the phospholipase Lecitase Ultra® and the α-amylase Stainzyme® and found a fundamental difference in their mode of action. In another exciting study on rhamnolipids, Silva et al. (2015), evaluated the potential larvicidal, insecticidal, and repellent activities of rhamnolipids and reported their positive effect against *Aedes aegupti* mosquitoes. Wang et al. (2014), for the first time report the complete pathway of the di-rhamnolipid synthesis process in the genus *Dietzia* and provided insights into the biosurfactant production, oil degradation and removal potential of *Dietzia maris* As-13-3.

From a simple idea of growing bacteria and fungi on immiscible substrates and producing surface-active compounds, to a hurl of more than 250 patents filed in close to three decades followed by a market value expected to reach $2,210.5 million by 2018, biosurfactant industry certainly stands on a substantial fundament. Such stimulating facts and figures are broadly discussed in the opinion article by Sekhon Randhawa and Rahman (2014).

Apart from their industrially diverse applications in the field of bioremediation, enhanced oil recovery, cosmetic, food, and medical industries biosurfactants can boast off their unique eco-friendly nature to attract consumers and give the chemical surfactants a tough competition in the global market. The pharmaceutical applications such as biological usage as antiviral, antitumor, antibiotic agents, as insecticides, fungicides, and immune-modulators or enzyme inhibitors have not been fully realized. With the stringent governmental regulations coming into effect in favor of production and usage of the bio-based surfactants, more and more companies are working on the commercialization of the production technology of biosurfactants and to bring down their higher prices. There is no dearth of astonishing applications of biosurfactants; the only challenge is their supply through bio-based production methods to meet the demands well in time.

AUTHOR CONTRIBUTIONS

PR initiated the research topic and co-ordinated the entire editorial process. There are 11 manuscripts accepted for publication in this research topic contributed by 55 authors from UK, Denmark, Greece, Germany, South Africa, India, Brazil, Bahrain, Portugal, and China. He has initiated peer review process by inviting experts from Germany, Spain, USA, Trinidad and Tobago, India, Denmark, China, Bahrain, Malaysia, Japan, and UK. The reviewers' co-operation and timely responses to complete the research topic is highly commendable. KS was not assigned as Topic Editor for this research topic but she has contributed as Co-author in this Editorial commentary. She is an expert on this topic and has been working specifically in this field, she has her adept inputs in conceptualizing the commentary, analysing the data, interpreting and drafting the commentary. KS has given her final approval of the version to be published and agrees to be held accountable for all aspects of the work related to the accuracy and integrity.

ACKNOWLEDGMENTS

PR wish to thank the BBSRC NIBB HVCfP Net Business Interaction Voucher (Ref No: BIV-HVCFP-APR15-011) and BBSRC NIBB CBMnet Proof of Concept award (Ref No: D0047), iCreate Entrepreneurs Accelerator Programme and Technology Future Institute's Research and Business Engagement allowances for supporting the editorial work.

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Conflict of Interest Statement: 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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 07 April 2015
doi: 10.3389/fmicb.2015.00274

Biosurfactant production from marine hydrocarbon-degrading consortia and pure bacterial strains using crude oil as carbon source

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OPEN ACCESS

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Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *02 January 2015*
Accepted: *19 March 2015*
Published: *07 April 2015*

Citation:
Antoniou E, Fodelianakis S, Korkakaki E and Kalogerakis N (2015) Biosurfactant production from marine hydrocarbon-degrading consortia and pure bacterial strains using crude oil as carbon source. Front. Microbiol. 6:274. doi: 10.3389/fmicb.2015.00274

Biosurfactants (BSs) are "green" amphiphilic molecules produced by microorganisms during biodegradation, increasing the bioavailability of organic pollutants. In this work, the BS production yield of marine hydrocarbon degraders isolated from Elefsina bay in Eastern Mediterranean Sea has been investigated. The drop collapse test was used as a preliminary screening test to confirm BS producing strains or mixed consortia. The community structure of the best consortia based on the drop collapse test was determined by 16S-rDNA pyrotag screening. Subsequently, the effect of incubation time, temperature, substrate and supplementation with inorganic nutrients, on BS production, was examined. Two types of BS – lipid mixtures were extracted from the culture broth; the low molecular weight BS Rhamnolipids and Sophorolipids. Crude extracts were purified by silica gel column chromatography and then identified by thin layer chromatography and Fourier transform infrared spectroscopy. Results indicate that BS production yield remains constant and low while it is independent of the total culture biomass, carbon source, and temperature. A constant BS concentration in a culture broth with continuous degradation of crude oil (CO) implies that the BS producing microbes generate no more than the required amount of BSs that enables biodegradation of the CO. Isolated pure strains were found to have higher specific production yields than the complex microbial marine community-consortia. The heavy oil fraction of CO has emerged as a promising substrate for BS production (by marine BS producers) with fewer impurities in the final product. Furthermore, a particular strain isolated from sediments, *Paracoccus marcusii,* may be an optimal choice for bioremediation purposes as its biomass remains trapped in the hydrocarbon phase, not suffering from potential dilution effects by sea currents.

Keywords: biosurfactant, marine bacteria, *Alcanivorax*, rhamnolipid, sophorolipid, crude oil, bioaugmentation, *Paracoccus marcusii*

Introduction

Chronic release of oil in the sea from numerous natural and anthropogenic sources poses a continuous-serious threat for the environment (Nikolopoulou and Kalogerakis, 2010).

The majority of petroleum hydrocarbon input comes from natural seeps, while spillage from vessels or operational discharges have nowadays decreased significantly and, e.g., in North America only 1% of the oil discharges is related to the extraction of the oil. Approximately, 1.3 million tones of petroleum enters the marine environment each year (National Research Council (NRC) of the National Academies - Committee on Oil in the Sea, 2003; Diez et al., 2007; Ventikos and Sotiropoulos, 2014), while in the Gulf of Mexico alone after the Deep Horizon incident > 600,000 tones were released into the sea (International Tanker Owners Pollution Federation [ITOPF], 2013). Acute accidents such as the Deep Horizon result not only in increased public concern but also in mass mortality of marine and coastal life. Fortunately they are rare.

Oil pollution cleanup in marine environments with the use of biological means-bioremediation (Nikolopoulou and Kalogerakis, 2008; Nikolopoulou et al., 2013a,b), has emerged as a very promising 'green' alternative technology following first response actions (skimmers, boomers, fire, dispersion with chemical surfactants). Crude oil (CO) is biodegradable. Hydrocarbon-degrading bacterial consortia exist in nature and thrive in oil-polluted sites, while using petroleum hydrocarbons as source of carbon and energy for growth (Hassanshahian et al., 2012; McGenity et al., 2012; Thomas et al., 2014). The way hydrocarbon-degrading bacterial consortia and pure strains engineer their way into the oil spill for biodegradation is very complex and still under investigation. Bacterial cells produce a mixture of biosurfactant (BS) lipids with the help of which oil is dispersed into very fine droplets and thus the bioavailability of CO is increased.

Biosurfactants are surface-active compounds produced by microorganisms. They display a variety of surface activities (surface tension decrease from 72 to 30 mN/m Helvaci et al., 2004) that increase the bioavailability of organic pollutants, including CO components, and thus enhance biodegradation (Nguyen et al., 2008; Rahman and Gakpe, 2008; Whang et al., 2008; Banat et al., 2010, 2014; Nguyen and Sabatini, 2011; Randhawa and Rahman, 2014). BSs belong to a structurally diverse group of amphiphilic biomolecules with both hydrophilic and hydrophobic moieties. They generally are grouped either as low or high molecular weight BSs, the former consisting of glycolipids and lipopeptides and the latter of high molecular weight polymeric BS. Due to their biodegradability and low toxicity they are very promising for use in remediation technologies as an alternative to the synthetic surfactants (Nguyen et al., 2008). Microbial BSs can replace the currently used chemical surfactants that are more toxic in many applications, like combating oil spills, bioremediation enhancement, micro-extraction of PAHs, pharmaceutical products, and detergent industry (Nguyen et al., 2008; Banat et al., 2010; Nguyen and Sabatini, 2011). There is a need for ecologically friendly and biodegradable surfactants (ionic or non-ionic) for reliable environmental cleanup. Commercially viable BSs have to be economically competitive therefore the development of good microbial BS producing cultures is required (Banat et al., 2000, 2010, 2014; Nguyen et al., 2008; Rahman and Gakpe, 2008; Whang et al., 2008; Nguyen and Sabatini, 2011; Randhawa and Rahman, 2014). Nowadays BSs still have not been employed extensively in industry because of the high production cost.

Biosurfactant production challenges and solutions for increasing the production yield are very well presented by Banat et al. (2014). Problems that limit BS industrial production include the required renewable substrate media quantities, slow growth rate of organisms on the substrate, low yield and final product purification from substrate impurities. Although cost effective BS production is still a goal to be attained, other important issues currently under investigation include the development-isolation of BS producing microorganisms (consortia or strains), the fine-tuning of their production ability by changing their incubation conditions (temperature, time, nutrients) and/or substrate type toward achieving a high yield and the production of lipid mixtures with an attractive/desired structure.

The primary objective of this work was to investigate the BS production efficiency and quality of isolated consortia and pure strains (that have hydrocarbon-degrading capabilities) isolated from the sediment and water column of a hydrocarbon-contaminated marine area (Elefsina bay, Attica, Greece) with CO as sole carbon source. The fact that marine hydrocarbon degraders are often BS producers as well impelled us to investigate the BS production efficiency of specific marine hydrocarbon-degraders. The sampling and isolation of hydrocarbon degraders from Elefsina bay was part of the FP7 project ULIXES. In particular, the production of two BS types, rhamnolipids (RLs) and sophorolipids (SLs) by isolated consortia was investigated regarding the effect of incubation time, temperature, addition of nutrients N (as KNO_3) and P (as KH_2PO_4), and finally the carbon source. Therefore, isolation, screening, detection and characterization techniques were used in order to evaluate/confirm the BS chemical composition. In addition, promising pure strains were also tested for their BS production ability. The effect of substrate on the RL yield of the best BS producing strain was investigated. In an attempt to explain the BS production yield, we try to answer the following questions: how the RL production yield by marine microbes compares to the critical micelle concentration (CMC)? How this relates to the oil degradation? What is the role - spatial distribution of BS in the process (emulsion, cell hydrophobicity increase)?

Materials and Methods

Sampling Locations

Seawater and sediment samples were collected from six locations in Elefsina bay, Attica, Aegean Sea as shown in **Figure 1**. Elefsina bay is a major industrial area, where among other industrial complexes, there are two large petroleum refineries. Due to several accidents in the past and the slow seepage of CO from old storage tanks, there is sufficient evidence of low chronic pollution in the area. The sampling campaign aimed to isolate consortia and strains from both the water column and the sediment, enhancing the probability to isolate different strains of interest. The samples were collected downstream of the local

FIGURE 1 | The sampling campaign map. The locations of all the sampling sites are represented by red dots. The code(s) of the consortia isolated from each sampling site is given next to each dot. For consortia isolated from the water column, i.e., consortia E1–E8, the depth of the water sampled for initial inoculation is given in parenthesis next to the consortium code. The image was captured and modified using Google Earth.

current direction (West-to-East). An additional sediment sample (ESP) was collected at the area where a small stream joins the bay.

Preparation of Enrichment Cultures

Enrichment cultures were prepared by adding 10 ml of seawater or 10 g of sediment (for sediment samples) in 90 ml ONR7 (Yakimov et al., 1998), with the addition of 0.5% w/v filter-sterilized CO in 250 ml Erlenmeyer flasks. The cultures were incubated at 20°C, in an orbital incubator, agitated at 150 rpm. At each re-inoculation, 1 ml of culture from the early exponential phase was transferred to 99 ml of ONR7 medium. Plating count on marine agar for marine heterotrophs and OD measurements was carried out to establish reliable growth curves.

Screening of Marine Consortia by the Drop Collapse Test for the Isolation of Pure Biosurfactant Producing Strains

The drop collapse test was performed according to (Youssef et al., 2004). Scoring was performed by setting sterile deionised water as a negative control and a 10^{-4} dilution of "S-200 oil-gone" commercial BS solution (IEP Europe S.L., Madrid) as a positive (+++) control and comparing the diameter of droplets from the examined cultures. Scoring, of "−" to "+++" was performed by comparing the diameter of the droplet (X) to that of the water droplet (Y) and the positive control (Z). A "−" score was given if $X \leq Y$ whereas a "+++" score was given if $X \geq Z$. Finally, a "+" score was given if $Y < X \leq (Z\text{-}Y)/2$ and a "++" if $(Z\text{-}Y)/2 < X < Z$.

For the drop collapse test, consortia were incubated at 14°C (in an effort to mimic the original aquatic habitat temperature) for 6 weeks in ONR7/CO 0.5% w/v as a sole carbon source. Re-inoculations were performed weekly. The drop collapse test was performed once every week, just before the re-inoculation.

Initial Community Screening of Biosurfactant Producing Consortia by Pyrotag Sequencing

Total genomic DNA was extracted according to (Moore et al., 2004). DNA yield and quality was determined by agarose gel electrophoresis of 5 μl of DNA extract. DNA extracts were stored at 4°C until use.

PCR and pyrosequencing were performed in Research and Testing Laboratory (Lubbock, TX, USA) on an FLX Titanium platform, for the V4 hypervariable region of the 16S rRNA gene using primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) which are known to have reduced bias and cover a wide range of bacterial and archaeal phyla (Kuczynski et al., 2012). Noise filtering and chimera removal (using the AmpliconNoise package Quince et al., 2011), operational taxonomic unit (OTU) clustering (at 97% similarity, using uclust Edgar, 2010), OTU

table construction, Good's coverage index estimation (Good, 1953) and phylogenetic assignments (comparing against the latest Greengenes database release McDonald et al., 2012 with uclust) were performed in QIIME v1.8 (Caporaso et al., 2010). The samples for the whole project have been deposited in the NCBI short read archive (SRA) database under the BioProject accession number PRJNA190077.

Isolation and Characterization of Pure Biosurfactant Producing Strains

Hundred μl of each mixed culture taken at the early stationary phase were initially spread on Zobell marine agar or ONR7 agar/CO 0.5% w/v in triplicates, at a dilution of 10^{-4} and 10^{-6}. Colonies of distinct morphology were carefully picked and reinoculated on the same medium. Single colonies were then picked and the growth of each isolated strain was tested in both ONR7/CO 0.5% w/v and marine broth at 14°C.

For the characterization of pure isolates, 200 μl of each culture was centrifuged for 5 min at 10000 g and after aspiration of the supernatant, the resulting pellet was incubated for 15 min at 95°C with 50 μl STE buffer (100 mM NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8) and 1 μl of the resulting solution was used as a template for PCR. PCR was prepared in a laminar flow chamber under aseptic conditions and performed in an Eppendorf Mastercycler gradient. Negative controls (autoclaved ultra-pure water) were used in every reaction. A ∼1500 bp fragment of the bacterial 16S rRNA locus was amplified using the universal bacterial primers 27F (5′-AGAGTTTGATC(AC)TGGCTCAG-3′) and 1492R (5′-ACGG(CT)TACCTTGTTA CGACTT-3′; Weisburg et al., 1991), as described in Fodelianakis et al. (2014). Quantity and quality of the PCR products were evaluated by agarose (1.2%) gel electrophoresis. PCR products were then purified using the Nucleospin Gel and PCR cleanup (Machery-Nagel) commercial kit. Purified PCR products were sequenced in StarSEQ GmbH, Mainz, Germany from both the forward and reverse primers. The overlapping sequence (∼890 bp) was compared against the NCBI nr and 16S database using the BLAST algorithm in order to find the closest relative and the closest described species respectively.

Biosurfactant Production

Cultivation

The isolated BS producing consortiums or pure strains were inoculated in 200 ml of ONR7 medium supplemented with 0.5%w/v carbon source and incubated on an orbital shaker at 150 rpm for 5–6 and 10–12 days at different temperatures. The nutrients added were KNO_3 and KH_2PO_4 until the final ratio of C:N:P was equal to 100:10:1. The origin of the CO used as the carbon source for the experiments was Kazakhstan. The isolated single strains were cultivated at 14°C (aquatic habitat temperature) for 6 and 12 days, in 200 ml ONR7 medium with 0.5% w/v CO as the carbon source. To test RL production of strain E8Y (see Results) under different carbon sources, the following media were used: (i) Glucose 0.5% wt, (ii) glucose 0.25% wt and CO 0.25% wt, (iii) glucose 0.25% wt and heavy CO fraction (asphaltene-aromatics fraction with less than 7% saturates) 0.25% wt and (iv) 0.5% wt CO.

Liquid-Liquid Extraction

The extraction of crude BS extract, free from the aqueous culture medium, was performed by liquid–liquid extraction. RLs were extracted with equal volume of ethyl-acetate, after centrifugation at 13,000 × *g* for 15 min at 4°C to remove the bacterial cells and acidification of the culture medium at PH = 3 with 6 N HCl (Smyth et al., 2010), while SL extract was obtained from the whole culture using three times equal volume of ethyl-acetate (Nuñez et al., 2001). Anhydrous sodium sulfate was added to the ethyl acetate layer to remove residual water, filtered, was collected in a round-bottom flask and connected to a rotary evaporator to remove the solvent. The process yielded a viscous honey-colored BS.

Biosurfactant Purification

Purification of the produced BS crude extract was conducted using Silica Gel Column Chromatography. Silica gel 60 (240–425 mesh) was the stationary phase in all cases. Neutral lipids, Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10} purified fractions were obtained using $CHCl_3$:CH_3OH (50:3 v/v), $CHCl_3$:CH_3OH (50:5 v/v) and $CHCl_3$:CH_3OH (50:50 v/v) as the mobile phase, respectively. Acidic and lactonic types of SLs were obtained using $CHCl_3$:CH_3OH (98:2 v/v), $CHCl_3$:CH_3OH (83:17 v/v), $CHCl_3$:CH_3OH (71:19 v/v) and $CHCl_3$:CH_3OH (60:40 v/v) as the mobile phase (Smyth et al., 2010; third and fourth fraction carried the different types).

Biosurfactant Detection-Characterization

A RL mixture of Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10} (Aldrich Chemistry, R-95 RL 95%) was used as standard to compare the RLs produced.

Thin Layer Chromatography (TLC)

Biosurfactant detection was performed on the crude extract by TLC on pre-coated silica gel of standard 20 × 20 Kiesel-gel 60 F254 Merck plates using the appropriate solvent system and visualization agent for each BS. In the case of RLs, the solvent system used was chloroform : methanol : acetic acid (65:15:2, v/v/v), the spray reagent was antrone (Smyth et al., 2010), while SLs were detected via chloroform : methanol : water (65:15:2, v/v/v) and the development agent was *p*-Anisaldehyde (100°C for 5 min; Asmer et al., 1988).

Fourier Transform IR Spectroscopy (FT-IR)

Infrared spectroscopy is a simple method for structure analysis. Samples were liophilized and milled with KBr to form a uniform capsule and were characterized via FT-IR spectroscopy on a Perkin Elmer 2000 FTIR spectrometer operated in the absorbance mode at a resolution of 4 cm^{-1}.

Mass Spectrometry (LC-MS)

Rhamnolipid mixtures were separated and identified by liquid chromatography coupled to mass spectroscopy using an Agilent Technologies 6110 quadrupole LC–MS (Smyth et al., 2010). Samples were prepared with ACN:H_2O (80:20 v/v; LC grade) with a concentration of 10 mg/l and 100 μl of the same was injected into a C_{18} (150mm × 2.1mm × 5 μm) column. The

LC flow rate was 0.25 ml/min. For mobile phase, an acetonitrile–water gradient was used starting with 40% of acetonitrile for 4 min, followed by 40–90% acetonitrile in 20 min then return to the initial condition in 6 min. Ammonium acetate buffer was added.

Results

Mixed Culture Screening and Pure Strain Isolation

Eleven enrichment cultures, namely E1-E9, ESP and ESPI were obtained from the respective seawater and sediment samples. Those cultures were then screened by the drop collapse test for BS production. As presented in **Table 1**, consortium E8 achieved the highest overall scores within the given time period, followed by consortium E4. An additional consortium, namely EB8, was subsequently created from the E8 consortium by re-inoculating material only from the oil-culture interface, to further test if the BS production could be enhanced further by these isolates (adaptation of the MATH test isolation method).

Subsequently, the community structure of consortia E8, EB8 and E4 was examined with 16S rRNA gene pyrotag sequencing. Consortium E9 was also included in this analysis due to its different origin (sediment) and therefore its potential to contain different BS producing strains. Good's coverage estimates ranged between 0.97 and 0.99, indicating that the sampling depth was enough for an adequate description of the bacterial diversity of each of the examined consortia.

Phylogenetic analysis of the pyrotag reads revealed the presence of seven families among the examined consortia; Rhodobacteraceae (0.07–2.5% per sample reads), Rhodospirillaceae (5.3–54.5% per sample reads), Shewanellaceae (0–4% per sample reads), Alcanivoracaceae (36.2–68.5% per sample reads), Halomonadaceae (0–22.4% per sample reads), Oceanospirillaceae (0–42.4% per sample reads) and Pseudomonadaceae (0.4–21.6% per sample reads; **Figure 2**). For some reads (0.8–5.4% per sample) phylogenetic assignment down to the family level was not possible (**Figure 2**).

TABLE 1 | Drop collapse test results for each consortium.

Sample	Origin	W1	W2	W3	W4	W5	W6
E1	Water	−	−	−	−	+++	−
E2	Water	+	+	+	+	+	++
E3	Water	−	+	−	−	+	−
E4	Water	++	++	+	++	++	++
E5	Water	−	−	−	++	+++	−
E6	Water	−	−	+	−	+	−
E7	Water	−	−	−	−	−	−
E8	Water	++	++	++	+++	++	++
E9	Sediment	+	−	+	++	−	++
ESP	Sediment	−	+	+	−	+	+
ESPI	Sediment	−	−	+	+	+	+

W1, W2, W3, W4, W5, and W6 stand for scores on weeks 1, 2, 3, 4, 5, and 6 respectively.

Upon further examination we found that within-family evenness was very low; the majority of reads within each family could be assigned to a single OTU. These OTUs were the most probable to be isolated and tested for their ability to produce BSs. The representative sequences of the most abundant OTUs within each family were then compared against the NCBI 16S database using BLAST in order to find the closest cultivated representative to each abundant OTU. **Table 2** summarizes the distribution of reads within each of the seven families and the BLAST results for the most abundant OTUs within each family.

Pure Strain Isolation and Biosurfactant Production Screening

As shown in **Table 2**, the number of possible isolates obtained from consortia E4, E8 and E9 was limited, as these were dominated by a handful of dominant strains. Thus, the chance of isolating strains other than those was statistically disfavoured. In order to increase the total number of possible BS producers, two more sediment samples from Elefsina, namely ESP and ESPI, were included. Isolation and purification of single strains was subsequently performed as described in Experimental Procedures.

Fifty pure cultures were obtained in total and their taxonomy was identified (see Experimental Procedures). Twelve of these strains were actually different. The ability of each different strain to grow in rich (marine broth) and minimal (ONR7a) medium with CO 0.5% w/v as a sole carbon source was then examined. All taxonomically different isolates were consequently screened for BS production by the drop collapse test in the medium(s) were growth was possible. The isolated strains' phylogenetic identity, growth ability and drop collapse test scores are shown in **Table 3**. Strains E8Y, E4D, E4F (*Alcanivorax borkumensis* SK2) in ONR7a/CO 0.5% w/v and ESP-A (*Paracoccus marcusii*) in ONR7a/CO 0.5% w/v achieved the highest scores. BS production of these strains was then quantified.

Biosurfactant Production by Mixed Bacterial Community – Effect of Time, Temperature, and Carbon Source

EB8 consortium was isolated as the most promising BS producing mixed culture based on the drop collapse test. The EB8 isolated community growth curves under the different incubation conditions are shown in **Figure 3**. All samples exhibited the same trend, reaching the stationary phase within 48 h. Bacterial growth reached the highest level when molasses and CO were used as a carbon source at 30°C, without addition of N and P sources.

In **Figure 4** the BS production from BE8 isolated consortium is presented for different incubation conditions. We observe that RL concentration remained constant and low at $\sim 20 \pm 10$ mg/l in the culture broth, independent of incubation conditions. The BS production yield of BE8 was about double compared to the BS production yield of the E8 consortia ($\sim$10 mg/l of purified RL), at 20°C with CO as carbon source. Addition of molasses led to increased biomass production of BE8 that decreased as temperature increased from 20 to 30°C but did not enhance BS production. In the presence of CO as carbon source, biomass

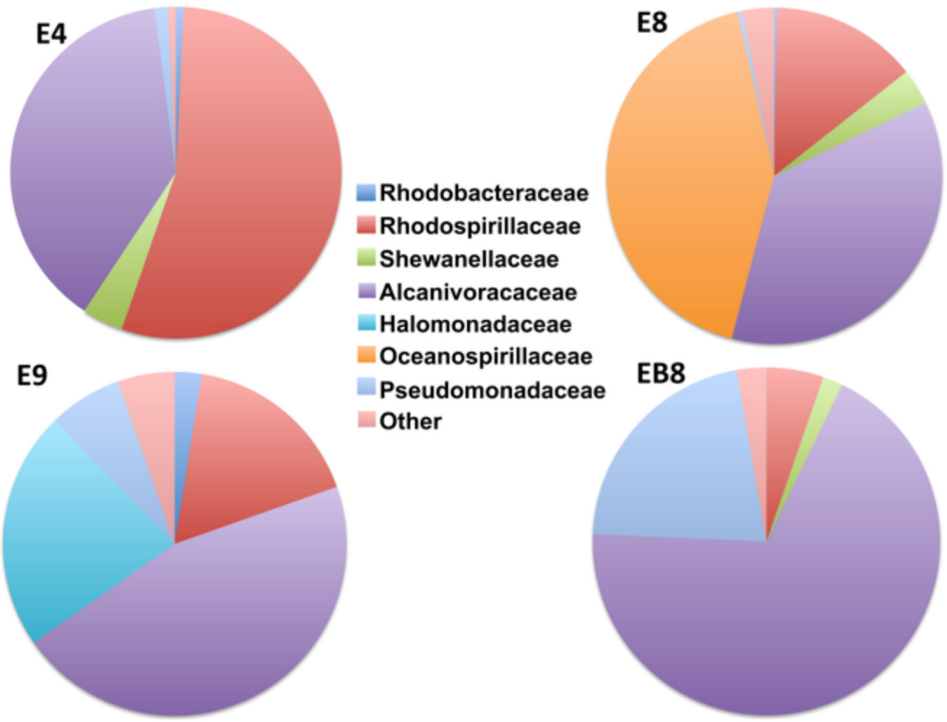

FIGURE 2 | The distribution of the bacterial Families among the screened consortia. The "Other" category stands for reads for which assignment down to family level was not possible.

TABLE 2 | Within-family read distribution and closest relative of the most abundant OTUs.

Family	# of within-family OTUs	% of the most abundant OTU	Closest Relative of the most abundant OTU (% of similarity)
Rhodobacteraceae	3	91	*Roseovarius crassostreae* (99)
Rhodospirillaceae	18	96	*Thalassospira lucentensis* strain QMT2 (98)
Shewanellaceae	3	98	*Shewanella frigidimarina* strain NCIMB 400 (99)
Alcanivoracaceae	29	95	*Alcanivorax borkumensis* SK2 (99)
Halomonadaceae	3	93	*Halomonas marina* (99)
Oceanospirillaceae	11	97	*Marinomonas vaga* strain 40 (99)
Pseudomonadaceae	3	96	*Pseudomonas pachastrellae* strain KMM 330 (99)

concentration remained constant at all temperatures and approximately at 0.5 g/l. Nutrients addition (N and P) did not have a significant effect on biomass growth or BS concentration.

Sophorolipid production is shown to be in the same range as that of the RLs (20 ± 10 mg/l). Similar to RLs, the production of SLs remained constant over time and does not depend on the carbon source, temperature or biostimulation with N and P.

Biosurfactant Production by Single Strains

Both RL and SL BS production using the isolated strains E8Y, E4F, E4D, and ESP-A, were investigated, focusing now on the RLs production. The results are shown in **Figure 5**. Interestingly, we observed that biomass remained more or less constant from day 6 to day 12 while RL production yield was also in the same levels for the case of E8Y, E4D, and E4F strains, i.e., 50 ± 10 mg/l (more than 100% increase in the production of purified RL). Strain E4F exhibited the highest production yield of BS at the lowest biomass content. Strains ESP-A, E4F, and E8Y were further tested for SL production. A low production yield of 20 mg/l in all cases was observed.

The biomass of strain ESP-A remained within the CO phase and did not precipitate by centrifugation. The corresponding BS production was low compared to the rest of the other three isolated strains; however, this strain may have significant advantages for "sustainable" bioaugmentation in the open sea environment (no dilution by seawater currents).

It must be noted that the purified BS production yield from the crude BS extract was low of the order of 0.01–0.03 g BS/g crude extract, when CO was used as carbon source. This is a significant disadvantage of the use of CO as carbon source as it contaminates the crude extract with CO impurities, and a purification step with column chromatography becomes necessary.

TABLE 3 | Code, phylogenetic identity, growth ability and drop collapse test results of the isolated pure strains.

Strain code	Closest Relative	Growth in marine broth	Growth in ONR7a/ crude oil 0.5% w/v	Drop collapse test in marine broth[1]	Drop collapse test in ONR7a/ crude oil 0.5% w/v[1]
XP2	*Pseudomonas pachastrellae* strain KMM 330	Yes	No	+	n/a
XP3	*Marinomonas vaga* strain 40	Yes	No	–	n/a
XP4	*Thalassospira lucentensis* strain QMT2	Yes	No	–	n/a
XP5	*Thalassospira lucentensis* strain QMT2	Yes	No	–	n/a
XP6	*Roseovarius crassostreae*	Yes	No	+	n/a
E8Y	*Alcanivorax borkumensis* SK2	No	Yes	n/a	+++
E4D	*A. borkumensis* SK2	No	Yes	n/a	+++
E4F	*A. borkumensis* SK2	No	Yes	n/a	+++
ESP-A	*Paracoccus marcusii*	Yes	Yes	+	+++
ESP-C	*Sulfitobacter pontiacus* ChLG-10	Yes	No	+	n/a
ESPI-G	*Pseudoalteromonas agarivorans* strain KMM 255	Yes	No	+++	n/a
ESP-B	*Paracoccus carotinifaciens* strain E-396	Yes	Yes	–	–

[1] *The median score value of three biological replicates (separate cultures) is reported.*

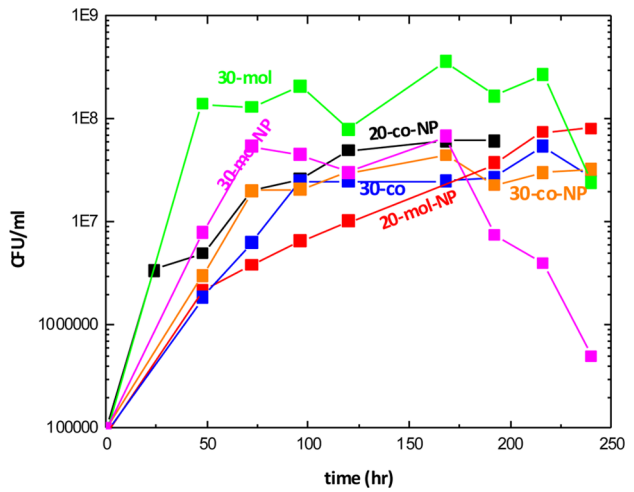

FIGURE 3 | Growth curves, colony forming units vs. time, under different conditions. (where mol, molasses and crude oil; co, crude oil; NP, additional nutrients and the number in the legend represents temperature).

Rhamnolipid Production by Strain E8Y – Effect of Carbon Source

In order to understand the mechanism that drives BS producing strains toward BS production, and hence enable us to fine-tune the production yield, we investigated BS production using different carbon substrates as energy source.

As shown in **Figure 6**, strain E8Y, a marine bacterial isolate, produced low concentrations of BS 50 ± 20 mg/l in the presence of the different carbon sources. In the absence of hydrocarbons, production of RLs decreased in half. Furthermore, the sample with CO as carbon source was made in duplicate and the BS concentration, extracted from the whole culture (0.5% wt CO-2), was compared to the one extracted by the culture without the oil phase (0.5% wt CO-1). A small difference (∼10 mg) is observed with the whole culture giving a better yield (∼70 mg purified BS/l). In addition, the culture with the heavy oil fraction (HOF) was made also in duplicate, one with E8Y strain (E8Y + 0.5% wt HOF) and a second one with acclimated to the HOF substrate E8Y strain (acclimated E8Y + 0.5% wt HOF). A better yield is observed when the acclimated strain was used (∼70 mg/l compared to 55 mg/l). Biomass remained low in all cases in the range 0.5 to 1 g/l.

When the heavy CO fraction was used, BS production was the highest (∼70 mg/l) and comparable to the BS concentration produced when CO was used as carbon source (50 ± 20 mg/l). This result is quite interesting because the HOF remains at the surface of the culture broth at all times and the extraction of the BS does not involve oil hydrocarbons as the substrate can be readily removed from the culture medium. The hydrocarbon-free culture medium is extracted without substrate impurities and the BS product is easily purified and produced.

Characterization of the Purified Biosurfactants

All samples were characterized by the following techniques in order to confirm/identify the BS characteristics of the generated product.

Thin Layer Chromatography

Thin layer chromatography results confirmed the presence of RLs and SLs in the crude extract and the purified BS products (**Figure 7**). Thin-layer chromatogram of isolated RL had an R_f value of 0.74 (band/solvent front ratio). SLs showed five prominent bands of R_f values of 0.08, 0.2, 0.36, 0.5, and 0.6 which compare with those published by Asmer et al. (1988).

FT-IR Measurements

Fourier transform infrared characteristic peaks at 3350, 2930, 2860, 1400, 1638, confirmed the presence of glycolipid type BSs (**Figure 8A**). FT-IR is a powerful tool to study the different forms of BSs. **Figure 8A** shows FT-IR spectrums of the purified RL and SL. In the entire spectrum, similar absorption arising from the O-H stretching vibrations occurs in the region of 3350 cm^{-1}. The carbonyl functional group (C = O) had a peak in the region

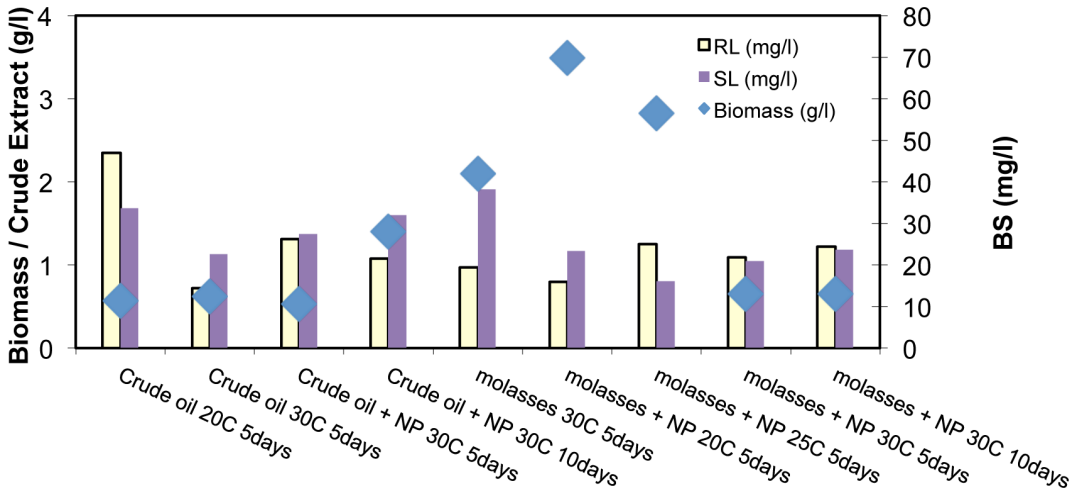

FIGURE 4 | Biomass concentration (g/l), and purified Rhamnolipid (RL) and Sophorolipid (SL; mg/l) for the different inoculation conditions and substrates of BE8 consortium (where NP means additional nutrients; and the number in the legend represents temperature).

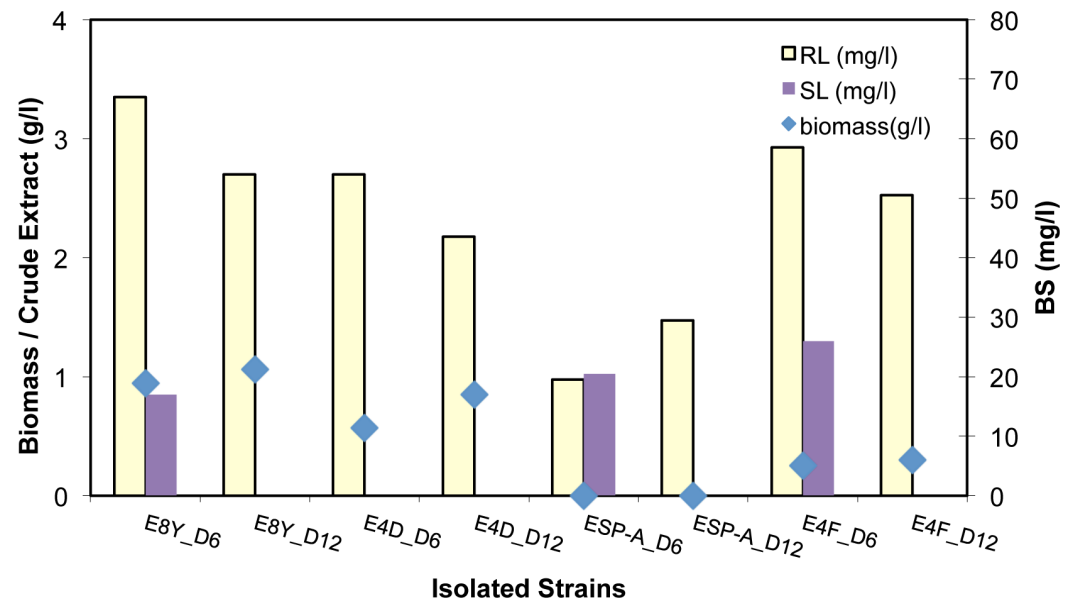

FIGURE 5 | Biomass (g/l), purified RL and SL concentration (mg/l) from the cultivation of strain E8Y, E4D, ESP-A, E4F for 6 and 12 days.

of 1744 cm^{-1}. The asymmetrical stretching (VasCH2) and symmetrical stretching (VasCH2) of methylene occurs at 2926–2930 and 2850–2860 cm^{-1}, respectively. The stretch of C-O band of C (-O)-O-C in acetyl esters appears at 1247 cm^{-1} (RL curve). The band at 1445 cm^{-1} that corresponds to the C-O-H in plane bending of carboxylic acid (–COOH; Silverstein and Webster, 1998).

Detection of Rhamnolipids with LC-MS

Rhamnolipids Rha-Rha-C_{10}-C_{10} and Rha-C_{10}-C_{10} were detected with pseudomolecular ion being 649 and 503 respectively (**Figure 8B**).

Discussion

The Biosurfactant Production Capacity of Marine Microbial Populations

The community structure of the four screened hydrocarbon degrading mixed consortia (E4, E8, BE8, E9) was rather simple. Overall, *Alcanivoracaceae* was the most abundant bacterial family with *A. borkumensis SK2* strain being the dominant strain. That specific strain is very well described in the literature (Yakimov et al., 1998; Golyshin et al., 2003; Schneiker et al., 2006) and it is one of the major players in hydrocarbon degradation in the water column; being commonly found

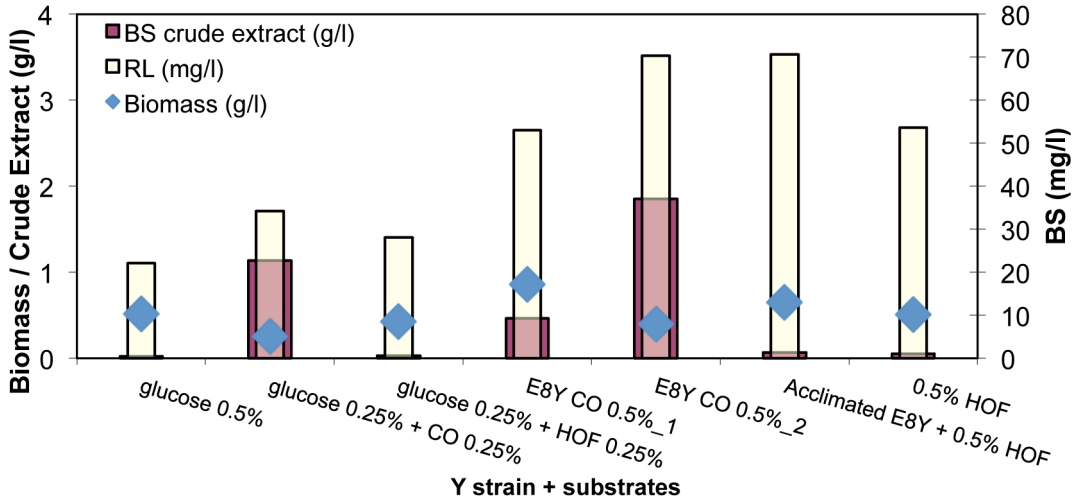

FIGURE 6 | Biomass (g/l), BS crude extract (g/l), purified RL (mg/l) concentration produced by the cultivation of strain E8Y for 12 days with substrates pure or mixtures of glucose, crude oil (CO), and heavy oil fraction (HOF). In sample E8Y CO 0.5% wt_1 BS was extracted by the culture without the oil phase. In sample E8Y CO 0.5% wt_2 BS was extracted by the whole culture.

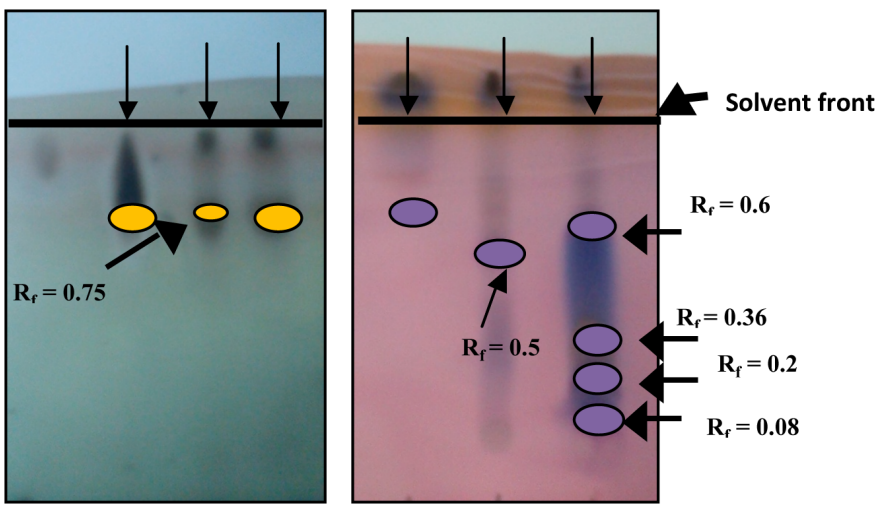

FIGURE 7 | Rhamnolipids (left) and Sophorolipids (right) TLC detection.

in enrichment cultures and contaminated areas (Yakimov et al., 1998; Golyshin et al., 2003; Schneiker et al., 2006). Members of the *Rhodobacteraceae, Rhodospirillaceae, Halomonadaceae, Oceanospirillaceae, Pseudomonadaceae,* and *Shewanellaceae* families have also been reported to encompass oil-degraders and BS producers (Cui et al., 2008; Fredrickson et al., 2008; Mnif et al., 2009; Raaijmakers et al., 2010; Jiménez et al., 2011; Kostka et al., 2011; Ibacache-Quiroga et al., 2013). However, the single strains isolated from these families in this study did not show significant BS production (drop collapse test). This fact may indicate that these strains were acting mainly as hydrocarbon degraders and not BS producers in the mixed cultures. On the contrary, the three tested *A. borkumensis SK2* strains were found to produce BS, indicating that those were the main producers in the mixed cultures. These findings highlight the functional plasticity of microbial communities in the presence of hydrocarbon contaminants; hydrocarbon degradation is not a trait "bound" by taxonomy, as the cassettes of genes that encode for hydrocarbon degradation are typically found within mobile genetic elements and can be transmitted horizontally (Top and Springael, 2003). Thus, a microbial community can adapt and regulate its functions depending on the community composition and the presence of different substrates. Despite the above, there is a "core set" of bacteria (such as members of the *Alcanivorax*, *Marinobacter*, and *Cycloclasticus* genera) that are commonly isolated from marine hydrocarbon-degrading consortia as they are obligate oil-degraders (Yakimov et al., 2007).

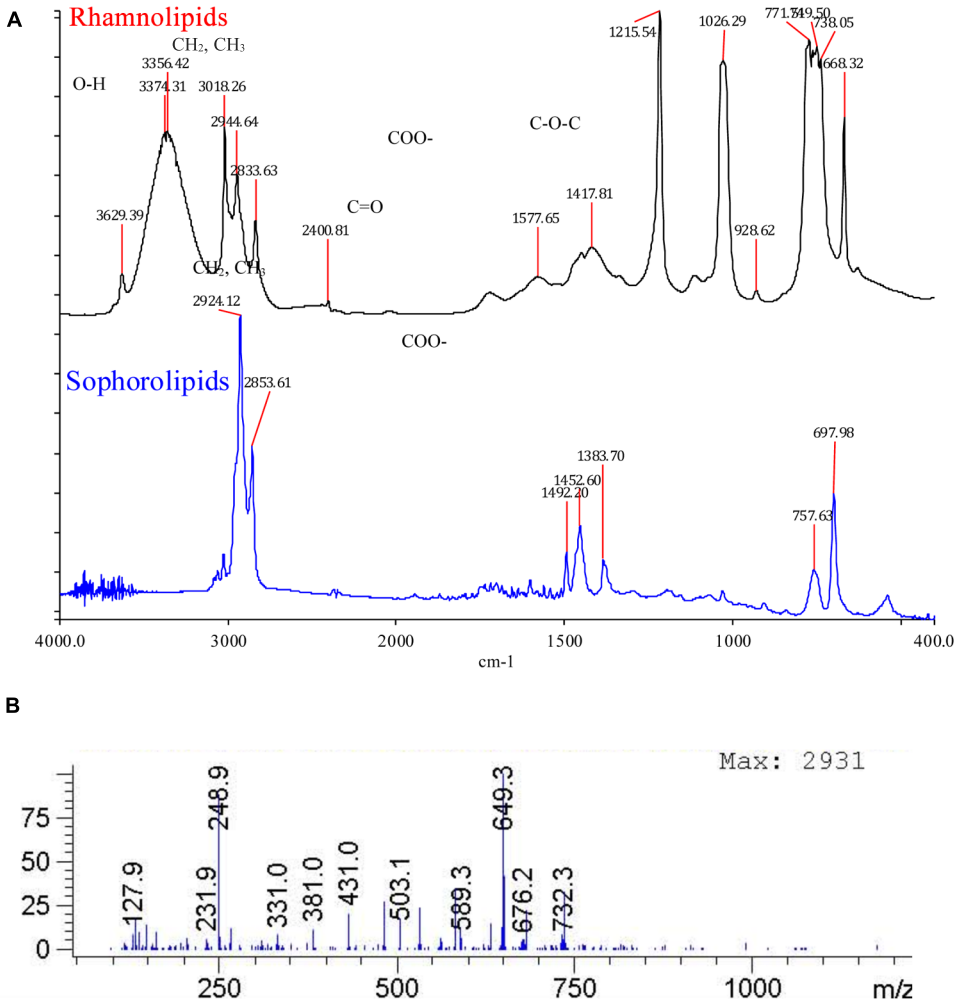

FIGURE 8 | (A) Fourier transform infrared (FT-IR) spectrum of isolated rhamnolipids and sophorolipids. The characteristic peaks at 2930 (CH_2, CH_3), 2830–2850 (CH_2, CH_3), 1725 (C = O), 1450 (COO^-), 1300-1100 confirm the presence of glycolipid type biosurfactant. **(B)** Rhamnolipids Rha-Rha-C10-C10 and Rha-C10-C10 detected with pseudomolecular ion being 649 and 503 respectively.

The EB8 consortium that was created using material from the oil-water interface of E8 consortium, consisted mainly of *Alcanivoracaceae* and *Pseudomonadaceae* as opposed to the "mother" E8 consortium that also contained *Oceanospirillaceae* in a high proportion. That indicates that members of the latter family were mainly present in the planktonic form in the water phase of the culture. A mixture of mono-, di-rhamnolipid and SL glycolipids was produced by the EB8 consortium. BS concentration in all cases was low, in the range of 20 ± 10 mg/l (comparable to the concentration produced by the mixed consortia E8 at 20°C with CO as the carbon source) and remained constant over time (after the stationary phase is reached). A temperature increase from 20 to 30°C or addition of glucose (molasses) to the carbon source had no significant effect on the BS production even though biomass increased (from 0.5 to 3.5 g/l) in the presence of molasses and temperature increase. Biostimulation with N and P had no major effect on the biomass or the BS concentration. To the best of our knowledge a constant BS production by hydrocarbon degrading-BS producing single strain or consortia regardless of biomass and culture conditions has not been reported as of yet. Rahman et al. (2003) report BS production yield that is not related to the biomass concentration (high yield-low biomass or the opposite). In this work the BS production yield depends on the amount of the non-soluble substrate. In the case of marine *Bacillus* sp. the BS product to biomass ratio varies with substrate variation (Mukherjee et al., 2008). This was also observed in this work.

There are only few reports available about production of BSs by bacterial consortiums isolated from hydrocarbon contaminated soil or marine water column (Rahman et al., 2003; Darvishi et al., 2011; Trejo-Castillo et al., 2014) and even fewer that at the same time use CO as the carbon source. In particular, Rahman et al. (2003) and García-Rivero et al. (2007) used crude and diesel oil as sole carbon source respectively. Furthermore, the consortium they used is a mixed culture comprising from the best single strains that had a specific characteristic, e.g., good BS production

or hydrocarbon degradation as opposed to ours that has been subcultured directly from environmental samples. Darvishi et al. (2011) and Rahman et al. (2003) reported BS production yield of 1.67 g/L and 4.9 g/L by mixed consortia, respectively. There is an evident difference of an order of magnitude in the BS production yield and this is probably due to our consortium being totally different in composition or the initial inoculum amount being substantially different (we started with a total inoculum of 10^7 CFU). Our consortium composed mainly by members of the Rhodobacteraceae, Rhodospirillaceae, Shewanellaceae, Alcanivoracaceae, Halomonadaceae, Oceanospirillaceae and Pseudomonadaceae families (**Figure 2**), whereas Rahman et al. (2003) used a consortium of five isolates (*Micrococcus* sp. GS2-22, *Bacillus* sp. DS6- 86, *Corynebacterium* sp. GS5-66, *Flavobacterium* sp. DS5-73 and *Pseudomonas* sp. DS10-129), and Darvishi et al. (2011) used a consortium of two isolates *E. cloacae* and *Pseudomonas* sp.. In all cases, the common denominator is the *Pseudomonas* family members that are well-known hydrocarbon degraders and BS producers. Rahman et al. (2003) also observed that "when oil degraders were introduced individually, the amount of surfactant production was more when compared to the production of surfactant by mixed bacterial consortium," which is similar to our results. This indicates that there may be a competition between the bacteria for nutrient substrate. However, BS production by the mixed bacterial consortium reported here has not been reported earlier.

When single *A. borkumensis SK2* strains E8Y, E4D, E4F where incubated with CO as the sole carbon source at 14°C the RL production yield increased up to 50 ± 20 mg/l. This represented a more than 100% increase compared to the concentration that complex marine consortia E8 and EB8 produced. SL concentration remained low at 20 mg/l. *Alcanivorax* is known for its glycolipid production as well as hydrocarbon degrading capability (Yakimov et al., 1998; Golyshin et al., 2003). It has been reported in the literature that mainly laboratory strains of *Pseudomonas aeruginosa* (a well described class II pathogen and RL producer) are used to produce yields of 10–20 g/l of RLs, whereas SLs are already produced by manufacturers using *Candida bombicola* yeasts with yields greater than 100 g/l (Wei et al., 2005; Marchant and Banat, 2012; Banat et al., 2014). Daniel et al. (1998) reported a surprisingly high yield (422 g/l) of SLs production by *Candida bombicola ATCC 22214.* The BS yields obtained by marine BS producers and reported here are at least one order of magnitude lower compared to the ones in literature (Rahman et al., 2003; Darvishi et al., 2011).

Most of the published works, study the time course of BS production kinetics in optimal media and substrate conditions, using BS producers whereas in our study we explore the BS production yield by consortia from the marine environment, which produce BS and consume CO.

Best Hydrocarbon Degrader Strain for Bioaugmentation in Open Sea?

The ESP-A strain, *Paracoccus marcusii* obtained from marine sediment, produced low amounts of BS, ∼20 mg/l of each glycolipid, *while keeping its biomass trapped in the oil phase (CO).* This observation is particularly interesting from a bioremediation point of view. If this strain is used for bioaugmentation purposes, no dilution effects are expected due to sea currents. *Paracoccus marcusii* strains have been reported present in hydrocarbon contaminated areas as general hydrocarbon degraders (Harker et al., 1998; Chaerun et al., 2004), as a promising polyaromatic hydrocarbon (PAH) degrader (Pouli et al., 2008) as well as carotenoid producer (Hirschberg and Harker, 1999). In oil spills, where PAHs are the predominant compounds, bioaugmentation with *Paracoccus marcusii* strains maybe an optimal choice.

Effect of Substrate Type and Concentration on BS Production

The BS production by *A. borkumensis SK2* strain (E8Y) was investigated for possible enhancement through the use of alternative media. In particular, the tested media were: the substrate hydrocarbon concentration was modified from zero to 0.5% with the use of glucose as the alternate carbon source. No significant increase in the BS concentration was observed by E8Y. This strain when fed with glucose (either entirely 0.5% w/v or partially 0.25% w/v) did not increase at all production, on the contrary RL concentration decreased approximately in half.

This may be an indication that the specific strain needs to be stressed by the presence of a non-water soluble substrate (like CO) to produce "only" the required amount of BS, otherwise acts as hydrocarbon degrader alone. Higher concentration was observed in all cases when as carbon source CO or a heavy fraction of CO was used. Interestingly, when a heavy CO fraction is used as the sole carbon source the final product is not contaminated by the substrate as the latter remains as a different phase in the culture broth and it can be readily removed. One of the main issues the BS industry faces, is the final product contamination with substrate impurities (Banat et al., 2014), when a water insoluble substrate is used (i.e., CO). Here, strain E8Y when fed with soluble substrates, like glucose, BS production was not promoted. Although water-soluble substrates may be attractive for processing (clean facilities), the fact that the same substrate can be easily used over and over again and the ability to be readily replaced in one piece, makes HOFs of CO (high in asphaltene concentration) quite attractive.

Implications in Bioreactor Operation for BS Production

The above observations lead us to consider the case of bioreactor configuration/operation for BS production using a heavy CO product as carbon source. The latter stresses the marine bacterial isolates to produce BS without being able to solubilize it. Thus, the extraction of the RLs from the aqueous phase is easy and no purification of the BS oil extract is necessary (silica gel column chromatography) as no substrate is dissolved in this case. A comparative process flowchart is given in **Figure 9**.

Interfacial Behavior of the Rhamnolipids-Sophorolipids Lipid Mixture

How much is the "required amount of BSs that enables dissolution -degradation of the CO?" It is known that addition of RL

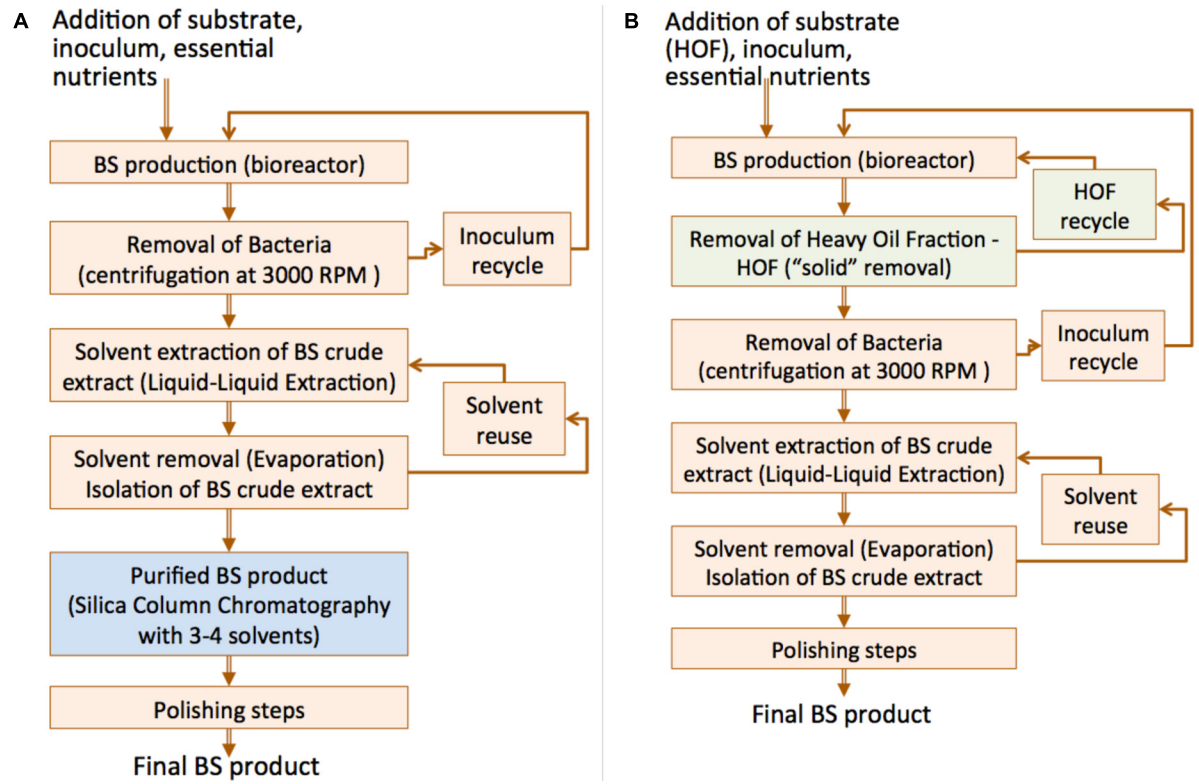

FIGURE 9 | Comparative process flowchart for BS production: (A) Typical substrates (i.e., crude oil) that cannot be readily removed from the culture broth and hence the chromatographic separation step is required; (B) Use of HOF (rich in asphaltenes) which can be readily removed from culture broth, overcomes the requirement for chromatographic separation step.

and/or SF increases substantially the solubility of diesel when in concentration higher than the CMC (Whang et al., 2008). In order to answer the above question we made rough calculations on the amount of BS that is required based on its use and interfacial behavior. The calculations were made with the use of RL data since RL has been proven as a very efficient bioremediation agent (Nikolopoulou et al., 2013b) while it increases the cell surface hydrophobicity (Al-Tahhan et al., 2000).

Assuming that RL is utilized as emulsifier around the oil droplets we calculated (using literature data Helvaci et al., 2004) the number of RLs attached to the oil droplet surface using 0.01–1 μl droplet size emulsion of CO (0.5% wt or 6 ml/l) in water. The amount was in the range of 0.1–0.01 mg/l depending on the oil droplet size (the smaller the emulsion droplet size, the higher the amount of RL needed). This amount is very close to the CMC value of RL 0.1–0.04 mM for 0 up to 1 M NaCl environments (Helvaci et al., 2004). Assuming 50% degradation and decrease of the oil droplet size from 1 to 0.5 μl, the amount of RL/l needed to cover the oil droplets interface remains low (0.012 mg/l). In the extreme scenario of 100% degradation of oil droplets (6 ml/l) 0.01 μl size (big interfacial area) the amount increases to 0.9 mg/l, still small quantity compared to the concentration of RL in the culture broth.

Rhamnolipids also attach on the microbial cell surface (Sotirova et al., 2009). Rough calculations show that for 10^7 cells per ml (at stationary phase) and 1 μm radius (as a nominal value) of the cell, 8 mg/l RLs could be attached on the microbial surfaces. It is quite interesting as, this value is comparable to the concentration of RLs produced by the mixed consortia E8 and EB8. Hence, the assumption that bacteria produce BS at the same rate they consume (bacteria consume the oil droplets with the BS attached) is probably wrong since most of the RL is attached on their cell surface and dissolved in the culture broth, while a very small amount is used for emulsification (0.01–0.1 mg/l) of the hydrocarbons.

Conclusion

The BS production capability (RLs and SLs) by marine hydrocarbon degraders isolated from Elefsina bay in Eastern Mediterranean Sea was investigated. The best-isolated microbial consortia based on the drop collapse test exhibited a relatively low productivity with average yields in the order of 20 mg/l. In addition isolated strains, consisting mainly of *Alcanivorax* species, performed significantly better in RLs production (50 ± 20 mg/l) in the presence of CO as substrate. A particular strain isolated from sediments, *Paracoccus marcusii,* may be an optimal choice for bioremediation purposes as it produces BSs, degrades hydrocarbons (especially PAHs)

and remains trapped in the hydrocarbon phase not suffering from potential dilution effects by sea currents. The HOF of CO emerged as a promising substrate for BS production by marine BS producers as it can be easily removed from the bioreactor and the BS extract has no impurities by the substrate. Thus, the need for additional column chromatographic separation is bypassed.

Acknowledgments

This work was funded by FP-7 Projects No. 266473, "Unraveling and exploiting Mediterranean Sea microbial diversity and ecology for xenobiotics' and pollutants' clean up" – ULIXES and No. 312139 "Integrated Biotechnological Solutions for Combating Marine Oil Spills" – KILL·SPILL.

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Conflict of Interest Statement: 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.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 12 December 2014
doi: 10.3389/fmicb.2014.00697

Cost effective technologies and renewable substrates for biosurfactants' production

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Diverse types of microbial surface active amphiphilic molecules are produced by a range of microbial communities. The extraordinary properties of biosurfactant/bioemulsifier (BS/BE) as surface active products allows them to have key roles in various field of applications such as bioremediation, biodegradation, enhanced oil recovery, pharmaceutics, food processing among many others. This leads to a vast number of potential applications of these BS/BE in different industrial sectors. Despite the huge number of reports and patents describing BS and BE applications and advantages, commercialization of these compounds remain difficult, costly and to a large extent irregular. This is mainly due to the usage of chemically synthesized media for growing producing microorganism and in turn the production of preferred quality products. It is important to note that although a number of developments have taken place in the field of BS industries, large scale production remains economically challenging for many types of these products. This is mainly due to the huge monetary difference between the investment and achievable productivity from the commercial point of view. This review discusses low cost, renewable raw substrates, and fermentation technology in BS/BE production processes and their role in reducing the production cost.

Keywords: biosurfactants, bioemulsifiers, fermentation, renewable, substrates, sustainable

INTRODUCTION

Our daily routine basic activities are mostly dependent on the use of some kind of surfactants or emulsifiers including toothpaste, personal hygiene, cosmetic products, and other pharmaceutical by-products, most of which contains surfactants and emulsifier as one of their ingredients. The market for such products is therefore huge and demands are ever increasing. However, due to the non-biodegradability, ability to accumulate and toxicity of some of the chemical petroleum based product to the environment, there has been a general desire to find replacement surfactants to the chemically synthesized compounds with biological products (Satpute et al., 2010a,b; Marchant and Banat, 2012a,b). Such biological biosurfactant/bioemulsifiers (BS/BEs) are mainly of microbial origin and are generally more environmental friendly benign products.

Biosurfactants and bioemulsifiers amphiphilic surface active abilities which are due to the presence of hydrophobic and hydrophilic moieties within their molecules which allows them to aggregate at interfaces (between immiscible liquids, for example water and oil). BS/BE reduce surface and interfacial tension (IFT) in liquids or different phases of matter, like gas, liquid, and solid. Such properties play an important role in various fields like bioremediation, biodegradation, oil recovery, food, pharmaceutics, and many other applications in different industrial sectors (Cameotra and Makkar, 2004; Banat et al., 2010; Fracchia et al., 2014; Franzetti et al., 2014). The structural and functional novelty of such surface active molecules is attracting the attention of many researchers throughout the world. Their synthesis processes take place on water soluble and insoluble substrates by *de novo* pathway and/or assembly from other substrates (Satpute et al., 2010c).

The use of cheaper, renewable substrates from various industries such as agricultural (sugars, molasses, plant oils, oil wastes, starchy substances, lactic whey), distillery wastes, animal fat, oil industries have been reported and reviewed thoroughly by several researchers (Makkar et al., 2011). Various cheaper substrates such as soybean oil not only act as nutrients for the microbial growth but also act as an important source for isolation of potential BS producing microorganisms (Guerra-Santos et al., 1986; Lee et al., 2008). Rhamnolipids (RHL), one of the common BSs are usually produced on soybean oil soapstock, spent soybean oil, or chicken fat as a carbon source (Nitschke et al., 2004, 2005, 2010). Improvement in the fermentation technology, strain selection and use of cheaper, renewable substrates have a vital role in enhancing the production processes of BS industries (Marchant and Banat, 2012b; Marchant et al., 2014). However, large scale production for most microbial surface active agents has not reached a satisfactory economical level due to their low yields. In addition to this, high cost input is required for downstream processing to recover and purify microbial surfactants (Rodrigues et al., 2006a,b;

Smyth et al., 2010a,b). Such obstacles may be overcome by isolating potential BS/BE producers that can use the renewable substrates to raise the quality as well as quantity of BS. We can make use of waste material as better substrates for BS/BE production. Several alternative strategies for production at commercial scale have been reviewed by Helmy et al. (2011).

This review aims to provide comprehensive information on various economical, renewable substrates that are used for production of BS and how these substrates have can support BS fermentation technology. We also give a brief glance on the kinetics of BS production and fermentation technology that has been improved since last two decades.

NEED FOR CHEAPER, RENEWABLE SUBSTRATES IN BIOSURFACTANT INDUSTRY

Most biotechnological products processes need high monetary inputs and securing an optimum yield of product at the lowest expense through usage of low cost material (Cazetta et al., 2005). However, very low quantities of surface active agents are usually produced by microorganisms and the downstream processing of biotechnological products costs ∼60–80% of the total production expenditure. This is why most of the marketable products based on BS and BE are quite expensive. Therefore, it essential to reduce the production costs of BS/BE through the use of inexpensive and renewable substrates (Desai and Banat, 1997; Banat et al., 2000; Makkar et al., 2011). A diversity of carbon (water soluble and water insoluble) and nitrogen sources have been used for BS production which may consequently vary in structure or production site within the cell (intra or extra cellular, cell associated) depending upon the substrate composition particularly the carbon source (Guerra-Santos et al., 1986; Fiechter, 1992). In addition to usual water soluble carbon sources, a variety of unusual carbon sources such as ethanol, blended gasoline, hydrocarbons like heptadecane, hexadecane etc. have been used (Shreve et al., 1995; Patricia and Jean-Claude, 1999; Prabhu and Phale, 2003; Cunha et al., 2004).

Increased public awareness of issues related to environmental pollution strongly influences the development of technologies that facilitates cleaning hazardous contaminants. This has given imputes for finding suitable cheap BS products that can be used in the treatment of such contaminations. Kapadia and Yagnik (2013) introduced an alternative approach using solid state fermentation to obtain a more economical viable production process worth implementing on a commercial scale. Some of the suggested strategies included the use of more cheaper materials, optimization of environmental conditions and screening for overproducing strain to attain the maximize productivity (Satpute et al., 2008). The efforts taken toward this direction are significant to claim BSs as the molecules of the future. It is important to note that the results obtained to date show encouraging potentials to drive a beginning for the BS production industry.

USE OF COST EFFECTIVE RENEWABLE SUBSTRATES FOR BIOSURFACTANT PRODUCTION

Different relatively cheap and abundant substrates are currently available for use as carbon sources from various industrial sectors (**Table 1**). Many of these substrates have been reported as suitable substrates for growth and production of a wide range of microbial amphiphilic molecules (see **Tables 2** and **3**). These substrates are described in detail as follows.

AGRO-INDUSTRIAL WASTE, CROPS RESIDUES

Products such as bran, straw of wheat, straw of rice, hull of soy, corn, rice, sugar cane molasses, beet molasses, bagasse of sugarcane, cassava flour and its wastewater are representative candidates of agro-industrial waste (Nitschke et al., 2004; Rashedi et al., 2005a; Benincasa, 2007; Thavasi et al., 2014). Some waste material like rice water (by-product from domestic cooking and rice processing industry), corn steep liquor (corn processing industry) and cereals, pulses processed waste water are rich in starch content. Agro-industrial waste contains high amount of carbohydrates, lipids and hence, can be used as a rich carbon source for microbial growth. Among the agro-industrial waste products, molasses had attracted considerable attention by many researchers.

Table 1 | Summary of various cheaper/renewable substrates available from different industrial sectors.

Source industry	Waste/residues as *cheaper, renewable substrate*
Agro-industrial waste, crops residues	*Bran, beet molasses, Bagasse of* sugarcane straw of wheat, cassava, cassava flour wastewater, rice straw of rice, hull of soy, corn, sugar cane molasses
Animal fat	Waste
Coffee processing residues	Coffee pulp, coffee husks, spent of free groundnut
Crops	Cassava, potato, sweet potato, soybean, sweet sugar beet, sorghum
Dairy industry	Curd whey, cheese whey, whey waste
Distillery industry	Industrial effluents
Food processing industry	Frying edible oils and fats, olive oil, potato peels rape seed oil, sunflower, vegetable oils
Fruit processing industry	Banana waste Pomace of apple and grape, carrot industrial waste, pine apple
Oil processing mills	Coconut cake, canola meal, olive oil mill waste water, palm oil mill, peanut cake, effluent, soybean cake, soapstock, waste from lubricating oil

Table 2 | Summary of various renewable substrates used for production of microbial amphiphilic molecules by *Acinetobacter, Bacillus,* and *Candida* sp.

Organism	Renewable substrate	Biosurfactant/bioemulsifier type	Reference
Acinetobacter	Renewable resources	Surface active polymers	Rosenberg and Ron (1998)
Acinetobacter calcoaceticus	Soap stock oil (SSO)	Expolysaccharide	Shabtai (1990)
Bacillus subtilis	Molasses	Surfactin	Makkar and Cameotra (1997)
B. subtilis ATCC 21332; *B. subtilis* LB5	Cassava flour wastewater	Lipopeptide	Nitschke and Pastore (2004, 2006)
B. subtilis	Potato cassava	Surfactin	Noah et al. (2002)
B. subtilis	Potato cassava	Surfactin	Noah et al. (2005)
B. subtilis	Potato waste	Surfactin	Thompson et al. (2000)
B. subtilis	Potato waste	Surfactin	Thompson et al. (2001)
Bacillus sp.	Lubricating oil	Lipopeptide	Mercadé et al. (1996)
Bacillus subtilis ATCC 21332	Potato waste	Surfactin	Fox and Bala (2000)
B. subtilis	Peat hydrolysate	Surfactin	Sheppard and Mulligan (1987)
B. subtilis NB22	Solid state fermentation	Peptide antibiotic iturin	Ohno et al. (1993)
B. subtilis (recombinant)	Solid state fermentation	Lipopeptide antibiotic surfactin	Ohno et al. (1995)
B. subtilis NB22 (recombinant)	Wheat bran	Lipopetide-surfactin	Ohno et al. (1992)
Candida antarctica, C. apicola	Oil refinery waste	Glycolipids	Deshpande and Daniels (1995)
C. bombicola	Animal fat	Sophorolipid	Deshpande and Daniels (1995)
C. bombicola ATCC 22214	Turkish corn oil and honey	Sophorolipids	Pekin et al. (2005)
C. lipolytica 1055 and 1120	Babacu oil	Bioemulsifier	Sarubbo et al. (1997)
C. lipolytica IA1055	Babassu oil	New biemulsifier: carbohydrate, lipid, protein	Vance-Harrop et al. (2003)
C. bombicola	Soy molasses-based medium	Sophorolipids	Solaiman et al. (2004, 2007)
C. bombicola ATCC 22214	Whey and rapeseed oil	Sophorolipid	Daniel et al. (1998a,b)
C. bombicola	Canola oil	Biosurfactant	Zhou and Kosaric (1995)
C. lipolytica	Industrial residue	Biosurfactant	Rufino et al. (2007)
C. lipolytica	Canola oil	Biosurfactant	Sarubbo et al. (2007)
Candida sp. SY16 95 45	Soybean oil	Mannosylerythritol lipid	Kim et al. (2006)
Yeast	Oil refinery waste	Glycolipids	Bednarski et al. (2004)

Molasses are concentrated syrups by-products of sugar cane and beet processing industries. This cheap substrate contains 75% dry matter, 9–12% non-sugar organic matter, 2.5% protein, 1.5–5.0% potassium and ≈1% calcium, magnesium, and phosphorus. Other components like biotin, pantothenic acid, inositol, and thiamine at 1–3% are also present giving it a thick, dark brown colored appearance. The high sugar content ranging approximately between 48 and 56% represents a good substrate for growth as well as production of microbial bioactive compounds for various microorganisms. When molasses are used as substrate, it needs to be clarified otherwise some of the components from molasses itself may impart unfavorable color to the desired products reducing their quality (Raza et al., 2007a,b). Molasses clarification process, however, can be quite costly as it involves dilution with water, acidification, pH adjustment to 7.0 using CaO powder and addition of $K_4Fe(CN)_6$ as a coagulant is carried out. During this process, heating up to 90°C for 1 h and cooling overnight at room temperature permits settlement of suspended solids and fibrous particles which can be removed by centrifugation (Raza et al., 2007a,b).

Achieving cost effective BS production depends on the development of cheaper processes and the provision of low cost substrate

Table 3 | Summary of various renewable substrates used for production of microbial amphiphilic molecules by *Pseudomonas* and other strains.

Organism	Renewable substrate	Biosurfactant/bioemulsifier type	Reference
Cladosporium resinae	Jet fuel JP8	Biosurfactant	Muriel et al. (1996)
Corynebacterium kutscheri	Waste motor lubricant oil and peanut oil cake	Biosurfactant	Thavasi et al. (2007)
Peudomonas cepacia	Sunflower oil	Bioemulsifier	Fiebig et al. (1997)
P. aeruginosa LB1	Oil wastes	Rhamnolipid	Nitschke et al. (2005)
P. aeruginosa	Whey	Rhamnolipid	Koch et al. (1988)
P. aeruginosa	Molasses	Rhamnolipid	Raza et al. (2007a)
P. aeruginosa AT10	Soybean oil refinery wastes	Rhamnolipid	Abalos et al. (2001)
P. aeruginosa GS9-119 *P. aeruginosa* DS10-129	Sunflower and soybean oil	Rhamnolipid	Rahman et al. (2002)
P. aeruginosa GS3	Molasses	Rhamnolipid	Patel and Desai (1997)
P. aeruginosa strain BS2	Distillery and whey waste	Rhamnolipid	Dubey and Juwarkar (2001)
P. aeruginosa strain BS2	Distillery and curd whey wastes	Rhamnolipid	Babu et al. (1996)
P. aeruginosa strain BS2	Curd whey and distillery waste	Rhamnolipid	Dubey and Juwarkar (2004)
P. aeruginosa strain BS2	Fermented distillery wastewater	Rhamnolipid	Dubey et al. (2005)
P. aeruginosa strain LBI	LB1 soapstock	Rhamnolipid	Benincasa et al. (2002)
P. aeruginosa strain LBI	LB1 soapstock	Rhamnolipid	Benincasa et al. (2004)
Pseudomoas sp. DSM 2874	Rapeseed oil	Mixture of four types of glycolipids (rhamnolipid 1–4), L-(+)-rhamnose and (R, R)-3-(3-hydroxydecanoyloxy) decanoic acid	Trummler et al. (2003)
Pseudomonas sp.	Jet fuel JP8, diesel oil	Biosurfactant	Bento and Gaylarde (1996)
Pseudomonas sp.	Used olive, sunflower oil	Rhamnolipid	Haba et al. (2000)
P. aeruginosa	Vegetable oil refinery wastes	Biosurfactant	Raza et al. (2007b)
P. aeruginosa FR	Palm oil	Biosurfactants	Oliveira et al. (2006)
Pseudomonas sp. JAMM	Olive oil mill effluent (OOME)	Rhamnolipids	Mercadé et al. (1993)
Rhodococcus sp.	Waste lubricating oil	Trehalose glycolipids	Mercadé et al. (1996)
Trichosporon montevideense CLOA 72	Dairy industry effluents	Glycolipid	Monteiro et al. (2009)
Tsukamurella sp. DSM 44370	Natural vegetable oil	Glycolipid	Vollbrecht et al. (1999)

raw material. Most earlier research concentrated on *Pseudomonas* sp. and *Bacillus* species while using molasses, whey, CSL as carbon and energy sources (Makkar and Cameotra, 1997, 1999; Patel and Desai, 1997; Makkar et al., 2011). However, there is a threat that the commercial products may get contaminated with those cheaper substrates products that are used as raw materials for production process. When the pure products are not available, there is difficulty to use them for intended application purposes. Various industrially important products like citric acid, xanthan gum, baker's yeast, acetone, alcohol, vitamins, amino acids, and organic acids are also produced successfully using molasses as a substrate.

India has an economy dependent on agro industries producing large volume of agro-industrial wastes which are mostly suitable for use as substrate. Some of the research laboratories are particularly involved in the use of molasses for production of various microbial metabolites. Makkar and Cameotra (1997) reported BS production from two *Bacillus subtilis* cultures in minimal medium supplemented with molasses as carbon source. Optimum BS production with good emulsification activity (EA) was achieved in

late stationary phase. Patel and Desai (1997) also worked on production of RHL BS from *Pseudomonas aeruginosa* GS3 by using molasses and corn steep liquor. Molasses of 7% (v/v) and corn steep liquor of 0.5% (v/v) were appropriate for optimum BS production. Cells produced BS in the stationary phase with a rhamnose sugar concentration of 0.24 g/L. Joshi et al. (2008) also used molasses and other carbon sources to produce BSs from several *Bacillus* strains under thermophilic conditions.

At international level several researchers have contributed in this area. Raza et al. (2007a) produced RHL BS from *P. aeruginosa* mutant strains using blackstrap molasses with or without supplementary nitrogen source and reported a yield of 1.45 g/L RHL after 96 h incubation. Another interesting work contributed by Benincasa (2007) suggests that the RHL produced from agro industrial wastes has an important role for hydrocarbon biodegradation in contaminated soil. Such studies have proved the importance of agro industrial wastes in bioremediation processes. Onbasli and Aslim (2009) used sugar beet molasses for RHL BS production from *Pseudomonas* strains and showed that among 18 strains of *Pseudomonas*, *P. luteola* B17 and *P. putida* B12, gave high yield of RHL at 5% (w/v) molasses. Cultures isolated from oily sites also utilize sugar beet molasses effectively for BS production. Rashedi et al. (2006), reported RHL BS producing *P. aeruginosa* isolated from Iranian oil wells. They used waste dates as sole carbon for the production of RHL using fed-batch culture and reported improved yields of BS. It is important to note that yield of the BS production increases with the increased concentration of molasses; maximum production, however, was reported using a medium containing 7% (v/v) of molasses. Other than above mentioned sources of molasses (sugar cane and beet), soy molasses are the most commonly used wastes from industrial sectors in the production of sophorolipid (SL) type BSs (Deshpande and Daniels, 1995; Solaiman et al., 2007). Molasses produced during the production process of soybean oil have been reported as a good carbon sources for SLs type BS from *Candida bombicola* (Solaiman et al., 2004). About 21 g/L yield was obtained as compared with glucose and oleic acid (79 g/L) in fermentation process. Such studies may not show benefits in enhancing the yield of metabolite but may be useful ways to reduce the accumulation of waste disposals from oil industry.

Researchers have worked with various combinations of carbon and nitrogen sources in BS production technology. Joshi et al. (2008) used molasses along with cheese whey as substrate for BS production from *Bacillus* sp. At the temperature of 45°C, the strain shows maximum BS production using molasses at 5.0–7.0% (w/v). Similar reports on BSs produced from probiotic bacteria have also been described. Rodrigues et al. (2006b) carried out studies with two microbial cultures namely, *Lactococcus lactis* 53 and *Streptococcus thermophilus* for BS production with conventional synthetic medium. They reported maximum BS production of 0.8 g/L for *S. thermophilus* and 0.7 g/L for *L. lactis* 53. Molasses have been found to enhance the yield of BS when compared to other conventional synthetic media. Thus, authors have suggested that there is not only an increase in the (about 1.2–1.5 times) mass of BS per gram of cell dry weight but also about 60–80% reduction in medium preparation costs. Therefore, molasses has been proved as an alternative economical medium for commercial BS production processes.

Cost variation in commercial production process has been calculated for molasses and soybean oil used as substrates (Joshi et al., 2008). Although this work is reported long time back, it showed that the substrate alone would place the BS production cost at a competitive setting when compared to chemical surfactants such as alcohol ethoxylate and alkylphenoletoxylate types for use in enhanced oil recovery (EOR). The point here to be highlighted is when molasses are used as a substrate BS production becomes more expensive than the chemically synthesized surfactant. On the other hand, the sophorose lipids employed in cosmetics is valuable as compared to the use of the synthetic surfactant, for the same cosmetic application. Usually various agricultural wastes like barley bran, trimming vine shoots, corn cobs, and *Eucalyptus globulus* chips have been used for simultaneous lactic acid and BS production. *Lactobacillus pentosus* has been tried in BS fermentation process by using hemicellulosic hydrolyzates after nutrient supplementation. The highest value of reduction (21.3 units) was found when using hemicellulosic sugar hydrolyzates obtained from trimming vine shoots, that corresponds to 0.71 g of BS per gram of biomass and 25.6 g of lactic acid/L. Whereas, barley bran husk hydrolyzates produces 0.28 g of BS per g of biomass and 33.2 g of lactic acid/L (Moldes et al., 2007).

ANIMAL FAT AND OIL INDUSTRIES AS SUBSTRATES

Meat processing industries such as food and leather produces significant quantities of animal fat, tallow and lard. Demand for animal fats is considerably less than vegetable oils and much of it becomes a problem for utilization as well as for their disposal. In comparison with other renewable substrates, animal fat and oil has not been much explored (**Figure 1**). An alternative option for such products is using them as raw material or substrates for production of commercial imperative compounds. Animal fat has been reported to act as a stimulator for the production of SLs BS from *C. bombicola* yeast (Deshpande and Daniels, 1995). One of the main outcome of their investigation indicated that this yeast grows poorly in presence of fat alone in the production medium. Mixture of glucose (10% w/v) and fat (10% v/v), however, enhances the growth of the yeast and the production of SLs (120 g/L). Recently, Santos et al. (2013) reported maximum glycolipid BS production using animal fat combined with corn steep liquor as compared to other carbon sources using yeast *Candida lipolytica* UCP0988. They also reported the product to have uses in bioremediation, oil mobilization, and recovery.

Production of BSs by fermentation of fats, oils, and their coproducts has also been reported (Solaiman et al., 2003). Nitschke et al. (2010) carried out BS production by using soybean oil waste (manipueira), along with molasses, whey and cassava flour, as substrates. Their observation suggests that cassava flour wastewater as a promising source of nutrients for BS production. These cheaper substrates were compared with conventional medium for BS production. Among eleven isolates tested, eight cultures reduced the surface tension (SFT) to levels below 30 mN/m using manipueira as substrate. They reported improved growth on manipueira agar for several isolates suggesting a high growth

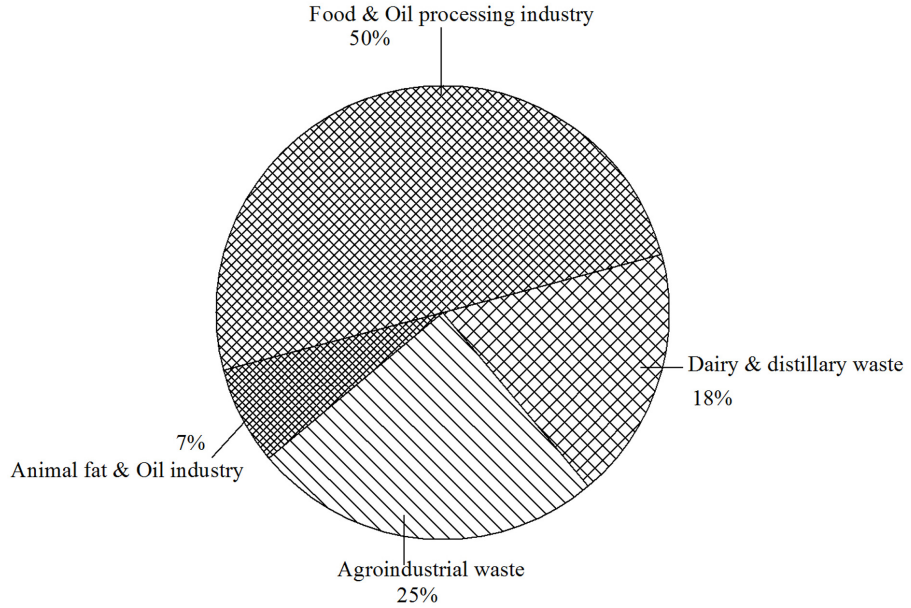

FIGURE 1 | Approximate percentage distribution for literature available on various renewable substrates used for biosurfactants production.

capacity and concluded that manipueira represents a potential alternative culture medium for BS production.

Industrial wastes, corn steep liquor and ground-nut oil refinery residue were also reported as low cost nutrients for the production of glycolipid type BS from *Candida sphaerica* (UCP 0995). The strain successfully mobilizes and recovers about 95% of motor oil adsorbed on sand sample which has vast applications in bioremediation processes (Luna et al., 2012). Several contributions are reported on usage of refinery wastes for production of microbial products. *Trichosporon mycotoxinivorans* CLA2 is BE-producing yeast strain which was isolated from the dairy industry effluents on mineral minimal medium containing refinery waste. This refinery wastes consists of diatomaceous earth impregnated with esters of having high organic matter content. Like molasses, pre-treatment of refinery waste is therefore, necessary for subsequent disposal in to the environment. Very few types of BEs have been produced from these residues (Monteiro et al., 2012).

DAIRY AND DISTILLERY INDUSTRIES BY-PRODUCTS

Dairy industries produce large quantities of whey that includes, curd whey, whey waste, cheese whey, lactic whey, all of which are easily available as raw substrate for microbial production of metabolites (Dubey and Juwarkar, 2001, 2004; Makkar and Cameotra, 2002; Dubey et al., 2005; Rodrigues and Teixeira, 2008). High amount (about 75%) of lactose is present in the lactic whey. Other components like protein and organic acids, vitamins provide good sources for microbial growth and BS production (Maneerat, 2005). Interesting studies have been reported regarding cloning the gene Lac ZY for lactose utilizing capability from *Escherichia coli* into *P. aeruginosa*. The cloned strain of *P. aeruginosa* grew well on whey and produces some RHL (Koch et al., 1988). Kosaric et al. (1984) have suggested the multi-organism strategy to decrease the cost at the commercial scale.

Other than RHL, a glycolipid type BS, considerable work has been reported on SLs. SLs has been produced by a two-stage (**Figure 2**) process starting from deproteinized whey concentrate (DWC) by using *Cryptococcus curvatus* ATCC 20509 and *C. bombicola* ATCC 22214 (Otto et al., 1999). These researchers compared the products from one-stage processes, by using different lipid based substrates. Two-stage batch cultivation process suggested that various physicochemical and properties of the SLs are greatly influenced by different carbon sources and not by the cultivation conditions. The same research group (Daniel et al., 1998b) had worked on the strains mentioned above using whey concentrates alone and in combination with rapeseed oil for production of SLs using single step batch cultivation. They developed sterilization method for whey by a combination of cross flow and sterile filtration. *C. bombicola* ATCC 22214 produced high (280 g/L) yield of SLs. Surprisingly, Daniel et al. (1998b) reported that *C. bombicola* ATCC 22214 does not consume whey lactose while it grows on oil or the lipidic substrates for SLs production. Daniel et al. (1998a) had also worked on two-stage batch cultivation concept reporting high yields (422 g/L) of SLs production using substrates like whey concentrate and rapeseed oil. The group also had grown the oleaginous yeast *C. curvatus* ATCC 20509 on DWCs in the first stage where they noted that lactose was consumed completely and biomass as well as an intracellular triglyceride, so-called single-cell oil (SCO), were produced. Crude cell extract resulted from cell disruption and heat sterilization were used for growth as well as SLs production by the yeast *C. bombicola* in a second stage (Daniel et al., 1999). The authors also showed that starting from DWC (50 g/L lactose), in the two stage fermentation process resulted in 12 g/L of extracellular SLs. In this two stage type of process, they

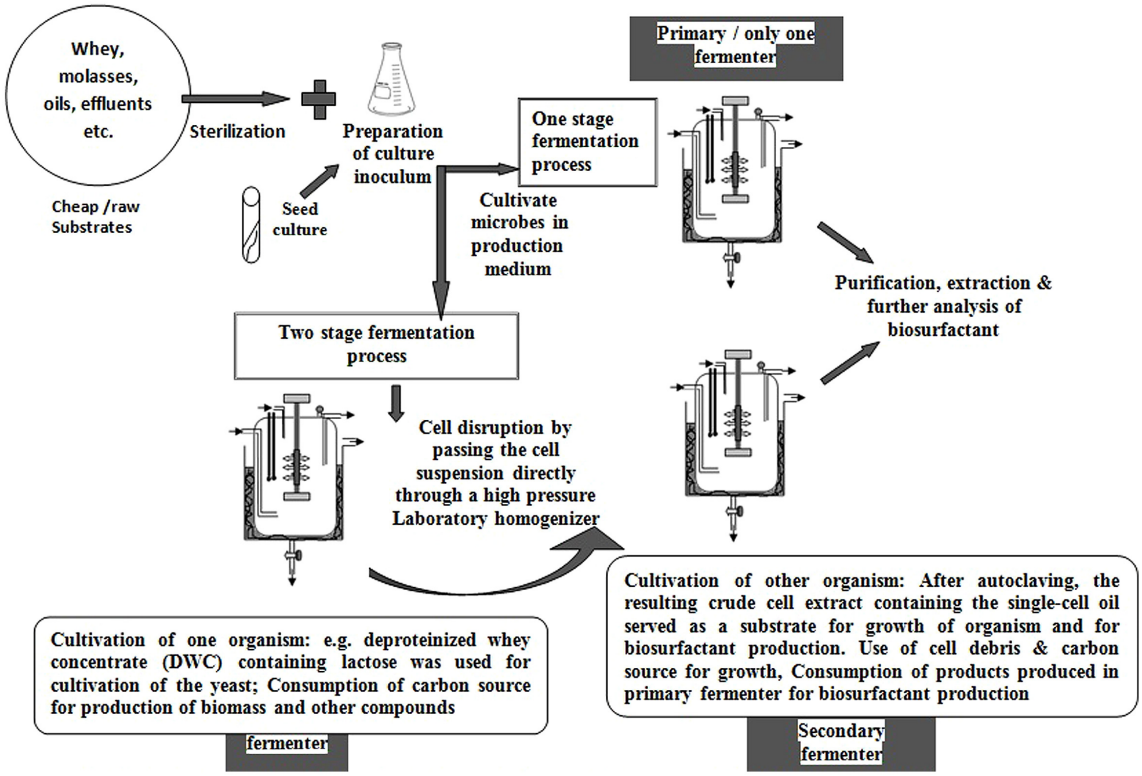

FIGURE 2 | Pictorial representation for one stage and two stage fermentation process for biosurfactant production. Several authors have used these approaches. We have constructed figure based on Daniel et al. (1999) experimental work.

reported that neither the growth of *C. bombicola* nor the productivity of SL from SCO is affected by the concentration of whey. In spite the amount of oil was the overall limiting factor of the process. Such kind of problems can be overcome by the addition of cheap oils during the production phase, to allow achieving high yields of SLs.

Wastewaters generated by dairy industries contain large quantities of fats and oils which are to some extent difficult to degrade (Willey, 2001; Cammarota and Freire, 2006). Like molasses, refinery wastes, pretreatment or clarification process for wastewater is a costly process. Daverey et al. (2009) described a process to utilize such valuable bioproducts for SLs production using yeasts *C. bombicola* through supplementing with sugarcane molasses and soybean oil. They reported about 38.76 g/L of SL with the synthetic dairy wastewaters containing 50 g/L sugarcane and 50 g/L soybean oil. Thus, authors therefore suggested utilizing real dairy industry wastewater for both the production of SLs. In addition to the above reports, Babu et al. (1996) reported on RHL production batch kinetic using distillery and whey waste in comparison to synthetic medium. Both the specific growth rates (μmax) and specific product formation rates (Vmax) were comparatively better in both waste media than in the synthetic media. Thus, their studies have proved that industrial wastes from distillery and whey are resourceful substrates for BS production. Similar observations were reported by Babu et al. (1996) and Dubey and Juwarkar (2001) for RHL production from *P. aeruginosa* by utilizing distillery effluent and whey wastes. Dubey and Juwarkar (2004) demonstrated 0.91 and 0.92 g/L of BS and use of *P. aeruginosa* strain in reduction of the pollution load up to 85–90%.

Although using dairy and distillery waste for various BS productions is possible, difficulties may arise when attempting to purify or collect such products. BSs recovery methods are diverse and include solvent extraction, precipitation, crystallization, centrifugation, and foam fractionation (Satpute et al., 2010a). However, Dubey and Juwarkar (2004) suggested that the various purification mentioned above cannot be effectively employed when using distillery wastewater as a nutrient medium for BS production. They produced BSs using *P. aeruginosa* strain BS2 and distillery wastewater and suggested that the substrate imparted color to the produced products which had a non-esthetic appearance and was difficult to recover from the fermentation medium. Therefore, they extended a new downstream technique involving adsorption-desorption processes using wood-based activated carbon (WAC). Therefore, WAC is one of the most efficient adsorbent among adsorbing materials like silica gel, activated alumina and zeolite. Polar solvent like acetone were also found to be efficient in recovering up to 89% BS from WAC. The authors recommended that WAC can be reused for BS recovery up to three cycles. The contribution by Dubey and Juwarkar (2004) has provided new approach for continuous recovery of BS from fermented distillery waste and concentrated foam. Such techniques can reduce the cost

involved in the solvent based purification methodology at the same time as providing efficient yield.

The use of distillery and whey waste as substrates for production of BSs is a useful recycling and reuse process. Babu et al. (1996) established maximum specific growth rates, specific product formation rates and proved to be superior to the synthetic medium. The provision of substrates from such wastes represents a huge contribution to future BS production industries. An exciting report has been published by Dubey et al. (2012) on distillery waste in combinations with curd whey waste, fruit processing waste and sugar industry effluent for growth and production of BS from newer microbes. They observed a positive impact of such combinations for BS production from *Kocuria turfanesis* strain BS-J and *P. aeruginosa* strain BS-P. The authors have suggested that we can replace precious water with other wastes required for diluting distillery waste for BS production. Instead of fruit processing waste, fruit juices like pineapple juice are also becoming attractive alternative carbon sources. Govindammal and Parthasarathi (2013a) carried out this studies and demonstrated 9.43 g/L yield from *P. fluorescens* MFS03 isolated from the crude oil enriched mangrove soil to improve the process economics.

OIL PROCESSING INDUSTRIES

Wastes from oil processing industries represent one of the alternative and easily available renewable substrates for production of microbial surface active molecules. Few examples are listed below. It is important to note that, the vegetable oil is one of the first substrates reported for high yield of SLs from *Torulopsis bombicola*. A SLs with the yield of 67 g/L has been reported by Cooper and Paddock (1984). Not only, sophorose lipid type BS but also other types of glycolipids have been studied extensively by using wastes from oil industries. Robert et al. (1989) and Mercadé et al. (1993) disclosed that the usage of vegetable oil from the distillation process and is effective for RHL production from *Pseudomonas* strains. Various oils along with water soluble carbon sources are proved to be good substrates for microbial surfactant molecules. This is evident from the following example. Babacu oil (5% v/v) supplemented with glucose (1% w/v) as carbon source provides a good source for growth and BE production. This work carried out by Sarubbo et al. (1999) suggested that two strains of *C. lipolytica* (1055 and 1120) produce BEs at the end of the exponential growth phase and beginning of the stationary growth phase.

Olive oil, sunflower has been proved as potential carbon and energy sources for production of microbial surfactants. The oils that contain low chain length (<C10) fatty acids undergoes modification for incorporation into surface active products. Haba et al. (2000) investigated the use of olive, sunflower oils in submerged culture condition by 36 microbial strains. They reported that several *Pseudomonas* strains usually grows well on waste olive or sunflower oil (2%) reducing SFT (<40 mN/m) of production medium while *Bacillus* strains do not use these substrates efficiently. Other strains like *Rhodococcus*, *Acinetobacter calcoaceticus*, and *Candida* neither use oils for growth nor for BS production. Abalos et al. (2001) also used a soybean oil refinery waste for production of RHLs using *P. aeruginosa* AT 10 strain and detected seven different homologs ($R_2C_{10}C_{10} + R_1C_{10}C_{10} + R_2C_{10}C_{12} + R_1C_{10}C_{12} + R_1C_{12}{:}1C_{10} + R_1C_{12}{:}_2 + R_1C_8{:}_2$) totaling ≈9.5 g/L. They also reported excellent antifungal properties against various filamentous fungi. A range of saccharic and lipidic feed stoke has been frequently used to produce SLs using *C. bombicola*. The fatty acid unsaturation, carbon chain length and source of low-cost industrial lipid feed-stocks influenced SLs production (Felse et al., 2007).

Rahman et al. (2002) used soybean oil, safflower oil and glycerol for production of RHLs using cultures of *P. aeruginosa*. Soybean oil supplements helps in increasing the biomass and RHL production to several fold that obtained just with safflower oil and glycerol. Increased amount of SL type BS has been produced by increasing the concentrations of safflower oil and glucose. Further yield can be enhanced with increased concentration of yeast extracts (Zhou et al., 1992). Use of such low cost renewable substrates in BS fermentation technology could be applied for bioremediation of hydrocarbon-contaminated sites and oil recovery process.

Among the above mentioned substrates, glycerol represents an important renewable carbon source as it is one of the main by-product of the biodiesel, biofuel production processes worldwide. For example, 1 kg of glycerol is generated from 10 kg of biodiesel when rapeseed oil is used (Meesters et al., 1996). Decanoic acid and rapeseed oil were used by Trummler et al. (2003) to grow *Pseudomoas* sp. DSM 2874 and produce mixture of four types of glycolipids (rhamnolipid 1–4), L-(+)-rhamnose and (R, R)-3-(3-hydroxydecanoyloxy). Fed-batch process with rapeseed oil produced mixtures of mono and di RHLs at a very high yield of 45 g l^{-1}. Another important work has been reported by Thanomsub et al. (2004) on glycolipid monoacylglycerols BS from *Candida ishiwadae*. This strain was isolated from plant material in Thailand on soybean cooking oil. Yeasts such as *C. bombicola* ATCC 22214 also efficiently used corn oil and honey for SLs production achieving higher yields when both grown on sugar and oil (Pekin et al., 2005).

In addition, Abouseoud et al. (2007) achieved BS production from *P. fluorescens* Migula 1895-DSMZ using olive oil as a carbon source with ammonium nitrate as a nitrogen sources. The products is reportedly a type of glycolipid with various properties like foaming, emulsifying and antimicrobial activities in addition to being highly stable at 120°C for 15 min, NaCl (10% w/v) and a wide range of pH values. *Tsukamurella* sp. DSM 44370 also used vegetable oil for its growth in addition to glycolipid BS production. Mutant strains of *P. aeruginosa* EBN-8 produced BS on canola, soybean, and corn oil refineries (Raza et al., 2007a,b). Canola oil refinery waste supplemented with sodium nitrate was reported best for microbial growth and RHL production with a yield of 8.50 g/L. Co-utilization of canola oil and glucose has also been carried out successfully for production of BS from *C. lipolytica* (Sarubbo et al., 2007). Oil wastes from cottonseed, soybean, palm oil, babassu, and corn oil refinery were studied as substituting low-cost substrates for RHL production by *P. aeruginosa* LBI strain. Marine microbial strains can also make use of oils (e.g., olive oil) other than aromatic hydrocarbons or crude oil for BS production. Khopade et al. (2012) reported potential BS producing strain, marine *Nocardiopsis* B4 isolated from the west coast of India. The BS is stable at higher temperature (100°C), wide range of pH and salt concentrations. Olive oil has been proved to

enhanced BS production. In some cases use of only pure carbon sources may not give the high yield of BS. However, disaccharides like lactose if supplemented with olive oil, the prominent difference can be seen in the intra- and extracellular lipids synthesized by the microbes. This concept has been showed from the work by Zhou and Kosaric (1993).

Palm oil mill effluent is also a promising substrate for BS production. Palm oil has also been used for BS production using *P. aeruginosa* SP4 (Pansiripata et al., 2010). A newly isolated BS-producing strain namely *Nevskia ramosa* NA3 has been reported for production of 1.0 g/L BS on palm oil mill effluent (Chooklin et al., 2013). Saimmai et al. (2012) also documented BS and BE producing microorganisms from palm oil contaminated industrial sites in palm oil refinery factory. Along with palm oil, they also included other sources like palm oil decanter cake and palm oil mill effluent. Use of such kind of different oil for screening and BS production process has successfully resulted five new genera namely, *Buttiauxella*, *Comamonas*, *Halobacterium*, *Haloplanus*, and *Sinorhizobium* for the first time. Such studies are significant for the future development of economically efficient industrial-scale biotechnological processes. Studies by Thaniyavarn et al. (2008) indicated the SL production by *Pichia anomala* PY1, a thermo tolerant strain isolated from fermented food. They used 4% soybean oil as carbon source at pH 5.5 and 30°C for 7 days. Comparative studies on media supplemented with both glucose or soybean oil lead to good BS production.

Comparative studies carried out by Govindammal and Parthasarathi (2013b) on glucose, petroleum based substrates, waste fried vegetable oil, and coconut oil cake for BS production from *Pseudomonas fluorescence* MFS03 isolated from mangrove forest soil. They proved that vegetable oil and coconut oil are reliable substrates for BS production. These oils contain high percentage of oleic acid.

Very recently, Saravanan and Subramaniyan (2014) isolated *P. aeruginosa* PB3A strain from oil contaminated soil and examined BS production on various substrates namely, castor oil, coconut oil, rapeseed oil corn oil, motor oil, sunflower oil, olive oil, olein, barley bran, cassava flour waste, rice bran peanut cake, potato waste, and wheat bran instead of routine carbon sources. Corn oil and cassava waste flour were found to be highly effective. Once again these studies have confirmed the potential role of agro-industrial wastes for BS production in place of synthetic media.

Sometimes the oils in the production medium needs to be supplied along with other ingredients like mineral salts, glucose. Bento and Gaylarde (1996) carried out BS production from *Pseudomonas* by growing in the production medium with sterile diesel oil, mineral salts, and glucose. Other oil sources like jet fuel JP8 also act as rich carbon source. Muriel et al. (1996) worked on Jet fuel JP8 for BS production from *Cladosporium resinae* where SFT of the production medium was lowered significantly with the increase in emulsion and foaming properties. Thavasi et al. (2009) reported *Azotobacter chroococcum* a BS producing strain isolated from marine environment able to grow on waste motor lubricant oil, crude oil, and peanut oil cake. Peanut oil cake was reported as best source for BS production with a yield of 4.6 g/L and an ability to emulsify various hydrocarbons effectively. Studies from Thavasi et al. (2007) described the outlook for BS production by using relatively cheap and abundantly available resources such as peanut oil cake and waste motor lubricant oil. This fact is supported from the studies reported on production and characterization of glycolipopeptide BS from *Corynebacterium kutscheri*. Studies showed optimum growth (9.8 g/L) and BS production (6.4 mg/ml) in fermentation medium with peanut oil cake. This glycolipopeptide emulsifies crude oil, waste motor lubricant oil, kerosene, diesel, peanut oil, xylene, naphthalene, and anthracene which have applications in various hydrocarbons in bioremediation processes. This study has proved potential role of BS in bioremediation process. Peanut oil has been used by probiotic bacterial system (*Lactobacillus delbrueckii*) for production of BS (Thavasi et al., 2011).

In addition to these inexpensive sources, spent yeast from fermentation industries has also been utilized in the production of high value product from a commercial point of view (Alcantara et al., 2012). Vance-Harrop et al. (2003) used babassu oil and D-glucose as carbon sources for the BS production from yeast strain *C. lipolytica* IA1055. This BS is composed of carbohydrate, lipid, protein in production medium prepared in natural seawater (diluted up to 50% v/v) supplemented with urea, ammonium sulfate, and phosphate. Most literature suggests exploitation of natural processes and developing economically viable production of BSs through the use of oil industry wastes. Bhardwaj et al. (2013) recently reviewed the production and applications of BSs from the oleo-chemical industrial wastes. Waste oils can be used for screening and selection of microbes for their waste oil utilizing capacity and BS production. Mercadé et al. (1996) carried out such studies where, lube oil was used to study 44 different cultures isolated from hydrocarbon-contaminated soil samples. Their studies showed that about 10% of the strains isolated shows BS production. These strains include *Rhodococcus* sp. for trehalose glycolipids and *Bacillus* sp. for lipopeptide type BS synthesis.

Spent oils are usually abundantly available oils that are quite difficult to dispose of due to environmental concerns including persistence and resistance to biodegradation (Mercadé et al., 1996). They include waste vegetable oil, used motor oil, lubricating oils, jet fuels all of which can act as cheaper source for microbial processes such as BS production. Usage of such kind substrates is usually encouraged as a pollution control strategies. Food processing industries use huge quantity of frying oils, where the composition vary depending on the number of times it has been used, modification in its composition, and finally need for pretreatment.

Studies carried out by Morita et al. (2007) on production of glycolipids by basidiomycete yeast *Pseudozyma antarctica*, on glycerol with the yield of 0.3 g/L of a BS. Another contribution on this aspect is shared by Ashby et al. (2005), where they used 40% of glycerol and 34% of hexadecane soluble compounds (92% of fatty acids and 6% of monoacylglycerol/triacylglycerol) and 26% of water for SLs synthesis by *C. bombicola*. About 60 g/L yields of SL was obtained from these studies. Several reports in literature support the use of glycerol as a carbon source for BS production (Guerra-Santos et al., 1986; Santa Anna et al., 2002; Rashedi

et al., 2005b). Not only *Candida* and/or *Pseudomonas* spp. utilize glycerol as carbon source. Fontes et al. (2012) reported a wild type *Yarrowia lipolytica* for BS production using residual glycerol or clarified cashew apple juice present abundantly in Brazil. High amount of olive oil mill wastewater is produced from the olive oil extraction procedures. Olive oil mill effluent (OOME) appears as a concentrated black color liquor that contains water-soluble polyphenols which usually represents an environmental challenge for disposal. However, OOME also contains some sugars (20–80 g/L), nitrogen compounds (12–24 g/L), organic acids (5–15 g/L), and residual oil (0.3–5 g/L). Mercadé et al. (1993) successfully used OOME for the production of RHL BS using the strain *Pseudomonas* sp. JAMM.

Oil cakes or soapstocks are semisolid or gummy product produced from processes oil seed where chemicals are used for extraction and refining the seed originated edible oils. The soapstock in spite of being a complex substrate has been successfully shown to produce highest yield of RHLs, along with different oily substrates, viz., sunflower oil, olive oil, soy bean oil. Yields up to 15.9 g/L were reported when using *P. aeruginosa* strain LBI grown in a salt medium containing soapstock (Benincasa et al., 2002). Soapstock has also been used efficiently for production of extracellular capsular polysaccharides (Benincasa et al., 2002). There have been examples of competent surfactant synthesis on soapstock and oil refinery wastes by *Candida antarctica* or *Candida apicola* with much higher yields than in the medium without addition of oil refinery waste (Bednarski et al., 2004). This shows the suitability of oil refinery waste for microbial surfactant production. Hydrophobic carbon sources like petroleum fractions, animal fat or vegetable oil have been utilized by several bacteria or yeast supplemented in cultured media for microbial surfactants (Hommel, 1990).

Two BEs namely, emulsan and biodispersan from *A. calcoaceticus* RAG-1 and *A. calcoaceticus* A2 were also produced by using soapstock as a carbon source (Shabtai, 1990). These two BEs show wide range of applications in stabilization of oil–water emulsion, the dispersion of large solid limestone granules and formation of micrometer-sized water suspension (Rosenberg and Ron, 1998). Soybean soapstock waste proved to be the best substrate with the yield of 11.7 g/L of RHLs that reduced the SFT in the culture broth to 26.9 mN/m with a critical micelle concentration (CMC) of 51.5 mg/L. Nitschke et al. (2005) reported production of mainly mono-RHL ($RhaC_{10}C_{10}$) when grown on hydrophobic substrates, while hydrophilic carbon sources lead to predominance of the di-RHL ($RhaRhaC_{10}C_{10}$) production. Pure soybean oil has been the predominant carbon source for many BSs production. Vollbrecht et al. (1999) tested similar oleic acid-rich oils, rapeseed oil and reported it to be efficient for BS production using *Tsukamurella* species DSM 44370. About 30 g/L glycolipid was produced from 110 g/L sunflower oil. The BS obtained showed high surface and interfacial activity and had some antimicrobial activities against some bacteria and a fungal strain.

FOOD PROCESSING BY-PRODUCTS

Most of the edible oils, vegetable oils, saturated, unsaturated fats are used by food processing industries. Today the majority of food markets are dependent on these oils and fats. In addition to this, medicinal, pharmaceutical, cosmetic industries also use them. Once the oils are processed for food production, the residues become harmful pollutants to our environment. Wastes from soybean, potato, sweet potato, sweet sorghum contains high amount of starch that acts as base material in fermentation process. Waste products like canola meal, coconut cake, peanut cake, soybean cake, also represent suitable candidates for cheaper substrates (Mercadé et al., 1996). Processed olive oil, sunflower, ground nut oil, rape seed oil; potato peels are useful as raw material for microbial products. A peat, composed of decomposed vegetable matter contains high amount of carbohydrates with main sugars like glucose, galactose, xylose, and amino acids provides excellent source for the growth of microbes. Other by-products from vegetable oil refining processes are also becoming one of the most targeted substrates for microbial BS production process.

In addition to the above mentioned relatively cheap substrates a number of abundantly available starch base substrates provide another alternative renewable carbon sources. One of the representative examples is the potato processing industry that produces significant quantities of starch-rich waste substrates suitable for BS production. In addition to approximately 80% water contents, potato waste also has carbohydrates (17%), protein (2%), fat (0.1%), vitamins, inorganic minerals, and trace elements. Thus, potato wastes are a rich source of various components which can support the growth of microorganisms for production of various commercially important products. A commercially prepared potato starch in mineral salts medium was investigated by Fox and Bala (2000). They reported BS production by *B. subtilis* ATCC 21332 and a significant reduction in SFT from 71.3 to 28.3 mN/m with a CMC value of 0.10 g/L using a methylene chloride extract of the BS. Thompson et al. (2000), put forward the use of potato effluents containing high-solids (HSs) and low-solids (LSs) substrates for production of surfactin for a *Bacillus* strain. They used 10 time diluted effluents with or without trace minerals amendments and used corn steep liquor successfully to produce surfactin with slightly lower yields LS substrate than from optimized potato starch medium.

Thompson et al. (2001) also showed that the LSs potato effluents can be used for surfactin production after heat treatment without the need for complete sterilization and after pretreatments to enhance yields. Such studies are significant for finding applications in low-value applications like environmental remediation or oil recovery. Like molasses, sometimes potato based substrates also need to undergo pretreatment procedures involving heating, removal of starch particulates and acid hydrolysis. Thermal and acid pretreatment would help in the removal of contaminant vegetative cells yet can have mixed results on slight improvement or reduction in yields (Thompson et al., 2001). Other contributions were reported by Noah et al. (2002, 2005) where potato process effluents were used for production of BS from *B. subtilis* sp. in continuous culture and air left fermentation conditions. Noah et al. (2002) worked on improving the process for utilization of potato related substrate. However, they observed the yield of BS was restricted by the oxygen availability and competition for indigenous bacterial population. The same research group (Noah et al., 2005) carried out studies on surfactin production from *Bacillus* sp.

by using purified potato starch and unamended LSs potato process effluent. Their studies highlighted that the process is oxygen limited and that recalcitrant indigenous bacteria in the potato process effluent hamper continuous surfactin production. They suggested the use of a chemostat operated in batch mode for surfactin production should be accomplished with the use of antifoam agents to prevent surfactant loss. They noted that antifoam does not interfere with recovery of surfactin and its efficacy and were able to achieve 0.6 g/L of surfactin from two different potato-processing facilities in comparison with Initial trials (0.9 g/L) from potato process effluent. Thus, they established that cassava wastewater produced from the cassava flour preparation, a renewable inexpensive and easily available carbon source can be used for surfactin production by *B. subtilis* and other biotechnological processes. Different unconventional carbon sources such as potato peel powder, corn powder, sugarcane bagasse and *Madhuca indica* were also used by Jain et al. (2013). They reported increased viscosity in cultures yet achieved maximum SFT reduction when compared to other substrates. They reported an unidentified BS production at a yield of 15.40 ± 0.21 g/L on corn powder base production medium from *Klebsiella* sp. strain RJ-03 and concluded that the use of such cheap substrates have a significant potential for commercialization for applications in bioremediation processes.

KINETICS OF BIOSURFACTANT PRODUCTION

It is well-established that along with environmental parameters such as pH, temperature, aeration, agitation, CO_2 level etc.; BS production is also dependent on the substrate composition and concentration in the media. These parameters interact with each other in a complex way to affect the kinetics of the BS production. C:N ratio plays an important role in the production process. Nitrogen limitation has been reported to enhance production (Abu-Ruwaida et al., 1991). Patel and Desai (1997) reported that BS production was enhanced under nitrogen limiting conditions. Temperature, pH, aeration, and salt concentrations are of course important parameters that influence production at commercial levels (Navon-Venezia et al., 1995).

Silva et al. (2010) used *P. aeruginosa* UCP0992 to investigate the effect of both carbon and nitrogen (source and concentration) on BS production at different cultivation conditions such as aeration, temperature, and agitation speed. Growth and BS production in mineral medium formulated with 3% glycerol and 0.6% $NaNO_3$, at 28°C during 120 h incubation at 200 rpm was monitored. They reported an almost parallel relationship between BS production, cell growth, consumption of glycerol, emulsification, SFT reduction, hexadecane, and other substrate utilization. They concluded that BS production is associated with growth starting shortly after inoculation with a two phase profile, the first up to 24 h and remaining constant until 48 h, while in the second phase, production increased at a slower rate up to 96 h with yields of 8.0 g/L. Biomass concentration was high (4.0 g/L) and glycerol consumption profile showed a similar pattern to SFT reduction, while, the hexadecane emulsification followed BS production. Such observations support the use of SFT and emulsification as indicative measures for the presence of BS molecules in the medium.

In studies carried out by Wei et al. (2005) on *P. aeruginosa* J4, isolated from wastewater of a petrochemical factory located in southern Taiwan, reported RHL production from different carbon substrates. Two complex media Luria Bertaini (LB) medium usually used as for *P. aeruginosa* strains and condensed molasses fermentation soluble (CMS) and a simpler glucose mineral salts (GMSs) medium were used to grow and produce RHL. RHL production was 1.7, 0.77 and 0.20 g/L on GMS, LB, and CMS media, respectively. It was also observed that high nitrogen content in a fermentation medium limits the BS production.

Wu et al. (2008) used an indigenous strain *P. aeruginosa* EM1 originating from an oil-contaminated site located in southern Taiwan to investigate RHLs improvement in GMS media by the response surface methodology. They changed carbon (glucose, sucrose, glycerol, olive oil, soybean oil, oleic acid, hexane) and inorganic nitrogen sources ($NaNO_3$ and NH_4Cl) and organic (yeast extract and urea). Maximum productivity of 136.4 and 71.8 mg/L/h was reported for glucose and glycerol, respectively. On the other hand, nitrate was the better inorganic nitrogen source (8.63 g/L) than ammonium ion (0.43 g/L) for RHL production. While organic sources were a very poor source of RHL production. The effect of C/N ratio on RHL production was thus investigated using two types of carbon sources (glucose or glycerol). The best RHL yield (6.8 g/L) occurred at a C/N ratio of 26 when glucose was used as the carbon source, whereas glycerol source yielded 7.5 g/L, at a C/N ratio of 52.

Literature surveys showed that, the Kinetics of biomass (BM), BS production, substrate utilization along with the fermentation duration required for growth of organisms are the most crucial parameters for commercial production processes. Raza et al. (2006) carried out kinetics for BS production for *P. aeruginosa* EBN-8 with different hydrocarbons viz., *n*-hexadecane paraffin oil, kerosene oil. Both *n*-hexadecane and paraffin oil, RHL production was 4.1 and 6.3 g/L respectively. Changing the carbon source and other parameter definitely affects the growth of organisms as well as the BS production.

Like *n*-hexadecane, other hydrocarbon namely *n*-octadecane 2% (v/v) has been proved to be supportive for the kinetic studies of BS production from *P. aeruginosa* OCD. Less than 5 days incubating conditions, in liquid Bushnell-Haas media with *n*-octadecane as the substrate resulted 0.98 mg/mL RHL in the culture broth at the stationary growth phase (Sahoo et al., 2011). Supplementation of multivalent cations viz., $ZnSO_4$ followed by $MnSO_4$ in the culture broth again, enhances the yield of BS production. This has been confirmed by authors thought monitoring the emulsification index assay.

Studies carried out by Khopade et al. (2012) included the kinetics of BS production up to 12 days for marine *Nocardiopsis* B4 under batch cultures condition. BSs from this halotolerant strains has potential for bioremediation of oil contaminated sites (oil spills). Investigation including measurement of SFT, emulsification assay, cell separation provides ample of information to understand the production of BS commercially.

Report available on probiotic bacteria like *L. pentosus* CECT-4023 has demonstrated strong BS production on cheese whey as an alternative medium. Carbon source viz., glucose, biomass and BS have been modeled according to reported models available in the literature (Rodrigues et al., 2006c). Their studies included four lactobacilli species for BS production by growing on De Man

et al. (1960) MRS broth for *lactobacilli* strains as well as medium supplemented with whey. With MRS medium the yield of BS from *Lactobacillus casei* reached 1.6 g/L. For both *Lactobacillus rhamnosus* and *L. pentosus* BS yields were reported at 1.7 g/L and for *Lactobacillus coryniformis* subsp. *torquens* it was found to be 1.8 g/L. Further investigation of *L. pentosus* CECT-4023 showed BS production using whey as an alternative medium with low yield of 1.4 g/L. The growth of *L. pentosus* CECT-4023 is less on whey medium which may be probably due to the lack of some nutrients, although similar BS concentrations were obtained. The authors suggested that with a culture medium optimization it could be possible to achieve higher BS concentrations.

Pacheco et al. (2010) suggested that higher concentrations of glycerol, sodium nitrate, and yeast extract lead to increased yield of BS from *Rhodococcus erythropolis* strain ATCC 4277. The authors also revealed that increasing the phosphate buffer within the range between 60 and 150 mmol/L increases the yield of BS (285 mg/L) due to maintained pH during the fermentation process. Well-established methodologies have been proposed for BS production from *C. lipolytica* through the usage of soybean oil refinery residue (6%) and 1% glutamic acid supplementation. Rufino et al. (2014) explored growth-associated production of crude BS with a yield of 8.0 g/L from *C. lipolytica* UCP 0988 after 72 h of incubation. Sarubbo et al. (2007) worked on canola oil and glucose as cheaper substrates and documented 8.0 g/L of yield of BS produced by *C. lipolytica*. Other strain of *C. sphaerica* produced yields of 4.5 g/L at up to 144 h culture conditions. *C. sphaerica* has been reported for BS production of about 9 g/L after 144 h (Luna et al., 2011, 2012).

Factorial experimental design has been proved to be very supportive for studying the kinetics for production of microbial metabolites. Rocha et al. (2014) reported the production of BSs using cashew apple juice from *P. aeruginosa* MSIC02. They used 24 full factorial experimental design, using temperature, glucose concentration from cashew apple juice, phosphorous concentration and cultivation time as variables. Kinetics of growth and production of BSs by *P. aeruginosa* indicated reduction in SFT up to 47.7 to 28.0 dyn/cm and indicated production of surface active molecules.

In a recent molecular biology investigation (Perfumo et al., 2013) for the expression of the rhlB and rhlC rhamnosyltransferase genes responsible for RHLs production of *P. aeruginosa* strains showed no significant differences in the genes or the quantity or composition of RHLs congeners obtained by manipulating growth conditions. Fixed sequential expression patterns for rhlB and rhlC rhamnosyltransferase genes were observed during growth. They reported that it was not possible to induce significant up-regulation by varying producer strains or growth media. Their results indicated that the RHLs genes are highly conserved molecules and that their expression has a rather stringent control. The authors conclude that there is little opportunity to manipulate and greatly increase the yield of RHLs in *P. aeruginosa* strains. They also concluded that manipulating growth and medium composition conditions has little effect in the strains obtained from widely different environmental situations. In addition RHLs production was not greater on water-insoluble substrates than water-soluble ones, as often claimed in the past.

FERMENTATION TECHNOLOGY: ROLE IN COMMERCIALIZATION OF BIOSURFACTANT PRODUCTION

When any industry is involved in the production of a particular compound, their main consideration is always achieving maximum profit from the minimum investment. BS industries are no exception to these policies and profitable applications are also of main concerns. The basic prerequisite of the BS/BE production industry is the type of substrate used in the production process. Since, some of the BS producing microbial communities are often isolated from oil, hydrocarbon-contaminated environments. Therefore, it is often assumed that water insoluble substrates are a necessity for the production of surface active agents. However, this fact may not always be true. Ample literature available suggests that water soluble carbon sources like glucose, fructose, sucrose etc., can be used in synthesizing the amphiphilic substances from a variety of microbial populations (Satpute et al., 2008, 2010a).

From an industrial point of view, using water soluble substrates is more attractive compared with using immiscible substrates. Therefore, the use of water soluble substrates particularly inexpensive industrial waste such as whey, molasses, distilleries effluent, waste oils would help to bring down the production cost in industries. Subsequently, such efforts make BS fermentation technology feasible. However, there are certain advantages and disadvantages of using low-priced substrates for BS production as shown in **Table 4**. Several challenging problems and possible strategies to overcome these problems are represented in **Figure 3** to enhance the commercial yield of BS. It is also noted that though

Table 4 | Advantages and disadvantages of cheaper substrates in biosurfactant production.

Advantages	Disadvantages
Commercial production cost can be reduced	Substrates contains undesired compounds
Many cheaper/renewable substrates are available	Processing or treatment of the substrates is required to use them as carbon, nitrogen, or energy source
Substrates are available in huge quantity	Final product itself get color or carry impurities from the substrates (e.g., molasses)
Enhanced the yield of biosurfactant/bioemulsifier	Special purification techniques needs to be employed to obtain the pure product, this increases the production of cost subsequently
Basic functional properties of the product do not change	Continuous supply of raw material with same composition may vary
Does not prove harmful to microorganisms	Raw substrates are may be very specific for different organisms
All components are eco-friendly and safe	A large quantity of raw substrates is essential, which may be difficult to get the continuous supply for the industrial process

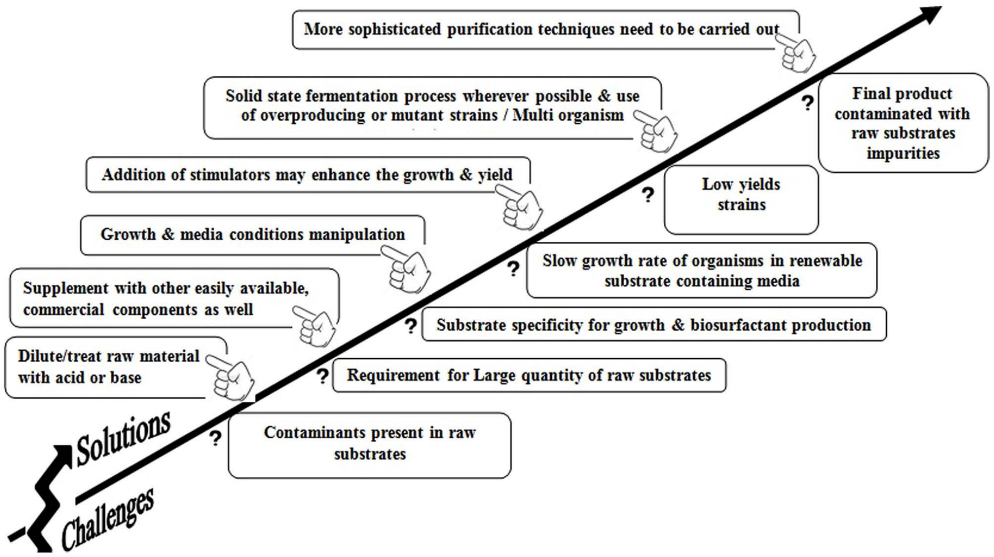

FIGURE 3 | Successful cram for increasing (he commercial yield of biosurfactant using renewable substrates.

there are numerous reports and patents on BEs and BSs production, they are rarely used in commercial production process (Shete et al., 2006). One of the foremost reasons is the use of chemical based media for BS growth and production process. These exclusive chemicals enhance the production cost of these amphiphilic molecules. Very few attempts have been offered for the usage of renewable substrates. The second most important factor to consider is a cost effective separation/purification of amphiphilic substances. The purification procedures are significant in terms of time requirement and could account for up to 60% of the total production cost and may result in a low yield (Desai and Banat, 1997; Banat et al., 2010). Under such circumstance, the utilization of crude quality product or the direct fermentation broth with or without affecting the activity and potency of the actual product may be a solution. This fact has been well-supported from the studies carried out by Thavasi et al. (2011) who concluded that for environmental applications the BSs need not be pure and could be synthesized from a mixed cheaper carbon sources. It is possible to create an economically and environmentally viable mitigation technology for the bioremediation process. Noteworthy achievements in the field of genetic engineering technologies have promoted some significant advances such as the alteration in the substrate requirement of producing organisms. One of the best examples was reported by Koch et al. (1988) where insertion of *lac* plasmid from *E. coli* in *P. aeruginosa* was carried out for utilization of whey from dairy industry to produce BS.

The next consideration that may contribute to cost reduction is the duration of fermentations for some BS production. RHLs fermentations in most literature continue for up to 100 h while most of the production may have occurred in the initial 48 h. Prolonging the production time for a little more yield achievement may not be a cost effective undertaking particularly as most gene expression for RHLs production take place in the initial 24 h of fermentation (Perfumo et al., 2013). Routine use of cheap renewable substrates (agro-industrial wastes) and competent methods for recovery and purification of BSs can assist optimized conditions for high yields fermentation process on commercial scale. Another important aspect that should be highlighted here is biological remediation technologies used in the process on a larger extent. We can felicitate this process through developing innovative techniques such as foams or micro-foams (colloidal gas aphrons-CGA) in conjunction with BSs (Pacwa-Płociniczak et al., 2011). Some of the important criteria that need to be considered for production of surface active agents in industries are as follows:

- The type of substrate/raw materials
- Continuous supply of ingredients of same composition
- Potential microorganisms
- Purification process used for the recovery of surface active compounds
- Monetary inputs
- Marketing
- Application potential

The new exciting development in this current area of research and priorities to the interdisciplinary research approaches in combination with the technologies of large-scale fermentation and genetic engineering, BSs will be commercially successful compounds of the future (Saharan et al., 2011).

Our future work in the area of BS should be on the economics of BS production processes, particularly using the alternative low-cost fermentative media and reasonable cheaper product recovery process.

CONCLUSION

Nature provides great immense possibilities for isolation of novel BEs or BSs producing microbial communities and products that

can be utilized in the various application fields such as petroleum industry, detergents, pharmaceutical companies, agriculture, and personal health care products. The use of economically cheaper substrates is paving the way for cost effective BS production process in industries. Large scale production of these surface active compounds is promising; however, product with pure quality needs further streamlined approaches. Enormous data has been generated on application oriented properties like SFT, IFT, EA, wetting, foaming, detergency, and flocculation leading to wider applications in various industrial sectors. Use in bioremediation technology has received better treatment as hydrocarbon-polluted sites can be treated effectively with crude BSs products or the producing organisms. Although number of developments have taken place, it is important to note that BS production should be followed by minimum monetary input using cheap low cost waste materials while maintaining quality and quantity wherever possible. In future, our research on BS must be targeted on the economics of the fermentation processes of BS and BE, predominantly carried out through the practice of alternative low-cost effective production media and recovery processes.

FUTURE PROSPECTS

Several food industries make use of various fats and oils that lead to the production of huge amount of high mass wastes, marine oils, soapstock, and free fatty acids from the extraction of seed oils. Searching for novel BS and BEs suitable for food industries has been steadily increasing and is expected to be a future prospect as more of these type molecules are included in food products. This is mainly driven by industries seeking to reduce dependency on plant emulsifiers produced by genetically modified crops. Other interesting areas include the use of BS producing microorganisms in composting. The most future potential application, however, are likely going to be related to oil industries application including bioremediation, cleaning, and microbial EOR both in oil sludge tank cleaning and oil well-recovery.

ACKNOWLEDGMENTS

Surekha K. Satpute, thanks Department of Science & Technology (DST), Government of India, Ministry of Science & Technology for financial support {SR/WOS-A/LS-1076/2014 (G)}.

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Conflict of Interest Statement: 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.

Received: 12 October 2014; accepted: 25 November 2014; published online: 12 December 2014.

*Citation: Banat IM, Satpute SK, Cameotra SS, Patil R and Nyayanit NV (2014) Cost effective technologies and renewable substrates for biosurfactants' production. Front. Microbiol. **5**:697. doi: 10.3389/fmicb.2014.00697*

This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 22 December 2014
doi: 10.3389/fmicb.2014.00696

Analysis of biosurfactants from industrially viable *Pseudomonas* strain isolated from crude oil suggests how rhamnolipids congeners affect emulsification property and antimicrobial activity

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Rhamnolipid biosurfactants produced mainly by *Pseudomonas* sp. had been reported to possess a wide range of potential industrial application. These biosurfactants are produced as monorhamnolipid (MRL) and di-rhamnolipid (DRL) congeners. The present study deals with rhamnolipid biosurfactants produced by three bacterial isolates from crude oil. Biosurfactants produced by one of the strains (named as IMP67) was found to be very efficacious based on its critical micelle concentration value and hydrocarbon emulsification property. Strikingly, antimicrobial, and anti-biofilm potential of this biosurfactant were higher than biosurfactants produced by other two strains. Thin layer chromatography analysis and rhamnose quantification showed that the rhamnolipids of IMP67 had more MRL congeners than biosurfactants of the other two strains. Emulsification and antimicrobial actions were affected by manual change of MRL and DRL congener proportions. Increase of MRL proportion enhanced emulsification index and antimicrobial property to Gram negative bacteria. This result indicated that the ratio of MRL and DRL affected the emulsification potentials of rhamnolipids, and suggested that high emulsification potentials might enhance rhamnolipids to penetrate the cell wall of Gram negative bacteria. In line with this finding, rhamnolipids of IMP67 also reduced the MIC of some antibiotics against bacteria, suggesting their synergistic role with the antibiotics.

Keywords: biosurfactant, rhamnolipid congeners, emulsification, thin layer chromatography, MRL-DRL proportion, antimicrobial action, biofilm disruption

INTRODUCTION

Amphipathic surface-active agents of biological origin are referred to as biosurfactants and they significantly reduce the surface tension of water from a value of 72 mN m^{-1} to as low as 22 mN m^{-1} (Mukherjee et al., 2006). They have affinity for both polar and non-polar media. Their several advantages over their chemical counterparts include mild production conditions from inexpensive substances (Mukherjee et al., 2006), lower toxicity (Poremba et al., 1991), better environmental compatibility and biodegradability (Zajic et al., 1977; Georgiou et al., 1990). They possess the property of retaining their activity at extremes of temperature, pH and salt concentration (Banat, 1995). They exhibit antibacterial, antifungal, and antiviral properties as well as anti-adhesive action against several pathogenic microorganisms (Cameotra and Makkar, 2004; Singh and Cameotra, 2004; Rodrigues et al., 2006; Das et al., 2009a). They also find application in enhanced oil recovery (Gautam and Tyagi, 2006) and bioremediation (Singh and Cameotra, 2004; Das et al., 2009b).

Different bacteria produce different classes of biosurfactants which serve varied purposes in the producer strains (Desai and Banat, 1997). Rhamnolipids are glycolipids biosurfactants produced by *Pseudomonas aeruginosa*, which emulsify oil and reduce the surface tension of water from 72 mN m^{-1} to around 25–30 mN m^{-1} (Itoh et al., 1971; Parra et al., 1989). They find applications in tertiary petroleum recovery (Parra et al., 1989), decontamination of marine oil pollution, soil remediation (Banat, 1995; Lotfabad et al., 2009) and crop protection (Stanghellini and Miller, 1997). They also show antimicrobial activity against Gram-positive and Gram-negative bacteria probably by interaction with the phosphatidylethanolamine moiety of biological membrane systems (Sanchez et al., 2006; Stipcevic et al., 2006). Rhamnolipid also affects the biofilm architecture in *P. aeruginosa* (Davey et al., 2003). Rhamnolipid production had been reported to start soon after inoculation and most of it was produced as a secondary metabolite, i.e., the production was under control of quorum sensing system and occurred after bacterial growth ceased (Haba et al., 2003). They are produced as homologues mainly rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate [monorhamnolipid (MRL)] and rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate [di-rhamnolipid (DRL; Ochsner and Reiser, 1995; Abdel-Mawgoud et al., 2010)]. MRL are precursors of DRL. Generally more DRLs are produced (Deziel et al., 1999) but predominance of MRLs had also been reported (Sim et al., 1997; Costa et al., 2006). Predominance of rhamnolipid congeners depends on the bacterial strain used, carbon substrate, age of culture and culture conditions (Bharali

and Konwar, 2011). The ratio of MRL to DRL is strain-dependent and changes during bacterial cultivation (Muller et al., 2011).

The present work reports the antimicrobial potentials and biofilm disruption potentials of rhamnolipid biosurfactant produced by three *Pseudomonas* strains isolated from crude oil. Under same culture conditions, one of the strains produced MRL and DRL congeners in almost equal proportion at a given point of time. The rhamnolipids of this strain also show the best antimicrobial potentials and emulsification property, while compared with the other strains that produced more DRL than MRL. The results suggested that the ratio of rhamnolipid congeners had significant contribution in the bioactivity profile. Multidrug resistance is now a worldwide problem. There is urgent need for novel antibacterial drugs or inhibitors and the present study suggested that the rhamnolipid biosurfactants could act synergistically with certain antibiotics.

MATERIALS AND METHODS

MICROBIAL CULTURE CONDITIONS AND THEIR MOLECULAR CHARACTERIZATION

The three rhamnolipid-producing strains used in the present work were isolated from the crude oil of Karamay W#8805, Xin-Jiang province, China. They were designated as IMP66, IMP67 and IMP68 respectively. Luria Bertanni (LB) medium was used for the preparation of the primary inoculum. The inoculum from LB was then transferred to PPGAS medium (Gunther et al., 2005) prepared with glycerol as the carbon source for biosurfactant production. The biosurfactant production medium was also prepared with olive oil and coconut oil as carbon sources. All cultures were incubated for a week at 37°C with an agitation speed of 200 rpm. An uninoculated medium was also incubated as a sterility control in each case. *P. aeruginosa* PAO1, known to be a rhamnolipid biosurfactant producer was grown as a positive control. DNA extraction was done from the bacterial cultures using Promega Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) as per the manufacturer's instructions. PCR amplification of the 16S ribosomal RNA gene was done with bacterial universal primers 27F and 1592R using a 35-cycle PCR (initial denaturation, 95°C for 5 min; subsequent denaturation, 95°C for 30 s; annealing temperature, 50°C for 1 min; extension temperature, 72°C for 1 min and final extension, 72°C for 5 min). PCR amplification products were analyzed by electrophoresis on 1% agarose gel. DNA sequencing was performed at Huada, Beijing, China and nucleotide sequence similarity searches were conducted by Genbank nucleotide collection BLAST.

STUDIES ON BACTERIAL GROWTH, BIOSURFACTANT PRODUCTION AND PIGMENT PRODUCTION

Fermentation broth samples were collected twice daily and checked for OD_{600nm}, surface tension and biosurfactant concentration. Biomass was estimated by the dry weight and also by the optical density of the fermentation broth at 600 nm measured with a UV-Visible spectrophotometer (Eppendorf, Germany). The surface tension of the cell free supernatants was measured with a digital surface tensiometer (Kruss K 100, Germany) working on the principles of Wilhelmy plate method. The validity of the surface tension readings was checked with pure water (72.2 ± 0.02) before each reading. Pyocyanin pigment production by the test strains was quantified by multiplying the optical density of the acidified culture supernatant at 520 nm with 17.072 (Raoof and Latif, 2010).

BIOSURFACTANT RECOVERY AND DETERMINATION OF CRITICAL MICELLE CONCENTRATION (CMC)

Biosurfactant was isolated from the culture broth obtained after the completion of each fermentation cycle by the standard technique (Das et al., 2008). Briefly, the fermentation broth was acidified and kept at 4°C overnight for complete precipitation of the biosurfactant. The precipitate was then centrifuged to get the crude biosurfactants as a pellet. Following solvent extraction, rhamnolipid concentration was quantified by the orcinol-sulphuric acid method with rhamnose as the standard (Arutchelvi et al., 2011).

The critical micelle concentration (CMC) of the biosurfactants from all the media was determined by taking 50 ml of water in a round bottomed vessel and adding weighed amounts of the biosurfactant gradually to the water. The surface tension was noted every time after mixing the biosurfactant with the water by stirring. This biosurfactant addition was continued and change in surface tension was noted until it reached a constant value. Finally the first concentration at which the surface tension became constant was determined as CMC. The test was repeated and the results were expressed as an average of three independent tests.

ASSAY OF EMULSIFICATION AND STABILITY STUDIES

The biosurfactants obtained from PPGAS cultures of the strains were checked for their ability to emulsify petrol and diesel. Equal volumes of aqueous biosurfactant solution (1 mg ml^{-1}) and hydrocarbons were mixed by vortex at high speed for 5 min. The resulting mixture was incubated at 25°C for 24 h and then the emulsification index (EI) value was calculated using the formula:

$$EI = (\text{Height of emulsion layer/Height of the total mixture}) \times 100$$

The emulsification of petrol and diesel by chemical surfactants like SDS and Tween 20 was also observed as a positive control.

Biosurfactant solutions were made at their respective CMCs. The solutions were heated for around 5 min to varied temperatures like 60, 70, 80, 90, and 100°C on a block heater. The surface tension of the biosurfactant was then checked after cooling them to room temperature. To monitor the effect of pH, biosurfactant solutions at CMC were made in separate tubes and the pH values were individually adjusted from 2.0 to 10.0 by HCl or NaOH respectively. The surface tension of the resultant solution in each of the test tubes was then checked. All the tests were repeated and results were expressed as an average of three independent tests.

DETECTION OF THE CHEMICAL NATURE OF THE BIOSURFACTANTS

Thin layer chromatography (TLC) was used to analyze the solvent extracted biosurfactants. The extracts were spotted on to

10 cm × 10 cm pre-coated silica gel TLC plates. Standard rhamnolipid was also spotted along with the samples. The solvent system used for the separation and analysis of the biosurfactants comprised of chloroform, methanol and acetic acid respectively, in a ratio of 65:15:2 (Wittgens et al., 2011). The developing jars were saturated with the solvent system for half an hour prior to the development. After development the plates were drained with detection agent consisting of 0.15 g orcinol, 8.2 ml 60% sulphuric acid and 42 ml water (Wittgens et al., 2011). The different biosurfactant spots were scrapped off from the TLC plates and suspended in a tube in a 2:1 mixture of chloroform and methanol. They were then vortexed at high speed and kept for extraction. After the extraction was complete, the contents of the tube were centrifuged to separate the silica from the solvent extracted biosurfactant. The biosurfactant extract was then transferred to fresh preweighed tubes and kept for evaporation of the solvent.The weights of the extracts were noted. Rhamnose estimation from all these extracts was then done and quantitation was done from the standard curve for detection of rhamnose.

DETERMINATION OF ANTIMICROBIAL ACTIVITY

Agar diffusion test was used to determine the susceptibility of a few bacterial strains like *Streptococcus epidermidis*, *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* to the biosurfactants. Overnight grown cultures of these bacterial strains were used to inoculate the agar plates. Biosurfactant solutions (1 mg ml^{-1}) from all strains were added aseptically into cups bored on these plates. The plates were incubated at 37°C for 24 h and zone of inhibition (ZOI) of microbial growth were measured. All tests were performed in triplicate and the inhibition zone diameter values represented the mean value ± SD.

Minimum inhibitory concentrations (MICs) of biosurfactants from all the cultures were determined by the broth microdilution assay (Das et al., 2008). Overnight grown cultures of the bacterial test strains, adjusted to a final density of 10^5 cfu ml^{-1} were used to inoculate 96-well microtiter plates containing serially diluted biosurfactant congeners. In these tests the uninoculated growth medium served as the negative control, the positive control was the test cultures and the sterility control was the growth medium with the biosurfactant congeners. Plates were incubated at 37°C for 24 h and then bacterial growth was monitored by absorbance at 600 nm in a microtiter plate reader. The minimum concentration of biosurfactant at which less than 50% growth of test organisms was observed, were defined as MIC_{50} values. All the tests for determination of MIC were performed in triplicate.

Minimum inhibitory concentration values of standard antibiotics like ampicillin, streptomycin, tetratcyclin, kanamycin, and tobramycin were determined against the same microbial strains used for checking antimicrobial activity of the biosurfactant. The procedure followed was the same as that mentioned earlier. Another test aimed at observing any change in MIC of antibiotics for the microbes was performed with a mixture of equal proportions of antibiotic and biosurfactant from IMP67 strain following the same procedure. MICs of the MRL and DRL congeners were also determined by following the same procedure as that for determining MICs of biosurfactants.

ANTIADHESIVE POTENTIAL OF THE BIOSURFACTANT

Microbial adhesion inhibition activity upon surface conditioning by the biosurfactant from IMP67 strain was tested using a previously reported anti-adhesion assay (Das et al., 2009a). Briefly, 200 µl of increasing concentrations of the biosurfactant (1–100 µg ml^{-1}) were poured in wells of a sterile 96-well flat-bottomed tissue culture polystyrene plate with lid. The plates were incubated for 18 h at 4°C and subsequently washed twice with PBS. Bacterial test strains, against whom the antimicrobial action was tested, were cultured overnight in LB broth at 37°C to obtain an inoculum of ∼10^5 cells ml^{-1} (according to Mc Farland turbidity standards). An aliquot of 200 µl was added and incubated in the wells for 24 h at 4°C. Unattached microorganisms were removed by washing the wells thrice with PBS. The remaining adherent microorganisms were fixed with 200 µl of 99% methanol per well, and after 15 min the plates were emptied and left to dry. Then the plates were stained with 200 µl of 2% crystal violet for 5 min. Excess stain was rinsed off by placing the plate under running tap water and the plates were allowed to dry. After the plates were air dried, the dye bound to the adherent microorganisms was resolubilized with 200 µl of 33% (v/v) glacial acetic acid per well. The optical density readings of each well were taken at 560 nm with a micro plate reader. The test was repeated thrice and the results expressed as the mean ± SD of three independent experiments.

Biofilm disruption activity of biosurfactant against bacteria adhering to a surface was done by a separate experiment, which was similar to the prior antiadhesive activity on biosurfactant-preconditioned surface. In this experiment, the sterile 96-well plates were incubated for a week with 200 µl inoculum of ∼10^5 bacteria ml^{-1} (according to Mc Farland turbidity standards) of the different bacterial strains tested for antiadhesive activity at 37°C. Control wells contained only sterile PBS. Unattached microorganisms were removed by washing the wells twice with PBS. 200 µl of the purified biosurfactant of different concentrations ranging from 1 to 100 µg ml^{-1} were added into the wells. The plates were incubated for about 24 h. The plates were washed thrice and the adherent population was fixed with 200 µl of 99% methanol per well. After 15 min the plates were emptied and left to dry. Then the plates were stained with 200 µl of 2% crystal violet per well for 5 min. Excess stain was then rinsed off by placing the plate under running tap water and the plates were kept to dry. After the plates were air dried, the dye bound to the adherent microorganisms was resolubilized with 200 µl of 33% (v/v) glacial acetic acid per well. The optical density readings of each well were taken at 560 nm with a micro plate reader. The test was repeated thrice and the results expressed as the mean ± SD of three independent experiments.

RESULTS

IDENTIFICATION OF STRAINS AND CHARACTERIZATION OF THEIR BIOSURFACTANT PRODUCTION

The bacterial strains, designated as IMP66, IMP67 and IMP68, were identified as *Pseudomonas* sp. pyr 41 (gene bank accession number:GU951459.1), *P. aeruginosa* LCD12 (GBAN:FJ194519.1) and *P. aeruginosa* D2 (GBAN:JN995663.1) respectively by 16S rRNA gene sequence analysis. Bacterial growth occurred in all the media with glycerol as the carbon substrate but no growth to very poor growth was observed with olive oil or coconut oil

as the sole carbon substrate. The observed preference for glycerol to oily substrates for biosurfactant production might be because of variation in preference of carbon source from strain to strain (Sandoval et al., 2001) although hydrocarbons and vegetable oils have been reported to be good sources of rhamnolipid production by *P. aeruginosa* strains (Maier and Soberon-Chavez, 2000; Perfumo et al., 2006).

Biosurfactant production initiated at different times in the cultures of different strains. Continuous stable foaming was the qualitative indicator of biosurfactant production. IMP66 and IMP67 strains produced biosurfactant after about 24 h of inoculation while biosurfactant production commenced in the culture of IMP68 after around 48 h of inoculation. The dry weights of the biosurfactants produced by different strains were measured. Although the IMP66 strain grew faster than IMP67 strain which in turn grew faster than IMP68 strain, the amount of biosurfactant produced was in the order IMP68 > IMP67 > IMP66 (**Table 1**). The biomass obtained in case of IMP67 culture was least in comparison to other test strains which suggested that the carbon flux might be mainly directed toward biosurfactant production. The pigment production through determination of the concentration of pyocyanin indicated that IMP68 strain was a hyperproducer of pyocyanin with respect to PAO1. IMP68 produced more than 40 μg ml^{-1} pyocyanin. The other two strains although found to be closely related to *P. aeruginosa*, did not produce much pyocyanin.

SURFACTANTS FROM THREE STRAINS DIFFER IN THEIR CONGENER PROPORTIONS

Thin layer chromatography detected all the biosurfactants to be rhamnolipid in nature (**Figure 1**). The spots at an R_f value of around 0.9 were that of MRLs while those obtained at R_f value of approximately 0.6 were that of DRLs (Guo et al., 2009). Extraction of MRL and DRL congeners of the biosurfactant from IMP67 after scrapping off from TLC plates showed that they were almost equal in weight. A similar result was given by rhamnose quantification (Arutchelvi et al., 2011) of TLC plate scrapings of MRL and DRL fractions. In other two strains, IMP66 and IMP68, DRL was more in proportion than corresponding MRL (**Figure 1**).

BIOSURFACTANTS OF IMP67 STRAIN ENDOWED WITH BEST PROPERTIES

The surface tension values of the cell free supernatants from cultures of all the three strains in the biosurfactant production medium recorded a surface tension value in the range of 29–31 mN m^{-1} (**Table 1**). Relative quantities of the biosurfactants produced by different strains were also determined and compared with *P. aeruginosa* PAO1 (**Table 1**). The CMC value of the biosurfactant produced by IMP67 strain was the least (**Table 1**). The lesser the value of CMC of a biosurfactant, the more is its activity which was also reflected in the emulsification potentials of this biosurfactant.

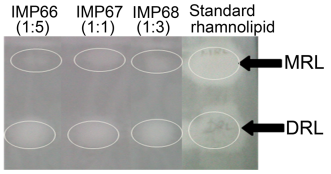

FIGURE 1 | Thin layer chromatography indicating the rhamnolipid nature of biosurfactants produced by the bacterial cultures under study. The numbers in parentheses indicate ratios of monorhamnolipid (MRL) and di-rhamnolipid (DRL) congener proportions. The white circles indicate the area with MRL or DRL. R_f for MRL and DRL are 0.9 and 0.6 respectively.

Petrol and diesel were emulsified by the IMP67 biosurfactant to high extents in comparison to the other biosurfactants (**Table 1**). This biosurfactant sustained heating upto 100°C as its solution did not show any change in surface tension even after heating at this temperature. Biosurfactants from other two strains were stable upto 80°C beyond which there was slight increase in the surface tension of their solutions. However, all the biosurfactants were stable toward changes of pH in the alkaline range but the surface tension of their solutions increased at around pH 2 because of biosurfactant precipitation. These results indicated that the biosurfactants from IMP67 strain can have great potential application in emulsification and crude oil recovery.

RHAMNOLIPIDS FROM IMP67 REVEALED BEST ANTIMICROBIAL AND ANTIADHESIVE ACTIVITY

The rhamnolipid biosurfactants produced by IMP67 strain inhibited both Gram positive and Gram negative bacterial strains tested, i.e., *E. coli, B. subtilis, S. aureus*, and *S. epidermidis*. It produced bigger ZOI in comparison to biosurfactants from other two strains as well as *P. aeruginosa* PAO1 (**Figure 2**). The MIC of this biosurfactant against the Gram positive and Gram negative bacterial strains was least when compared to biosurfactant from IMP66, IMP68, and PAO1 strains which proved its efficiency (**Table 2**).

The antiadhesive activity of the biosurfactant was tested against a panel of microorganisms. The activity was found to depend

Table 1 | Physicochemical properties of biosurfactants from bacteria under study.

Bacterial strains	Surface tension values (mN m^{-1})	Rhamnolipid content (g l^{-1})	CMC values (mg l^{-1})	EI values of petrol	EI values of diesel
IMP66	31.93 ± 0.04	0.8 ± 0.02	100	50	60
IMP67	29.85 ± 0.07	3.8 ± 0.1	50	70	80
IMP68	31.27 ± 0.06	4.2 ± 0.04	80	60	65
Pseudomonas aeruginosa PAO1	31.23 ± 0.07	2.8 ± 0.04	60	65	70

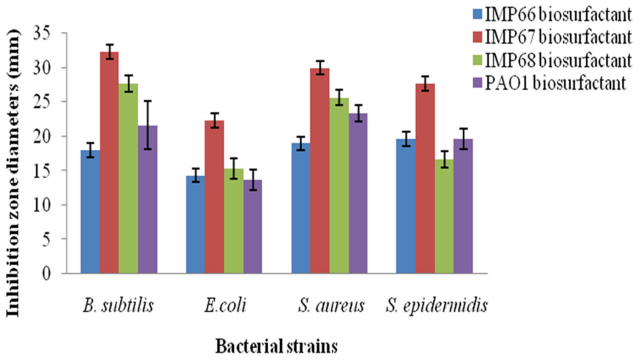

FIGURE 2 | Bigger antimicrobial inhibition zone diameters obtained with IMP67 biosurfactant in comparison to other biosurfactants.

Table 2 | Minimum inhibitory concentrations (MICs) of the biosurfactants.

Test bacterial strains	MICs of biosurfactants (μg ml^{-1})			
	IMP66	IMP67	IMP68	*P. aeruginosa* PAO1
Bacillus subtilis	16	4	6	10
Escherichia coli	32	4	16	30
Staphylococcus aureus	30	16	16	25
Streptococcus epidermidis	16	4	6	20

on both the concentration of biosurfactant and the microorganism tested. Anti-adhesion achieved by biosurfactant conditioning of surfaces was in the range of 50–80% at a concentration of 8–64 μg ml^{-1} (**Table 3**). Biofilm dislodging was also observed significantly (**Table 3**).

INCREASE OF MRL PROPORTION ENHANCED EMULSIFICATION POTENTIALS AND ANTIMICROBIAL PROPERTY OF RHAMNOLIPID BIOSURFACTANT

The IMP67 biosurfactant contained more MRL than that of the other strains. The biosurfactant of IMP67 also exhibited the best emulsification potentials and antimicrobial activity. Thus we hypothesized that there was a correlation between its bioactivities and proportion of congeners. The biosurfactant congeners extracted from thin layer chromatograms were mixed in various proportions and emulsification of hydrocarbons was conducted with them in order to note whether they promote any change in emulsification. The EI values obtained by varying the proportions of the congeners showed that when the proportion of the di-congener increased with respect to the mono-congener, some decrease occurred in the EI values (**Table 4**). In contrast, slight increase in emulsification indices was observed when the di-congener proportion increased with respect to the mono-congener (**Table 4**).

These different congener mixtures were also checked for their antimicrobial action and the variation in congener proportion varied the diameter of ZOI markedly (**Table 4**). The change in antimicrobial ZOI against the Gram negative bacterium tested was more prominent than that in the Gram positive one. The ZOI in Gram negative bacterium increased when the MRL was more in proportion than the DRL. Momo- to di-RL ratio in 5:1 exhibited the greatest EI values and the best antimicrobial activity against both Gram positive and negative bacteria. MRL congener alone showed bigger inhibition zone against bacteria and better emulsification activity than that of DRL (**Table 4**), although MIC analysis showed MRL and DRL congeners of IMP67 biosurfactant had similar MIC values against most of strains tested (**Table 5**). These results indicated that increase of MRL proportion enhanced antimicrobial activity of rhamnolipids from IMP67.

Table 3 | Anti-adhesive property of the IMP67 biosurfactant.

Test microorganisms	50% effective concentrations (EC_{50}) of rhamnolipids used for surface conditioning to promote anti-adhesion (μg/ml)	50% effective concentrations (EC_{50}) of rhamnolipids for biofilm disruption (μg/ml)
B. subtilis	8	64
E. coli	16	128
S. aureus	64	128
S. epidermidis	8	32

THE POTENTIAL OF RHAMNOLIPIDS BIOSURFACTANT TO ACT SYNERGISTICALLY WITH ANTIBIOTICS

Antimicrobial activity of biosurfactants was proposed by interaction with biological membrane systems. Thus we hypothesized that rhamnolipids can be used synergistically with other antibiotics to help their penetration into bacteria. MIC values of standard antibiotics against the test microbial strains were determined in combination with equal proportions of the IMP67 biosurfactant and the change in the MIC values showed a varied pattern in different strains (**Table 6**). In *B. subtilis*, a huge change in the MIC of tetracycline was observed when treated along with IMP67 biosurfactant while there was no change in the MICs of ampicillin, gentamicin, and kanamycin in conjugation with biosurfactant. When the test bacterium was *E. coli*, there was no change in MIC of gentamicin and streptomycin in collaboration with biosurfactant while all the other antibiotics got their MICs reduced (**Table 6**). MICs of ampicillin and kanamycin against both *S. aureus* and *S. epidermidis* reduced in presence of the biosurfactants from IMP67 while the MICs of other drugs did not change (**Table 6**). All these results suggested the efficacy of the biosurfactants as a potential drug synergist against the drug resistant bacteria.

DISCUSSION

Rhamnolipid biosurfactants produced by the strains under investigation were potent hydrocarbon emulsifiers which can make them suitable candidates for promoting environmental remediation. Although the IMP67 strain was found to be a close

Table 4 | Changes in emulsification index and ZOIs by varying proportions of congeners from IMP67.

Varied IMP67 biosurfactant congener proportions		Changes in emulsification index		Changes in antimicrobial ZOIs	
		Petrol	Diesel	Gram positive bacterium (*B. subtilis*)	Gram negative bacterium (*E. coli*)
MRL:DRL	1:1	70	80	33.0 ± 0.0	20.0 ± 0.0
	1:3	60	75	32.0 ± 0.0	18.66 ± 0.57
	1:5	50	60	30.0 ± 0.0	15.66 ± 0.57
	3:1	75	85	32.0 ± 0.0	27.33 ± 0.57
	5:1	80	90	30.0 ± 0.0	30.0 ± 0.0
	6:0	70	80	28.33 ± 0.57	30.0 ± 0.0
	0:6	50	50	25.66 ± 0.57	12.0 ± 0.0

Table 5 | Minimum inhibitory concentration values of individual congeners from IMP67.

Strains	*B. subtilis*	*E. coli*	*S. aureus*	*S. epidermidis*
MIC of MRLs ($\mu g\ ml^{-1}$)	4	4	16	4
MIC of DRLs ($\mu g\ ml^{-1}$)	2	4	16	4

Table 6 | Minimum inhibitory concentrations of biosurfactant from IMP67 in conjugation with standard antibiotics.

Test bacterial strains	MICs of antibiotics ($\mu g\ ml^{-1}$)		MICs of antibiotics with IMP67 biosurfactant ($\mu g\ ml^{-1}$)
B. subtilis	Tetracyclin	25	15
	Streptomycin	4	2
E. coli	Ampicillin	4	2
	Tetracyclin	10	4
	Tobramycin	6	4
	Kanamycin	8	6
S. aureus	Ampicillin	6	2
	Kanamycin	6	4
S. epidermidis	Ampicillin	4	1
	Tetracyclin	10	4
	Kanamycin	4	1

relative of *P. aeruginosa*, it did not produce copious amounts of pyocyanin and hence devoid of deleterious effects of this pigment. Most importantly, this strain is also a good biosurfactants producer, making it to be an industrially viable strain. Antimicrobial potentials of the rhamnolipid biosurfactants are well documented (Bharali and Konwar, 2011) but the antimicrobial potential of MRL and DRL congeners separately have not been tapped earlier to the best of our knowledge. The low MIC values of the IMP67 biosurfactant, as a whole, as well as its congeners separately, against the test bacterial strains in comparison to earlier reported ones (Bharali and Konwar, 2011) also advocate for the potency of these compounds. The higher antimicrobial potential of this biosurfactant is thought to be because of the equal congener proportions. As the MRLs and DRLs were present in almost similar amounts, there may be some sort of synergistic action on the overall antimicrobial property of the biosurfactant thus leading to the lesser MIC values of this biosurfactant against the strains tested. Reports on microbial production of varied biosurfactant structures under different growth conditions are there and structures were reported to promote variation in their action potentials. In the present work, detailed structural investigation needs to be done to get some insight in this direction. However, the degree of hydrophobicity also plays a role in exhibiting antimicrobial action. In this work, as the proportion of mono- to di-changed in the order of 1:1, 1:2 and so on, there was an enhancement in the polar nature of the compound and this resulted in the decrease of its antimicrobial action. On the other hand, when the proportion of di- to mono-rhamnolipid changed in the same manner, antimicrobial inhibition zone diameters increased, especially in case of the Gram negative bacteria. This enhancement may be accounted for by the increment in hydrophobic nature of the mixture which supposedly favored its increased penetration through lipids of the bacterial cell wall. Interestingly, DRL alone showed a decreased emulsification potential than the whole biosurfactant. Although it was reported that DRL was more useful in bioremediation (Zhang et al., 1997) but in this work in the IMP67 biosurfactant, MRL seemed to play a significant role in emulsification and antimicrobial potentials.

Anti-adhesion potential of IMP67 biosurfactant was also worth mentioning. Biosurfactant-conditioning of any surface, to which the bacteria might attach, inhibits bacterial adhesion in contrast to inhibition of bacterial adhesion either by exclusion or steric hindrance mechanism (Zá rate and Nader-Macias, 2006). In the present study, adhesion inhibition mediated by both surface conditioning and biosurfactant-mediated biofilm dislodging was observed which may find potential applications in protection of the surfaces of surgical instruments where no microbial load is desirable.

ACKNOWLEDGMENTS

Dr. Palashpriya Das acknowledges Chinese Academy of Sciences (CAS) for award of the International Young Scientist Fellowship. The authors thank Dr. Di Wang and Dr. Qing Wei at Institute of Microbiology, Chinese Academy of Sciences for their contribution to the revision of the manuscript. This work was supported by National Basic Research Program of China, 973 Program, grant 2014CB846002 (Luyan Z. Ma) and National Natural Science Foundation of China grant 31270177 (Luyan Z. Ma).

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Conflict of Interest Statement: The Review Editor Jun-Jie Zhang, declares that, despite being affiliated to the same institution as authors Palashpriya Das and Luyan Z. Ma, the review process was handled objectively and no conflict of interest exists. The authors declare that they have filed two provisional patent applications on the industrially use of strain IMP67 and IMP68 (PRC Patent Application No. 201310135130.1 and 201310135091.5 respectively). This does not alter our adherence to all policies on sharing data and materials.

Received: 10 September 2014; accepted: 24 November 2014; published online: 22 December 2014.

Citation: Das P, Yang X-P and Ma LZ (2014) Analysis of biosurfactants from industrially viable Pseudomonas strain isolated from crude oil suggests how rhamnolipids congeners affect emulsification property and antimicrobial activity. Front. Microbiol. ***5****:696. doi: 10.3389/fmicb.2014.00696*

This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 06 February 2015
doi: 10.3389/fmicb.2015.00059

Biosurfactant production by *Bacillus subtilis* using corn steep liquor as culture medium

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In this work, biosurfactant production by *Bacillus subtilis* #573 was evaluated using corn steep liquor (CSL) as culture medium. The best results were obtained in a culture medium consisting of 10% (v/v) of CSL, with a biosurfactant production of about 1.3 g/l. To the best of our knowledge, this is the first report describing biosurfactant production by *B. subtilis* using CSL as culture medium. Subsequently, the effect of different metals (iron, manganese, and magnesium) on biosurfactant production was evaluated using the medium CSL 10%. It was found that for all the metals tested, the biosurfactant production was increased (up to 4.1, 4.4, and 3.5 g/l for iron, manganese, and magnesium, respectively). When the culture medium was supplemented with the optimum concentration of the three metals simultaneously, the biosurfactant production was increased up to 4.8 g/l. Furthermore, the biosurfactant exhibited a good performance in oil recovery assays when compared with chemical surfactants, which suggests its possible application in microbial enhanced oil recovery or bioremediation.

Keywords: *Bacillus subtilis*, corn steep liquor, surfactin, bioremediation, microbial enhanced oil recovery

INTRODUCTION

Surfactants are an important class of chemical compounds included in many of the everyday products we use. They can be found in detergents, laundry formulations, household cleaning products, cosmetics, herbicides, or pesticides, and are also used in bioremediation, agriculture, food, pharmaceutical, textile, paper, or petroleum industries, among others (Geys et al., 2014; Rebello et al., 2014). Surfactants are amphiphilic molecules that, due to their structure, tend to accumulate at the interfaces between fluid phases with different polarities (e.g., oil-water or air-water), reducing surface and interfacial tensions (Gudiña et al., 2013). Most of the surfactants currently used are chemically synthesized from petrochemical resources and are only partially biodegradable, causing detrimental effects on the environment (Vaz et al., 2012; Rebello et al., 2014).

Nowadays, due to the increasing environmental awareness, the demand for surfactants obtained from bio-based resources is increasing. Biosurfactants, surface-active compounds synthesized by a variety of microorganisms, are attracting a pronounced interest owing to their potential advantages over their chemical counterparts, namely they exhibit a similar or better performance and have less impact on the environment than conventional surfactants, due to their lower toxicity and higher biodegradability. Furthermore, biosurfactants are usually effective at extreme environmental conditions and can be produced from renewable resources (Pereira et al., 2013; Gudiña et al., 2015). As a consequence, biosurfactants can replace some of the synthetic surfactants in many fields. However, the application of biosurfactants depends on whether they can be produced economically at large-scale. Presently, biosurfactants are not competitive with chemical surfactants from an economic point of view, since expensive substrates are required for their production and they present relatively low productivities, which hampers their widespread use and commercialization. As the culture medium can account for up to 30–50% of the overall production costs of biosurfactants, the replacement of expensive synthetic media by cheaper agro-industrial wastes and by-products can contribute to reduce their production costs and increase their competitiveness (Henkel et al., 2012; Al-Bahry et al., 2013; Gudiña et al., 2015).

Biosurfactants display a wide variety of chemical structures, including glycolipids, lipopeptides, phospholipids, fatty acids, or neutral lipids, among others (Gudiña et al., 2013; Geys et al., 2014). *Bacillus* species produce a broad spectrum of lipopeptide biosurfactants, which are cyclic molecules consisting of a fatty acid of variable length (hydrophobic moiety) linked to a short peptide chain (hydrophilic moiety) of seven or ten aminoacids. Among them, surfactin, a lipoheptapeptide produced by *Bacillus subtilis* strains, is one of the most effective biosurfactants known so far; it can reduce the surface tension (ST) of water up to 27 mN/m, with critical micelle concentrations (*cmc*) as low 0.01 g/l, and shows a high emulsifying activity; furthermore, it exhibits antimicrobial, antiviral, and antitumor activities (Gudiña et al., 2013). Consequently, surfactin can be potentially useful in numerous therapeutic, industrial, and environmental applications. In order to reduce the production costs, surfactin production by *B. subtilis* strains has been studied using different substrates, such as molasses (Abdel-Mawgoud et al., 2008; Joshi et al., 2008; Al-Bahry et al., 2013), potato peels (Das and Mukherjee, 2007), whey powder (Cagri-Mehmetoglu et al., 2012), cashew apple juice (Oliveira et al., 2013) or cassava waste water (Nitschke and Pastore, 2006). Furthermore, the effect of different nutritional factors has been studied to improve surfactin production by *B. subtilis* strains.

Among them, it has been reported that the addition of various metals (i.e., iron, magnesium, and manganese) to the culture medium contribute to increase its production, since those elements are co-factors of enzymes involved in the synthesis of surfactin (Thimon et al., 1992; Makkar and Cameotra, 2002; Wei et al., 2003).

In this work, biosurfactant production by a *B. subtilis* strain was evaluated using the agro-industrial by-product corn steep liquor (CSL) as a low-cost alternative substrate. Once established the optimum CSL concentration for biosurfactant production, the culture medium was optimized by supplementing it with different metals (iron, magnesium, and manganese) at different concentrations, first individually and then in different combinations. Finally, the applicability of the biosurfactant produced in oil recovery was evaluated.

MATERIALS AND METHODS

BACTERIAL STRAIN

The biosurfactant-producing strain *B. subtilis* #573 was isolated and identified in our laboratory in a previous work (Gudiña et al., 2012). The strain was stored at –80°C in Luria-Bertani (LB) medium supplemented with 20% (v/v) of glycerol. The composition of LB medium was (g/l): NaCl 10.0; tryptone 10.0; yeast extract 5.0; pH 7.0. Whenever required, frozen stocks were streaked on LB agar plates and incubated at 37°C for 24 h. The agar plates were stored at 4°C no longer than 3 weeks.

BIOSURFACTANT PRODUCTION BY *B. subtilis* #573 USING CSL

Corn steep liquor was kindly provided by COPAM [Companhia Portuguesa de Amidos, S. A. (Portugal)], and it was evaluated as a substrate for biosurfactant production by *B. subtilis* #573. Total carbohydrates and protein concentrations in CSL were determined using the phenol-sulfuric and Lowry methods, respectively (Lowry et al., 1951; Dubois et al., 1956).

Different culture media were prepared by dissolving CSL in demineralized water at different concentrations [5, 10, and 15% (v/v)]. Subsequently, the pH was adjusted to 7 using 1 M NaOH and the media were sterilized at 121°C for 15 min. Those media were evaluated for biosurfactant production by *B. subtilis* #573 in order to establish the optimum CSL concentration for biosurfactant production. The assays were performed in 500 ml flasks containing 200 ml of each medium. The flasks were inoculated with 2 ml of a pre-culture of *B. subtilis* #573 grown in LB medium at 37°C and 200 rpm for 24 h. Pre-cultures were prepared by inoculating 10 ml of LB medium with a single colony taken from an agar plate. The cultures were incubated at the same conditions (37°C and 200 rpm) until the maximum biosurfactant production was achieved. Samples were taken at different time points during the fermentation to determine biosurfactant production. The samples were centrifuged (4000 rpm, 20 min) and the cell-free supernatants were used to measure the ST and to determine the emulsifying activity (E_{24}), as described below. Whenever required, the culture broth supernatants were diluted 10 or 100 times with demineralized water and the surface tension (ST^{-1}, ST^{-2}) and emulsifying activities ($E_{24}{}^{-1}$, $E_{24}{}^{-2}$) were measured.

EFFECT OF METAL MEDIUM SUPPLEMENTATION ON BIOSURFACTANT PRODUCTION

Once selected the CSL concentration that led to the highest biosurfactant production by *B. subtilis* #573, the effect of the addition of different metals (iron, manganese, and magnesium) at different concentrations on biosurfactant production was evaluated in order to try to increase biosurfactant production by this isolate. The metal concentrations used were selected according to previous studies performed by different authors, as shown in **Table 1**. Control assays were performed using the culture medium without supplements. The cultures were performed at the same conditions described above and the biosurfactant production was evaluated along the fermentation. The cultures were maintained until the maximum biosurfactant production was achieved in each case.

BIOSURFACTANT RECOVERY

At the end of the fermentation, the cultures were centrifuged (4000 rpm, 20 min) to remove the cells. The cell-free supernatants were adjusted to pH 2 with 6M HCl and were subsequently incubated overnight at 4°C to promote the biosurfactant precipitation. Afterward, the precipitates (crude biosurfactant) were collected by centrifugation (9000 rpm, 20 min, 4°C). The crude biosurfactant was dissolved in a minimal amount of demineralized water and the pH was adjusted to 7 using 1 M NaOH. The biosurfactant solutions were freeze-dried and the products obtained were weighed and stored at –20°C.

SURFACE-ACTIVITY DETERMINATION

Surface tension measurements of culture broth supernatants and biosurfactant solutions were performed according to the Ring method described elsewhere (Gudiña et al., 2012). A KRÜSS K6 Tensiometer (KRÜSS GmbH, Germany) equipped with a 1.9 cm De Noüy platinum ring was used. To increase the accuracy of the ST measurements, an average of triplicates was determined. All the measurements were performed at room temperature (25°C).

Table 1 | Different salts and concentrations used to study the effect of iron, manganese and magnesium on biosurfactant production by *Bacillus subtilis* #573.

Compound	Medium	Concentration	Reference
$FeSO_4$	A	0.025 mM	Sen (1997) and Sen and Swaminathan (1997)
	B	0.3 mM	Wei et al. (2007)
	C	2.0 mM	Yeh et al. (2005)
$MnSO_4$	D	0.02 mM	Mnif et al. (2012)
	E	0.2 mM	Wei et al. (2007)
	F	2.0 mM	Sen (1997) and Sen and Swaminathan (1997)
$MgSO_4$	G	0.8 mM	Sen (1997), Sen and Swaminathan (1997) and Wei et al. (2007)
	H	2.0 mM	Sousa et al. (2012)
	I	4.0 mM	Akpa et al. (2001)

EMULSIFYING ACTIVITY DETERMINATION

Emulsifying activity was determined by the addition of 2 ml of *n*-hexadecane to the same volume of cell-free supernatants in glass test tubes. The tubes were mixed with a vortex at high speed for 2 min and then incubated at 25°C for 24 h. The emulsification index (E_{24}) was calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm). All the emulsification indexes were performed in triplicate.

CRITICAL MICELLE CONCENTRATION (*cmc*)

Critical micelle concentration is the concentration of an amphiphilic compound in solution at which the formation of micelles is initiated. Different concentrations of the freeze-dried biosurfactant produced by *B. subtilis* #573 were prepared in demineralized water, and the ST of each sample was measured at 25°C as described above. The *cmc* was determined by plotting the ST as a function of the logarithm of biosurfactant concentration, and it was found at the point of intersection between the two lines that best fit through the pre- and post-*cmc* data. All the measurements were done in triplicate.

OIL RECOVERY ASSAYS

The applicability of the biosurfactant produced by *B. subtilis* #573 in oil recovery was evaluated using artificially contaminated sand containing 12.5% (w/w) of Arabian Light crude oil [kindly provided by GALP (Portugal)]. Samples of 40 g of sand were mixed with 5 g of crude oil in 100 ml flasks by shaking, and allowed to age at room temperature for 24 h. Afterward 40 ml of biosurfactant solutions at different concentrations were added to each flask. The flasks were incubated at 90 rpm and 40°C for 24 h, and the oil removed was recovered from the surface and transferred to a graduated tube. In order to separate the water recovered together with the crude oil, the tubes were centrifuged (9000 rpm, 20 min, 25°C), and subsequently the volume of crude oil was measured. The amount of oil recovered (grams) was calculated according to its density (0.837 g/ml). Control assays were performed using demineralized water at the same conditions. All the experiments were performed in triplicate. Statistically significant differences of the assays performed with the different biosurfactant concentrations tested were evaluated by a one-way ANOVA ($P < 0.05$) applying the Tukey multiple-comparisons; a significant difference was considered if $P < 0.05$.

RESULTS

EVALUATION OF CSL AS A SUBSTRATE FOR BIOSURFACTANT PRODUCTION

Bacillus subtilis #573 was isolated and identified in a previous work as a promising biosurfactant producer (Gudiña et al., 2012). The applicability of the biosurfactant produced by this isolate [identified as a mixture of C_{13}-, C_{14}-, and C_{15}-surfactin (Pereira et al., 2013)] in oil recovery (Pereira et al., 2013) and its anti-tumor activity (Duarte et al., 2014) were also demonstrated. In order to optimize biosurfactant production by this isolate and reduce its production costs, CSL was evaluated as a low-cost alternative culture medium. CSL is a by-product of corn wet-milling, where starch is recovered from corn, and it is an important source of nitrogen for several biotechnological processes (Henkel et al., 2012; Gudiña et al., 2015). The CSL used in this work contained 75 g/l of carbohydrates and 5 g/l of protein.

In order to establish the optimum CSL concentration for biosurfactant production by this isolate, different culture media were prepared by dissolving CSL in demineralized water at different concentrations [5, 10, and 15% (v/v)], and subsequently were evaluated for biosurfactant production. The cultures were incubated at 37°C and 200 rpm, and biosurfactant production was evaluated along the fermentation by measuring the ST and the emulsifying activity of the cell-free supernatants. The cultures were maintained until the maximum biosurfactant production was achieved in each case. The results obtained are shown in **Table 2**.

Although with the three media similar ST values were obtained in the cell-free supernatants without dilution, more pronounced differences were observed in the cell-free supernatants 10 and 100 times diluted. With the medium CSL 5%, the best results regarding biosurfactant production were obtained after 24 h, whereas for the other two media these were obtained after 48 h. From **Table 2** it can be seen that the lowest ST values and the highest emulsifying indexes were obtained with the medium CSL 10%, thus indicating a higher biosurfactant production. Taking into account the price of CSL (about 40€/ton), the price of 1 liter of medium CSL 10% is about 0.004€. Therefore, the use of CSL as an alternative culture medium would greatly decrease the production costs of this biosurfactant at a higher production scale.

The cell-free supernatants of cultures performed in CSL 10% for 48 h were subjected to acidic precipitation to recover the biosurfactant produced, yielding 1.311 ± 0.109 g of biosurfactant per liter. The *cmc* calculated for this biosurfactant was 0.16 g/l. In view of the results obtained, the medium CSL 10% was selected to perform the following optimization assays.

EFFECT OF METALS ON THE BIOSURFACTANT PRODUCTION

Iron, manganese, and magnesium are co-factors of enzymes involved in the synthesis of surfactin by *B. subtilis*. Therefore, the

Table 2 | Surface tension values [ST, ST^{-1} and ST^{-2} (mN/m)] and emulsifying indexes [E_{24}, $E_{24}{}^{-1}$ (%)] obtained in cultures performed with *B. subtilis* #573 grown in media containing CSL at different concentrations [5, 10, and 15% (v/v)].

	CSL 5%	CSL 10%	CSL 15%
Time (h)	24	48	48
ST_0 (mN/m)	51.4 ± 0.4	52.8 ± 0.3	50.5 ± 0.4
ST (mN/m)	32.8 ± 0.5	30.7 ± 0.4	31.8 ± 0.5
ST^{-1} (mN/m)	50.3 ± 1.7	34.9 ± 0.9	40.0 ± 0.8
ST^{-2} (mN/m)	72.0 ± 0.0	59.8 ± 1.8	67.3 ± 1.5
E_{24} (%)	28.0 ± 3.7	55.0 ± 2.0	40.3 ± 1.7
$E_{24}{}^{-1}$ (%)	0.0	29.0 ± 6.6	0.0

The results presented correspond to the values obtained at the optimum time for each medium. ST_0: surface tension of the culture medium. Results represent the average of three independent experiments ± SD.

Table 3 | Surface tension values [ST, ST^{-1}, and ST^{-2} (mN/m)], emulsifying indexes [E_{24}, E_{24}^{-1} (%)] and biosurfactant concentrations obtained in cultures performed with *B. subtilis* #573 grown in CSL 10% supplemented with different metals at different concentrations.

Medium*(time)	ST (mN/m)	ST^{-1} (mN/m)	ST^{-2} (mN/m)	E_{24}(%)	E_{24}^{-1}(%)	[BS] (g/l)
A (72 h)	31.0 ± 0.7	33.7 ± 1.4	55.8 ± 3.0	54.5 ± 1.0	31.5 ± 2.5	1.915 ± 0.124
B (48 h)	30.6 ± 0.5	33.3 ± 1.0	55.1 ± 1.7	56.5 ± 0.6	25.3 ± 1.0	1.991 ± 0.100
C (72 h)	29.7 ± 0.5	32.6 ± 0.6	46.8 ± 1.0	57.0 ± 1.2	41.0 ± 2.0	4.170 ± 0.054
D (72 h)	30.4 ± 0.5	32.5 ± 1.0	48.2 ± 1.0	57.0 ± 3.5	32.0 ± 2.8	3.690 ± 0.135
E (72 h)	30.4 ± 0.5	33.8 ± 1.0	46.0 ± 0.9	59.0 ± 2.0	41.0 ± 2.6	4.467 ± 0.158
F (72 h)	30.7 ± 1.0	32.3 ± 0.5	47.6 ± 1.3	57.8 ± 0.5	31.5 ± 1.9	3.901 ± 0.061
G (72 h)	30.8 ± 0.9	34.1 ± 3.3	48.7 ± 1.2	58.0 ± 2.3	43.0 ± 6.0	3.519 ± 0.102
H (48 h)	30.0 ± 0.1	32.6 ± 1.5	49.0 ± 0.5	55.0 ± 1.2	28.0 ± 2.8	3.405 ± 0.167
I (48 h)	30.3 ± 0.5	32.5 ± 0.4	53.1 ± 1.7	56.3 ± 2.1	21.5 ± 1.9	2.381 ± 0.170
Control (48 h)	30.7 ± 0.4	34.9 ± 0.9	59.8 ± 1.8	55.0 ± 2.0	29.0 ± 6.6	1.311 ± 0.109

Control: CSL 10% without supplements.
*ST_0: 52.8 ± 0.3 mN/m. ST_0^{-1}: 60.3 ± 0.5 mN/m. ST_0^{-2}: 70.0 ± 0.8 mN/m. The results presented correspond to the optimum time for each medium. Results represent the average ± SD of three independent experiments. *The composition of the different culture media is shown in **Table 1**.*

concentration of these elements in the culture medium plays an important role in the biosurfactant production (Sen, 1997; Wei and Chu, 1998, 2002; Wei et al., 2003, 2007). The medium CSL 10%, selected in the previous phase as the best medium for biosurfactant production by *B. subtilis* #573, was supplemented with $FeSO_4$, $MnSO_4$, or $MgSO_4$ individually at different concentrations (as shown in **Table 1**), in order to study their effect on biosurfactant production. The metal concentrations used were selected taking into account previous works from other authors studying biosurfactant production by different *B. subtilis* strains. The medium CSL 10% without supplements was used as control. As in the previous assays, the cultures were incubated at 37°C and 200 rpm, and biosurfactant production was evaluated along the time. The cultures were maintained until the maximum biosurfactant production was achieved in each case. The results obtained are gathered in **Table 3**.

For the experiments conducted with the media B, H, and I, the lowest ST values were obtained at 48 h of growth, as it was previously observed in the assays performed with the medium CSL 10%. In the other media, the lowest ST values were achieved at 72 h of growth. Regarding the ST of the cell-free supernatants without dilution, similar values (between 29.7 and 31.0 mN/m) were obtained for all the media tested. However, greater differences were observed in the cell-free supernatants that were diluted 10 and 100 times (**Table 3**).

In the culture media supplemented with $FeSO_4$, the best result was obtained with the highest concentration tested (2.0 mM, medium C). In that case, the ST^{-2} was reduced up to 46.8 ± 1.0 mN/m at 72 h, which resulted in a biosurfactant production of 4.170 ± 0.054 g/l. Regarding manganese, the best result was obtained with $MnSO_4$ at a concentration 0.2 mM (medium E) at 72 h. The ST^{-2} was reduced up to 46.0 ± 0.9 mN/m, and the amount of biosurfactant produced was 4.467 ± 0.158 g/l. In the media supplemented with magnesium, similar results were obtained with $MgSO_4$ 0.8 and 2.0 mM (media G and H). The ST^{-2} was reduced up to 48.7–49.0 mN/m, and the amount of biosurfactant produced ranged between 3.4 and 3.5 g/l. In all the cases a direct relationship was found between the ST^{-2} values and the amount of biosurfactant produced (**Table 3**).

Regarding the emulsifying indexes, similar values (54.5–59.0%) were obtained with all the media assayed (**Table 3**). Emulsifying indexes higher than 50% entail that the hydrocarbon phase is completely emulsified. More pronounced differences were observed for the E_{24}^{-1} values. The highest E_{24}^{-1} values were obtained with the media C, E, and G, which is in agreement with the amounts of biosurfactant produced in each medium.

As it can be seen from the results obtained, the supplementation of the medium CSL 10% with the different metals resulted in an increase in biosurfactant production in all the cases when compared with the medium without supplements, although for each metal, that effect was dependent on the concentration used.

It has been reported that the combination of different metals can result in a positive interaction on surfactin production as compared with their individual effect (Sen, 1997; Makkar and Cameotra, 2002). Taking into account the results obtained with the medium CSL 10% supplemented with the different metals

Table 4 | Combinations of metal salts assayed for studying its effect on the biosurfactant production by *B. subtilis* #573.

Medium	Compound	Concentration
J	$FeSO_4$	2.0 mM
	$MgSO_4$	0.8 mM
K	$FeSO_4$	2.0 mM
	$MnSO_4$	0.2 mM
L	$MgSO_4$	0.8 mM
	$MnSO_4$	0.2 mM
M	$FeSO_4$	2.0 mM
	$MgSO_4$	0.8 mM
	$MnSO_4$	0.2 mM

individually, this medium was supplemented with different metal combinations, according with the concentrations that led to the best results in the individual assays for each compound, namely $FeSO_4$ 2.0 mM, $MnSO_4$ 0.2 mM, and $MgSO_4$ 0.8 mM. The different combinations assayed are summarized in **Table 4**. As in the previous assays, the cultures were incubated at 37°C and 200 rpm, and biosurfactant production was evaluated along the time. The medium CSL 10% without supplements was used as control. The cultures were maintained until the maximum biosurfactant production was achieved in each case. The results obtained are gathered in **Table 5**.

Regarding the combinations iron-magnesium (medium J) and iron-manganese (medium K), the best results regarding ST reduction and biosurfactant production were obtained at 24 h of growth. However, the amount of biosurfactant produced (2.704 ± 0.132 and 3.933 ± 0.205 g/l, respectively) was lower than the obtained when those metals were added individually, which suggests a negative interaction on the biosurfactant production. Regarding the combination magnesium-manganese (medium L), the best results were obtained after 48 h. In this case, the amount of biosurfactant produced (4.224 ± 0.157 g/l) was slightly lower when compared with the obtained with the optimum concentration of manganese, but better than the obtained with the optimum concentration of magnesium. Finally, a combination of the three metals was tested (medium M). The best results were obtained at 72 h, and the amount of biosurfactant produced (4.829 ± 0.193 g/l) was higher than the obtained when the different metals were added individually.

OIL RECOVERY ASSAYS

The oil recovery assays were performed using the freeze-dried biosurfactant produced by *B. subtilis* #573 in the medium CSL 10%, dissolved in demineralized water at different concentrations. The results obtained are shown in **Table 6**.

As it can be seen from **Table 6**, similar oil recoveries were obtained with the biosurfactant at a concentration of 2.5 and 5.0 g/l [the differences were not statistically significant ($P > 0.05$)], which suggests that the use of higher biosurfactant concentrations would not increase the oil recovery.

DISCUSSION

Biosurfactant production by *B. subtilis* isolates using low-cost substrates was studied by several authors. *B. subtilis* B20 and B30 produced between 0.3 and 2.3 g/l of biosurfactant using a mineral medium supplemented with date molasses as carbon source (Al-Bahry et al., 2013; Al-Wahaibi et al., 2014). Abdel-Mawgoud et al. (2008) reported a biosurfactant production of 1.12 g/l by *B. subtilis* BS5 using a mineral medium containing sugarcane molasses. *B. subtilis* LAMI005 produced 0.3 and 0.44 g/l of biosurfactant growing in media containing cashew apple juice (Oliveira et al., 2013) and raw glycerol derived from biodiesel production (Sousa et al., 2012), respectively. However, in these cases, other nutrients (e.g., carbon or nitrogen sources, salts) were added to the media, thus increasing their prices. On the contrary, in the present work, the only substrate used to prepare the culture media was CSL. Likewise, other authors reported biosurfactant production by different *B. subtilis* isolates using culture media containing exclusively agro-industrial by-products (e.g., cassava wastewater, sugarcane molasses, or rehydrated whey powder), with productivities between 0.3 and 3.0 g/l (Nitschke and Pastore, 2006; Joshi et al., 2008; Cagri-Mehmetoglu et al., 2012). However, to the best of our knowledge, this is the first report describing biosurfactant production by *B. subtilis* using CSL as culture medium. The amount of biosurfactant produced (about 1.3 g/l) was close to the values reported by other authors using different substrates.

It has been reported that the addition of iron, magnesium or manganese to the culture medium increased biosurfactant production by *B. subtilis* isolates (Thimon et al., 1992; Wei and Chu, 1998, 2002; Makkar and Cameotra, 2002; Al-Ajlani et al., 2007). Mn^{2+} at a concentration 0.01 mM increased biosurfactant production by *B. subtilis* ATCC 21332 from 0.33 to 2.6 g/l (Wei and Chu, 2002). Al-Ajlani et al. (2007) reported an increase in biosurfactant production by *B. subtilis* MZ-7 from 0.22 to 0.3 g/l by supplementing the culture medium with Fe^{2+} at a concentration 4.0 mM; and similar results were obtained by Thimon et al. (1992). Makkar and Cameotra (2002) increased biosurfactant production by *B. subtilis* MTCC 2423 growing in a mineral medium from 0.342 to 0.814 g/l by the addition of $FeSO_4$ (0.719 mM), and to 0.792 g/l by the addition of $MgSO_4$ (2.43 mM). However, in some cases, higher concentrations of those metals inhibited biosurfactant production (Makkar and Cameotra, 2002). In our case, the amount of biosurfactant produced by *B. subtilis* #573 in the medium CSL 10% was increased up to 3.2, 3.4, and 2.7 times due to the addition of iron, manganese, and magnesium, respectively, to the culture medium.

Table 5 | Surface tension values [ST, ST^{-1}, and ST^{-2} (mN/m)], emulsifying indexes [E_{24}, E_{24}^{-1} (%)] and biosurfactant concentrations obtained in cultures performed with *B. subtilis* #573 grown in CSL 10% supplemented with different combinations of metals.

Medium*(time)	ST (mN/m)	ST^{-1} (mN/m)	ST^{-2} (mN/m)	E_{24} (%)	E_{24}^{-1} (%)	[BS] (g/l)
J (24 h)	29.3 ± 0.4	31.6 ± 0.5	51.6 ± 2.0	55.5 ± 1.9	34.0 ± 3.7	2.704 ± 0.132
K (24 h)	29.3 ± 0.3	31.5 ± 0.8	47.4 ± 1.9	56.5 ± 1.0	34.0 ± 2.3	3.933 ± 0.205
L (48 h)	29.7 ± 0.3	31.3 ± 1.0	46.6 ± 1.3	57.0 ± 1.2	26.0 ± 1.6	4.224 ± 0.157
M (72 h)	29.1 ± 0.6	31.3 ± 0.9	45.1 ± 1.9	59.5 ± 0.9	36.1 ± 1.7	4.829 ± 0.193
Control (48 h)	30.7 ± 0.4	34.9 ± 0.9	59.8 ± 1.8	55.0 ± 2.0	29.0 ± 6.6	1.311 ± 0.109

Control: CSL 10% without supplements. ST_0: 52.8 ± 0.3 mN/m. ST_0^{-1}: 60.3 ± 0.5 mN/m. ST_0^{-2}: 70.0 ± 0.8 mN/m. The results presented correspond to the optimum time for each medium. Results represent the average ± SD of three independent experiments.

The composition of the different culture media is shown in **Table 4.*

Table 6 | Percentages of oil recovered with the biosurfactant produced by *B. subtilis* #573 in the medium CSL 10% at different concentrations.

[BS] g/l	Oil recovered (%)
5.0	25.1 ± 1.7
2.5	26.3 ± 0.0
1.0	15.0 ± 0.8
Control	0.0 ± 0.0

Results represent the average ± SD of three independent experiments.

Sen (1997) reported a strong and positive interaction between iron and manganese in the biosynthesis of surfactin by *B. subtilis*. Wei et al. (2007) increased biosurfactant production by *B. subtilis* ATCC 21332 from 1.74 to 3.34 g/l through the optimization of the trace elements composition (Mg^{2+}, K^{+}, Mn^{2+}, Fe^{2+}, and Ca^{2+}). Makkar and Cameotra (2002) also reported an increase on biosurfactant production by *B. subtilis* MTCC 2423 from 0.342 to 1.230 g/l when the culture medium was supplemented with the optimum concentrations of magnesium, iron, and calcium simultaneously, and this effect was higher than the obtained when the different metals were added individually. In our case, a combination of $FeSO_4$ 2.0 mM, $MgSO_4$ 0.8 mM, and $MnSO_4$ 0.2 mM increased biosurfactant production 3.6 times as compared with the medium without supplements.

Regarding the oil recovery assays, in a previous work, the biosurfactant produced by this isolate in a mineral medium (containing sucrose as the carbon source and ammonium nitrate as the nitrogen source) recovered 18.8 ± 1.1% of oil at a concentration of 1 g/l (Pereira et al., 2013). That value is higher than the obtained in the current work (15.0 ± 0.8%) at the same conditions. However, the *cmc* calculated for the biosurfactant produced in the mineral medium by this isolate was 0.03 g/l (Pereira et al., 2013), whereas for the biosurfactant produced in CSL 10% it was 0.16 g/l. This can explain the differences observed in the percentages of oil recovered in both cases. Nevertheless, the results obtained in this work are still better comparing with the oil recoveries obtained with the chemical surfactants Enordet and Petrostep at the same concentrations and conditions (9–12%; Pereira et al., 2013), thus suggesting that this biosurfactant can be useful for application in microbial enhanced oil recovery or bioremediation.

CONCLUSION

Biosurfactant production by *B. subtilis* #573 was evaluated using CSL as an alternative low-cost culture medium. The use of such substrate led to a surfactin production of about 1.3 g/l, which is in good agreement with the values reported so far in the literature. To our knowledge this is the first report on the use of CSL for the production of surfactin. Besides, if an industrial scale is envisaged, the use of CSL as culture medium would have a huge impact in the surfactin production costs. Additionally, the amount of biosurfactants produced using this alternative medium was increased about 3 to 4 times when metal salts were used as supplements, thus representing an even higher impact on the production costs. It is important to mention that the surfactin produced from these alternative media retained all its previously reported properties, including its great potential for microbial enhanced oil recovery or bioremediation applications.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of the work. Eduardo J. Gudiña, Elisabete C. Fernandes and Ana I. Rodrigues performed the experimental work and wrote part of the manuscript. José A. Teixeira and Lígia R. Rodrigues participated in the design of the study and drafted the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

The authors acknowledge the financial support from the Strategic Project PEst-OE/EQB/LA0023/2013 and project ref. RECI/BBB-EBI/0179/2012 (project number FCOMP-01-0124-FEDER-027462) funded by Fundação para a Ciência e a Tecnologia, and from AdI (Agência de Inovação, S. A.), through the project BIOCLEAN – "Development of biosurfactant-based products for surfaces cleaning and desinfection in the food industry" (QREN Ref. 30215).

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Conflict of Interest Statement: 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.

Received: 09 November 2014; accepted: 17 January 2015; published online: 06 February 2015.

Citation: Gudiña EJ, Fernandes EC, Rodrigues AI, Teixeira JA and Rodrigues LR (2015) Biosurfactant production by Bacillus subtilis using corn steep liquor as culture medium. Front. Microbiol. ***6****:59. doi: 10.3389/fmicb.2015.00059*

This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 14 August 2014
doi: 10.3389/fmicb.2014.00423

Sulfur source-mediated transcriptional regulation of the *rhlABC* genes involved in biosurfactants production by *Pseudomonas* sp. strain AK6U

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Despite the nutritional significance of sulfur, its influence on biosurfactants production has not been sufficiently studied. We investigated the expression of key biosurfactants production genes, *rhlABC*, in cultures of *Pseudomonas* sp. AK6U grown with inorganic or organic sulfur sources. AK6U grew with either inorganic sulfate ($MgSO_4$), dibenzothiophene (DBT), or DBT-sulfone as a sole sulfur source in the presence of glucose as a carbon source. The AK6U cultures produced variable amounts of biosurfactants depending on the utilized sulfur source. Biosurfactants production profile of the DBT cultures was significantly different from that of the DBT-sulfone and inorganic sulfate cultures. The last two cultures were very similar in terms of biosurfactants productivity. Biosurfactants yield in the DBT cultures (1.3 g/L) was higher than that produced by the DBT-sulfone (0.5 g/L) and the inorganic sulfate (0.44 g/L) cultures. Moreover, the surface tension reduction in the DBT cultures (33 mN/m) was much stronger than that measured in the DBT-sulfone (58 mN/m) or inorganic sulfate (54 mN/m) cultures. RT-qPCR revealed variations in the expression levels of the *rhlABC* genes depending on the sulfur source. The DBT cultures had higher expression levels for the three genes as compared to the DBT-sulfone and inorganic sulfate cultures. There was no significant difference in the expression profiles between the DBT-sulfone and the $MgSO_4$ cultures. The increased expression of *rhlC* in the DBT cultures is indicative for production of higher amounts of dirhamnolipids compared to the DBT-sulfone and inorganic sulfate cultures. The gene expression results were in good agreement with the biosurfactants production yields and surface tension measurements. The sulfur source mediates a fine-tuned mechanism of transcriptional regulation of biosurfactants production genes. Our findings can have an impact on industrial production of biosurfactants and other biotechnological processes like biodesulfurization.

Keywords: gene expression, surface tension, RT-qPCR, rhamnolipids, dibenzothiophene, biodesulfurization

INTRODUCTION

Biosurfactants are surface active natural compounds produced by many microorganisms. As compared to petrochemicals-derived (synthetic) surfactants, biosurfactants are characterized by superior physicochemcial properties in addition to their environmental compatibility (Desai and Banat, 1997). They are structurally diverse, amphipathic, and can lower surface and interfacial tension. An effective biosurfactant can reduce the surface tension of water from 72 to 35 mN/m (Soberón-Chávez and Maier, 2011). They can also emulsify various hydrocarbons (Sekhon et al., 2012).

Biosurfactants have attracted an increasing interest as efficient and eco-friendly substitutes to synthetic surfactants in many environmental, industrial, agricultural, and biomedical applications. These include, bioremediation (biodegradation), soil washing, biocontrol and spray application of fertilizers, enhanced oil recovery, de-emulsification, cosmetics, pharmaceuticals, antimicrobial agents, foods, beverages, etc. (Urum and Pekdemir, 2004; Mulligan, 2005; Rodrigues et al., 2006; Perfumo et al., 2010; Rodrigues and Teixeira, 2010).

Although there has been an increasing number of reports describing the production and characterization of efficient biosurfactants, to date biosurfactants are still not able to economically compete with synthetic surfactants. This is mainly due to high production costs (Cameotra and Makkar, 1998; Deleu and Paquot, 2004). One way to reduce the prohibitive high costs of biosurfactants is to enhance the strain productivity by, for instance, optimizing the growth conditions. The carbon source in the growth medium is of particular interest. Inexpensive carbon sources have been used for biosurfactants production to minimize the overall production costs.

Many studies reported improved biosurfactants yield with the application of industrial carbon-rich wastes (Makkar et al., 2011; Merchant and Banat, 2012). Various cheap substrates have the potential of enhancing biosurfactants production. These include vegetable oils, and oil wastes, animal fat, molasses, lactic whey, starchy substrates, etc. (Haba et al., 2000; Abalos et al., 2001; Dubey et al., 2005; Sekhon et al., 2012).

In contrast to the carbon source, the effect of the sulfur source on biosurfactants production has not received the proper

attention, although it is an essential component of the growth medium. In an earlier study, Pruthi and Cameotra (2003) reported that 80 ppm of inorganic sulfur was the optimal concentration for biosurfactants production by *Pseudomonas putida*. However, the authors did not test different types of sulfur sources and did not discuss the observed effect of sulfur concentration on biosurfactants production. Recently we have isolated a *Pseudomonas* sp. strain AK6U which can simultaneously produce rhamnolipid biosurfactants and utilize organosulfur compounds as sole sulfur sources. Interestingly, we have noticed that biosurfactants production was significantly enhanced in cultures containing organosulfur substrates as compared to cultures containing inorganic sulfate as a sole sulfur source (unpublished). Consequently, an interesting question arose. Is the increase in biosurfactants production due to promotion in the expression of the relevant genes or enhanced activity of the involved biosynthetic enzymes?

Here we conducted further investigations to unravel the reason behind the observed increase in biosurfactants production. We wanted to find out if it is due to enhanced expression of the *rhlABC* genes which encode key enzymes of rhamnolipid biosurfactants biosynthesis. RhlA catalyzes the synthesis of hydroxyalkanoic acid dimers which represent the hydrophobic moiety of rhamnolipid biosurfactants produced by many *Pseudomonas* spp. RhlB and RhlC are rhamnosyltransferases which catalyze the transfer of dTDP-L-rhamnose to either hydroxyalkanoic acid moiety (to produce monorhamnolipid) or to an existing monorhamnolipid molecule to produce dirhamnolipid, respectively (Deziel et al., 2003). We adopted real time quantitative polymerase chain reaction (RT-qPCR) with gene specific primers to investigate the expression of the *rhlABC* genes in the presence of different sulfur sources.

MATERIALS AND METHODS

CULTURE MEDIA AND THE BACTERIAL STRAIN

The AK6U strain was isolated from soil polluted with diesel, benzene, and used lubricating oil. The strain was recovered from a mixed culture enriched in chemically defined medium (CDM) containing glucose as a carbon source and DBT as a sulfur source (unpublished). Lauria-Bertani (LB) agar and broth media were prepared according to the instructions of the supplier. Sulfur-free CDM was prepared from stock solutions according to Gilbert et al. (1998). The CDM was supplemented with vitamins solution and trace elements (Pfenning, 1978; Van Hamme et al., 2000). The carbon source was glucose (10 mM) and the sulfur source was either $MgSO_4 \cdot 7H_2O$ (1 mM) or an organosulfur compound (0.1 mM). The tested organosulfur substrates were dibenzothiophene (DBT) and dibenzothiophene sulfone (DBT-sulfone). DBT is one of the most common organosulfur compounds found in petroleum and diesel. It is also the most frequently adopted model compound for the biodesulfurization studies (Monticello and Finnerty, 1985; Monot and Warzywoda, 2008). DBT-sulfone is an intermediate of the biodesulfurization 4S pathway (Monot and Warzywoda, 2008). Stock solutions of the organosulfur compounds were prepared in ethanol (100 mM for DBT) or acetone (50 mM for DBT-sulfone). $MgCl_2$ (1 mM) was added instead of $MgSO_4$ when organosulfur compounds were added as a sole sulfur source.

GROWTH OF AK6U ON DIFFERENT SULFUR SOURCES AND BIOSURFACTANTS PRODUCTION

Precultures were grown in sulfur-free CDM (100 mL in 250 mL Erlenmeyer flasks) containing glucose as a carbon source and either $MgSO_4$ or an organosulfur compound as a sole sulfur source. At the mid-log phase, culture samples were drawn and inoculated into 400 mL of the same medium in 1 L Erlenmeyer flasks (in triplicates). The inoculum size was 1–2% v/v (6–8 mg dry cell weight/L). Growth was monitored by measuring culture turbidity (optical density at 600 nm, OD_{600}) after time intervals until the cultures reached the stationary phase. Uninoculated flasks containing the same medium were included as controls. The biomass yield was measured as dry cell weight by drying cell pellets at 105°C for 15 h. All liquid cultures were incubated in an orbital shaker (180 rpm) at 30°C. Culture foaming was monitored as a preliminary indication of biosurfactants production. Cells were harvested at the late exponential growth phase by centrifugation in precooled centrifuge at 10,000 rpm for 10 min (Beckman centrifuge J2–21, USA). Cell pellets were washed once with 0.1 M ice–cold phosphate buffer (pH 7) and the washed cell pellets were stored at −20°C. All the cell harvesting procedures were performed on ice.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Cultures grown on different sulfur sources were analyzed by high performance liquid chromatography (HPLC) to monitor the utilization of the organosulfur compounds. Culture samples (500 μL) were centrifuged at 14,000 rpm for 5 min and the cell-free supernatants were extracted once with one volume of ethylacetate. The organic phase (300 μL of ethylacetate) was removed and transferred to clean (1.5 mL) eppendorf tubes. Ethylacetate was evaporated in a vacuum concentrator (speed-vac) at room temperature. The residue was dissolved in 50 μL ethanol and analyzed using a Thermo-Dionex UHPLC 3000 (Thermo, USA), and an Acclaim^{TM} 120 C18 column (5 μm, 120 A, Thermo, USA). The mobile phase was 60% acetonitrile-water pumped at a flow rate of 1 mL/min. Detection was performed with a photodiode array at 233 and 248 nm.

MEASUREMENT OF SURFACE TENSION

Culture samples (20 mL) were retrieved after time intervals and the cells were removed by centrifugation for 5 min at 10,000 rpm (4°C). The cell-free supernatants were then filtered under vacuum through 0.22 μm membrane filters to remove residual cells. The surface tension of cell-free culture supernatants was measured with a Kruss K100MK3 tensiometer (Kruss, Germany) equipped with a platinum plate at room temperature via the Wilhelmy plate method (Walter et al., 2010). The instrument was calibrated by adjusting the measurement so that the surface tension of water is 72 mN/m at room temperature.

RECOVERY OF THE CRUDE BIOSURFACTANTS

Cell-free culture supernatants (100 mL) were collected by centrifugation (14,000 rpm, 10 min) at the late-log growth phase and filtered through 0.22 μm membrane filters (Millipore, USA) to remove residual cells. After acidification to pH 2 with 25% HCl, the supernatants were kept at 4°C overnight. The biosurfactants were

extracted twice with one volume of chloroform-methanol (2:1) in a separating funnel. The organic phases were pooled and evaporated under vacuum (Buchi rotary evaporator V850, Switzerland) at 40°C. The residue was weighed and the crude biosurfactants yield was estimated as g/L.

ISOLATION OF RNA AND cDNA SYNTHESIS

Total RNA was isolated from 0.003 g ± 0.0001 of cell pellets harvested from different cultures using RNeasy Mini kit (Qiagen, Germany). The isolated RNA was treated twice with DNaseI. One treatment was done on-column and the second treatment was performed on the eluted RNA. Agarose gel electrophoresis (0.7%) was used to check the quality of RNA. The concentration and purity of RNA were estimated using Biophotometer plus (Eppendorf, Germany). To check DNA contamination, we performed PCR with gene-specific primers (**Table 1**) using the isolated RNA as a template. The PCR conditions were: 5 min at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 58°C, 30 s at 72°C, and final extension step 5 min at 72°C. cDNA was synthesized from a normalized RNA quantity (800 ng) with High Capacity cDNA Reverse Transcription kit (ABI, USA) according to manufacturer's instructions. cDNA was used as a template in RT-qPCR assays as explained in the following.

RT-qPCR

The primers used in this study are shown in **Table 1**. Primers specific for the *rhlAC* genes were derived from the corresponding sequences harbored in the genome of the *Pseudomonas aeruginosa* PAO1 strain (Genbank accession number NC_002516). Primers specific for the 16S rRNA gene were designed based on the partial sequence of the 16S rRNA gene of the AK6U strain (Genbank accession number AB922602). Delta–delta C_T relative quantification of gene expression was used to investigate the change in gene expression in cultures grown with different sulfur sources. 16S rRNA gene was included as an endogenous control and the results were expressed as fold change in expression. For each condition, three cDNA preparations (obtained from three independent cultures) were tested in RT-qPCR. For each cDNA replicate, two RT-qPCR assays were performed using Rotor-Gene Q (Qiagen, Germany). Each RT-qPCR assay (20 μL) contained 10 μL Rotor-Gene master mix (SYBR-Green PCR kit, Qiagen, Germany), 0.5 μM of each primer, 1 μL of cDNA and the rest was completed with nuclease-free water. PCR conditions were five minutes at 95°C followed by 40 cycles of 15 s at 95°C, 20 s at 58°C, and 30 s at 60°C.

STATISTICAL ANALYSIS

One way analysis of variance (Tukey test with $p < 0.05$) was performed with the JMP statistical software (version 10.0.2, SAS Corporation, Chicago, IL, USA).

RESULTS

BIOSURFACTANTS PRODUCTION BY AK6U UTILIZING DIFFERENT SULFUR SOURCES

AK6U grew in minimal medium containing either $MgSO_4$, DBT, or DBT-sulfone as a sole sulfur source in the presence of glucose as a carbon source (**Figures 1–3**). HPLC analysis of culture samples after time intervals revealed the utilization of the organosulfur substrates DBT and DBT-sulfone (**Figures 4** and **5**). Biosurfactants production was indicated by culture foaming and changes in the surface tension of the growth medium over time. The DBT cultures exhibited stronger foaming than either the $MgSO_4$ or the DBT-sulfone cultures that revealed similar foaming profile.

The surface tension was measured in cell-free culture supernatants. Interestingly, the surface tension decreased with time in all cultures (**Figures 1–3**). However, the extent of surface tension reduction was different depending on the utilized sulfur source (**Table 2**). The surface tension of the DBT culture was significantly different from that of the DBT-sulfone and the $MgSO_4$ cultures ($p < 0.05$). There was no significant difference in surface tension between the DBT-sulfone and the $MgSO_4$ cultures ($p > 0.05$). The DBT cultures recorded the strongest reduction in surface tension (from 72 to 33 mN/m). In contrast, surface tension reduction in the $MgSO_4$ and DBT-sulfone cultures was much weaker. The minimal surface tension in the $MgSO_4$ culture was 54 mN/m, whereas that of the DBT-sulfone cultures was 58 mN/m. The lowest surface tension in the DBT culture was attained towards the end of the exponential growth (after 25 h of incubation). The $MgSO_4$ and DBT-sulfone cultures reached the minimal surface tension value in a shorter time. This was also towards the end of exponential growth in all cultures. No further reduction in surface tension was observed during the stationary growth phase.

Table 1 | Primers used in this study.

Primer name	Primer sequence (5′–3′)	Target gene	Product size (bp)	Reference
16S-F	CACCGGCAGTCTCCTTAGAG	16S rRNA	203	This study
16S-R	AAGCAACGCGAAGAACCTTA			
rhlA-F	TGGACTCCAGGTCGAGGAAA	*rhlA*	263	This study
rhlA-R	GAAAGCCAGCAACCATCAGC			
Kpd1	GCCCACGACCAGTTCGAC	*rhlB*	226	Bodour et al. (2003)
Kpd2	CATCCCCCTCCCTATGAC			
rhlC-F2	GTCGAGTCCCTGGTTGAAGG	*rhlC*	211	This study
rhlC-R2	CGTGCTGGTGGTACTGTTCA			

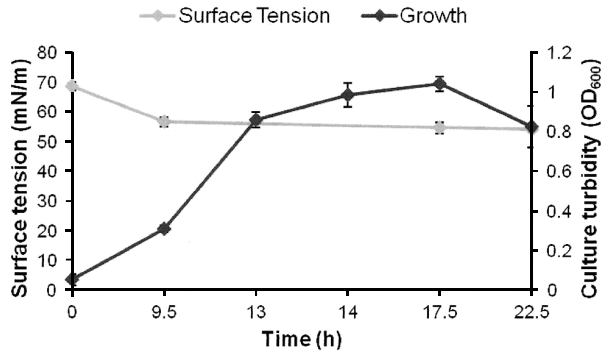

FIGURE 1 | Growth and biosurfactants production by AK6U in sulfur-free minimal medium containing $MgSO_4$ as a sole sulfur source and glucose as a carbon source. Biosurfactants production was monitored by measuring the surface tension of the cell-free culture supernatants. Data are averages of measurements from three cultures ± SE.

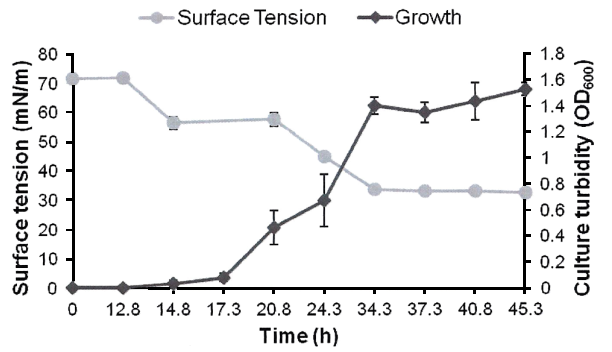

FIGURE 2 | Growth and biosurfactants production by AK6U in sulfur-free minimal medium containing DBT as a sole sulfur source and glucose as a carbon source. Biosurfactants production was monitored by measuring the surface tension of the cell-free culture supernatants. Data are averages of measurements from three cultures ± SE.

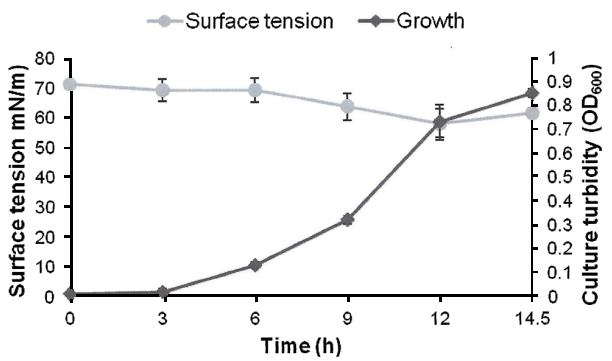

FIGURE 3 | Growth and biosurfactants production by AK6U in sulfur-free minimal medium containing DBT-sulfone as a sole sulfur source and glucose as a carbon source. Biosurfactants production was monitored by measuring the surface tension of the cell-free culture supernatants. Data are averages of measurements from three cultures ± SE.

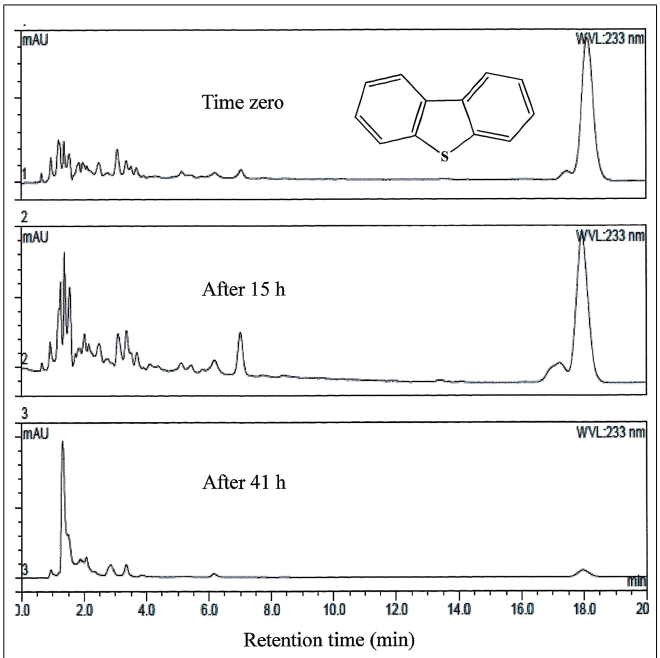

FIGURE 4 | HPLC analysis showing the utilization of DBT by AK6U grown in sulfur-free minimal medium containing DBT as a sole sulfur source and glucose as a carbon source. The chemical structure of DBT is shown and refers to the major peak in the chromatogram (retention time 18 min).

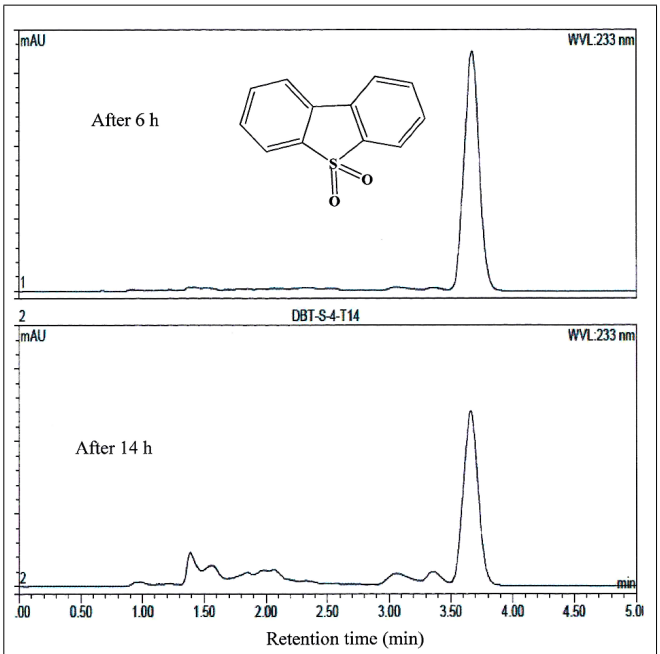

FIGURE 5 | HPLC analysis showing the utilization of DBT-sulfone by AK6U grown in sulfur-free minimal medium containing DBT-sulfone as a sole sulfur source and glucose as a carbon source. The chemical structure of DBT-sulfone is shown and refers to the major peak in the chromatogram (retention time 3.7 min).

Table 2 | Characteristics of biosurfactants production by AK6U.

Sulfur source	Minimal surface tension* (mN/m)	Biosurfactants yield (g/L)
$MgSO_4$	54.3 ± 0.2 A	0.44
DBT	32.8 ± 0.2 B	1.3
DBT-sulfone	58.2 ± 0.7 A	0.5

**Data are means of three replicates ± SE. Values labeled with different letters are significantly different ($p < 0.05$).*

A crude form of the produced biosurfactants was recovered from cell-free culture supernatants via acidification and solvent extraction. In addition to the variability in surface tension reduction, the AK6U cultures produced different amounts of biosurfactants depending on the utilized sulfur source (**Table 2**). The DBT cultures produced the highest biosurfactants yield (1.3 g/L). This was ca threefold higher than that recovered from the $MgSO_4$ and DBT-sulfone cultures. The latter two cultures produced similar quantities of crude biosurfactants.

RNA ISOLATION AND cDNA SYNTHESIS

Total RNA was successfully isolated from cell pellets harvested from different AK6U cultures. The integrity of the isolated RNA was verified by gel electrophoresis. The second DNaseI treatment removed genomic DNA contamination as revealed by gel electrophoresis. This was confirmed by using the isolated RNA as a template with gene-specific (*rhlB*) primers in PCR. This PCR failed to give any amplicons which confirms the absence of genomic DNA. cDNA was synthesized from the isolated RNA and was used as a template in PCR also with primers specific for the *rhlB* gene. Specific bands with the expected size were visible on the agarose gels.

EXPRESSION OF THE *rhlABC* GENES

We applied RT-qPCR to investigate the influence of the sulfur source on the expression of the *rhlABC* genes in AK6U cultures grown on different sulfur sources. As shown in **Figure 6**, the *rhlABC* genes expression profile was different among the AK6U cultures depending on the utilized sulfur source. In general, the DBT culture revealed the highest expression level for the three genes. The difference in gene expression levels between the DBT culture on one hand and the $MgSO_4$ and DBT-sulfone cultures, on the other hand, was statistically significant ($p < 0.05$). However, there was no significant difference in gene expression level between the $MgSO_4$ and the DBT-sulfone cultures ($p > 0.05$).

As compared to the $MgSO_4$ culture, the DBT culture had 3.2-fold higher expression for *rhlA*, 2.15-fold higher expression for *rhlB*, and 3.6-fold higher expression for *rhlC* (**Figure 6**). Compared to the DBT-sulfone culture, the DBT culture had 5.9-fold higher expression for *rhlA*, 5.3-fold higher expression for *rhlB*, and 3-fold higher expression for *rhlC*. The lowest expression level of the *rhlAB* genes was measured in the DBT-sulfone cultures. Moreover, the expression level of each of the *rhlABC* genes was variable in the same culture. For instance, in the DBT culture,

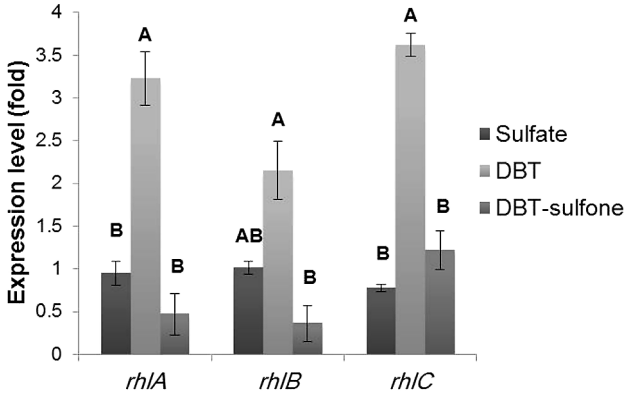

FIGURE 6 | Results of RT-qPCR showing the relative expression levels of the *rhlABC* genes in AK6U cultures grown with different sulfur sources in the presence of glucose as a carbon source. The expression level was calculated relative to that of the $MgSO_4$ culture as a control group. Data are means of at least two replicates ± SE. Significantly different values ($p < 0.05$) are indicated by different letters above the bars.

the expression level of *rhlA* and *rhlC* was higher than that of *rhlB*.

Interestingly, the *rhlC* expression level in the DBT-sulfone culture was 1.2-fold higher than that of *rhlA*. There was no significant difference between the expression levels of *rhlA* and *rhlB* in the DBT-sulfone culture ($p > 0.05$). In the $MgSO_4$ culture, the expression level of *rhlB* (1.01) was significantly different from that of *rhlC* (0.77).

DISCUSSION

Both inorganic sulfate and organosulfur substrates were adopted to test their effect on the expression of the *rhlABC* genes. The tested organosulfur compounds, namely DBT and DBT sulfone, are structurally similar and differ in their polarity (hydrophilicity). DBT-sulfone is more polar than DBT due to the presence of the two oxygen atoms (sulfonyl group). Growth of AK6U on either DBT or DBT-sulfone as a sole sulfur source indicates its ability to obtain sulfur from these two substrates in a process known as biodesulfurization (Gallagher et al., 1993; Chen et al., 2008).

The observed culture foaming is a preliminary indication of biosurfactants production (Ismail et al., 2013; Pasternak and Kotwzan, 2013). Results of the surface tension measurements were in agreement with the culture foaming trends. The DBT culture had the lowest surface tension. This provides a direct evidence for biosurfactants production. It further confirms that the DBT cultures produced much more biosurfactants than the DBT-sulfone or the $MgSO_4$ cultures. Ibacache-Quiroga et al. (2013) reported biosurfactants production by *Cobetia* sp. growing with DBT, however, as a carbon and energy source. It appears that the DBT-sulfone and the $MgSO_4$ cultures have similar biosurfactants productivity. This was inferred from the similar foaming and surface tension profiles. The extraction of more biosurfactants from the DBT culture is in line with suggestion that it produces higher amounts of biosurfactants as compared to the DBT-sulfone and the $MgSO_4$ cultures. Altogether, the results clearly point to

the influence of the sulfur source on biosurfactants production. Moreover, biosurfactants appear to play a significant role in the biodesulfurization of organosulfur compounds like DBT (Amin et al., 2013; Dinamarca et al., 2014).

The differences in biosurfactants production by AK6U can be attributed to variations in the expression profiles of the *rhlABC* genes. That is the answer to the main question of this study. RT-qPCR revealed different expression patterns among the AK6U cultures depending on the utilized sulfur source. This clearly shows that the sulfur source plays a regulatory role in the expression of the biosurfactants-related genes *rhlABC*. Upregulation of *rhlC* expression in the DBT and DBT-sulfone cultures indicates that they produce more dirhamnolipids than the $MgSO_4$ cultures. Interestingly, the gene expression profiles are in very good agreement with and corroborate the results of the surface tension measurements, biosurfactants yield, and culture foaming trends. Moreover, all support the suggested involvement of biosurfactants as a facilitator of DBT biodesulfurization.

Biosurfactants production is known to be controlled by a sophisticated and highly organized regulatory network at both the transcriptional and post-transcriptional level (Dusane et al., 2010; Abdel-Mawgoud et al., 2011; Reis et al., 2011). Factors that were shown to play a key role in biosurfactants production include quorum sensing and stress conditions like nutrients deprivation. This might explain the influence of the sulfur source on the expression of biosurfactants-related genes. The cells produce biosurfactants to overcome the low bioavailability of some essential nutrients like sulfur. Obviously, DBT is less accessible than inorganic sulfate (water soluble) and DBT-sulfone (more polar than DBT).

Although the literature lacks comprehensive studies addressing the influence of the sulfur source on biosurfactants production, other essential nutrients were shown to be involved. For instance, biosurfactants production increases under nitrogen-limiting conditions (Abdel-Mawgoud et al., 2011; Reis et al., 2011). The nitrogen source indirectly affects the *rhlAB* operon via the nitrogen metabolism sigma factor regulator RpoN. Moreover, RpoN has a direct impact on the biosurfactants biosynthesis regulator RhlR. Also phosphate deprivation upregulates RhlR-controlled genes including those related to rhamnolipid biosurfactants production (Reis et al., 2011).

Based on the results of this study, it is evident that the sulfur source affects the transcriptional regulation of the *rhlABC* genes. Nonetheless, our data do not show if the regulation mechanism is direct or indirect. Moreover, it is not clear whether the sulfur source itself or a metabolite thereof is involved in the regulation process. Since the change in biosurfactants production occurred early in the exponential phase, it can be proposed that the sulfur substrate *per se* provoked this change.

Variations in biosurfactants productivity due to the provision of different sulfur sources can be perceived as a part of a global response of AK6U to sulfate starvation. This can be reconciled in terms of the difference in bioavailability of DBT, DBT-sulfone and $MgSO_4$. In the presence of DBT as a sole sulfur source, the cells are stressed due to the very low aqueous solubility of DBT (sulfur-limiting conditions). Consequently, they produce biosurfactants to facilitate the uptake and sulfur utilization process.

Bacteria growing in the absence of easily accessible sulfur sources respond by synthesizing specific sulfate-starvation induced proteins (Kertesz and Wietek, 2001; Tralau et al., 2007). These include proteins related to the uptake and utilization of different sulfur sources, high-affinity transport systems for sulfate and cysteine, as well as antioxidants. Expression of the genes related to sulfate starvation is under the control of the global regulators (sigma factors) RpoN and RpoS (Tralau et al., 2007). Interestingly, both of these proteins play a role in the regulation of the biosurfactants production genes in *P. aeruginosa* (Reis et al., 2011). This may highlight the link between sulfur metabolism and biosurfactants production.

Considering the close structural similarity between DBT and DBT-sulfone, one might expect to see similar biosurfactants production profiles in cultures containing either of them as a sulfur source. Since this was not the case, we assume that AK6U has a well controlled sulfur source-mediated regulatory mechanism. This enables the cell to efficiently sense the bioavailability of the sulfur substrate and fine-tune biosurfactants production according to its needs.

The sulfur source can play a role in biosurfactants production via transcriptional regulation of the *rhlABC* genes. The AK6U strain appears to have the ability to fine-tune biosurfactants production according to the bioavailability of sulfur. AK6U overcomes the low aqueous solubility of DBT by producing biosurfactants. Overall, our findings provide a template for further studies aiming at better understanding of the underlying mechanisms through which the sulfur source influences biosurfactants production. This should have an impact on the industrial production of biosurfactants as well as other biotechnology-based processes such as biodesulfurization and bioremediation.

ACKNOWLEDGMENT

The authors wish to acknowledge the financial support provided by the Arabian Gulf University (Grant number BIO01).

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Conflict of Interest Statement: 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.

Received: 30 June 2014; accepted: 25 July 2014; published online: 14 August 2014.

*Citation: Ismail W, El Nayal AM, Ramadan AR and Abotalib N (2014) Sulfur source-mediated transcriptional regulation of the rhlABC genes involved in biosurfactants production by Pseudomonas sp. strain AK6U. Front. Microbiol. **5**:423. doi: 10.3389/fmicb.2014.00423*

This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology.

frontiers in Microbiology

REVIEW
published: 19 March 2015
doi: 10.3389/fmicb.2015.00212

Surfactants tailored by the class *Actinobacteria*

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Globally the change towards the establishment of a bio-based economy has resulted in an increased need for bio-based applications. This, in turn, has served as a driving force for the discovery and application of novel biosurfactants. The class *Actinobacteria* represents a vast group of microorganisms with the ability to produce a diverse range of secondary metabolites, including surfactants. Understanding the extensive nature of the biosurfactants produced by actinobacterial strains can assist in finding novel biosurfactants with new potential applications. This review therefore presents a comprehensive overview of the knowledge available on actinobacterial surfactants, the chemical structures that have been completely or partly elucidated, as well as the identity of the biosurfactant-producing strains. Producer strains of not yet elucidated compounds are discussed, as well as the original habitats of all the producer strains, which seems to indicate that biosurfactant production is environmentally driven. Methodology applied in the isolation, purification and structural elucidation of the different types of surface active compounds, as well as surfactant activity tests, are also discussed. Overall, actinobacterial surfactants can be summarized to include the dominantly occurring trehalose-comprising surfactants, other non-trehalose containing glycolipids, lipopeptides and the more rare actinobacterial surfactants. The lack of structural information on a large proportion of actinobacterial surfactants should be considered as a driving force to further explore the abundance and diversity of these compounds. This would allow for a better understanding of actinobacterial surface active compounds and their potential for biotechnological application.

Keywords: biosurfactant, emulsifier, glycolipid, lipopeptide, trehalose lipid, *Rhodococcus*, rhamnolipid

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *23 January 2015*
Accepted: *02 March 2015*
Published: *19 March 2015*

Citation:
Kügler JH, Le Roes-Hill M, Syldatk C and Hausmann R (2015) Surfactants tailored by the class Actinobacteria. Front. Microbiol. 6:212. doi: 10.3389/fmicb.2015.00212

Microbial Surfactants and their Applications

Microbially derived compounds that share hydrophilic and hydrophobic moieties, and that are surface active, are commonly referred to as biosurfactants. Many have been detected and described, and the majorityare molecules of low molecular weight. Within this group of low molecular weight microbial surfactants, the classes of lipopeptides or glycolipids, where fatty acid or hydroxy fatty acid chains are linked to either peptides or carbohydrates, have been extensively studied (Hausmann and Syldatk, 2014). The combinations of different types of hydrophilic and hydrophobic moieties within surfactants are innumerable and highly biodiverse.

Due to their amphiphillic structures, surfactants act as emulsifying agents, resulting in low surface tensions of interphases. Often, microorganisms produce them when growing on hydrophobic carbon sources or when exposed to growth limiting conditions. It is hypothesized, that biosurfactants play a role in the uptake of various hydrophobic carbon sources thus making nutrients bioavailable, as well as the protection of bacteria from harsh environmental conditions (Ristau and Wagner, 1983; Vollbrecht et al., 1998; Philp et al., 2002). Some biosurfactants show antimicrobial effects and the distinction of secondary metabolites as antibiotics or biosurfactants is often not strict.

Biosurfactants, compared to chemically derived surfactants, are independent of mineral oil as a feedstock, they are readily biodegradable and can be produced at low temperatures. Furthermore, they are described to be less toxic, effective at low concentrations and show effects in bioremediation. Industrial interest in biosurfactants is not solely based on the bio-acitivity of these molecules, but is also due to the broader ecological awareness linked to their application, which in turn is driven by sustainability initiatives and green agendas (Marchant and Banat, 2012). Biosurfactants can be applied in various areas such as the nutrient-, cosmetic-, textile-, varnish-, pharmaceutical-, mining-, and oil recovery industries (Henkel et al., 2012; Marchant and Banat, 2012; Müller et al., 2012).

An example of an actinobacterial biosurfactant that has already entered the market and found industrial application, is the lipopeptide antibiotic daptomycin. This antibiotic is used in the treatment of diseases caused by Gram positive pathogens and has been marketed as Cubicin® by Cubist Pharmaceuticals. Other promising studies for the potential application of actinobacterial biosurfactants are in environmental applications such as bioremediation: Oil spills were successfully dispersed by biosurfactants produced by a *Gordonia* sp. (Saeki et al., 2009), a *Dietzia* sp. (Wang et al., 2014) and a *Rhodococcus* sp. (Kuyukina and Ivshina, 2010); and trehalose lipids were applied in microbial enhanced oil recovery and the cleaning of oil storage tanks (Franzetti et al., 2010). In medical applications, the production of biosurfactants are generally considered safer than synthetically produced compounds due to high enzymatic precision during synthesis. Antiproliferation activities of cancerogenic cells could be induced by application of various glycolipids (Isoda et al., 1997; Sudo et al., 2000). In cosmetic applications, the use of trehalose lipids is favored above that of sodium dodecyl sulfate as it causes less irritation (Marques et al., 2009).

Different types of biosurfactants or bioemulsifiers have been described to be produced as secondary metabolites within the class *Actinobacteria*, and to the best of our knowledge, all of the producing species belong to the order *Actinomycetales* (**Figure 1**). The following section of the review will focus on the different types of actinobacterial biosurfactants reported in literature as well as their key structural features and bio-activities.

Metabolite Production within the Class *Actinobacteria*

Over the past few decades, there has been an increased interest in the discovery of bioactive metabolites with novel bioactive

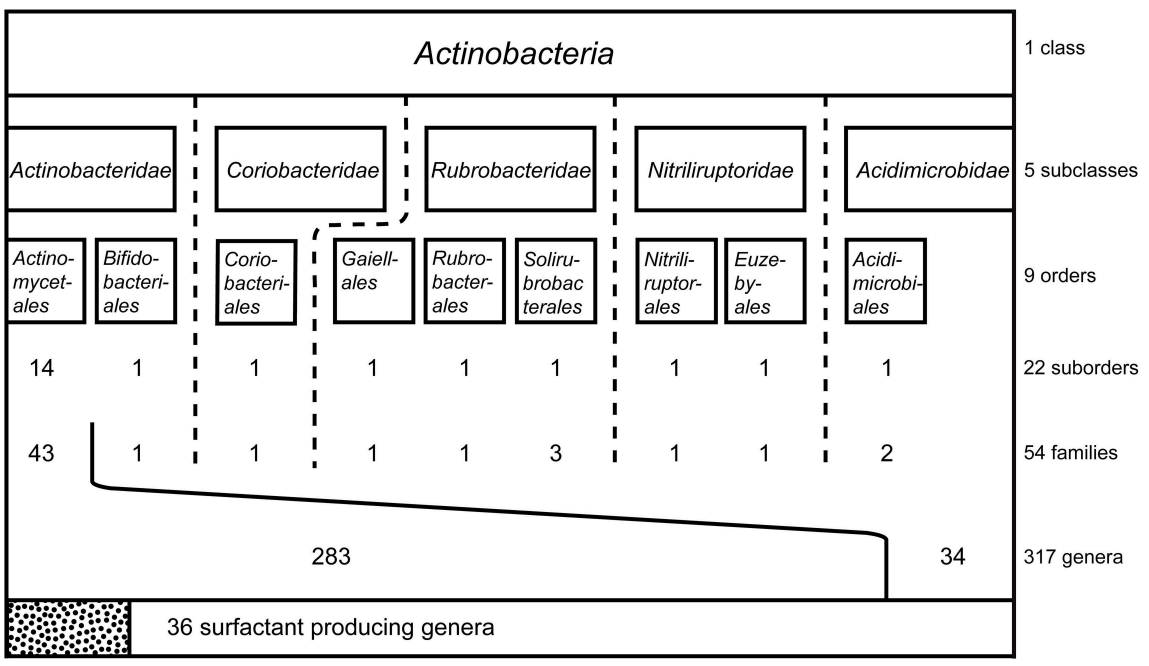


FIGURE 1 | Systematic classification of the class *Actinobacteria* including subclasses and orders. Suborder, families and genera examined for the production of biosurfactants and bioemulsifying compounds are displayed in numbers. Thirty six surfactant-producing genera are reported, all belonging to the largest order within the *Actinobacteria*: *Actinomycetales*.

properties and their potential for application in medical- or industrial-based processes. Microbial products are still considered to be the most promising source for the discovery of novel chemicals or therapeutic agents (Berdy, 2005). In addition, vast microbial genetic resources remains untapped and can lead to the development of novel bioactive metabolites.

In contrast to primary metabolites, secondary metabolites often accumulate and have miscellaneous chemical compositions that are species-specific. These secondary metabolites often exhibit bioactivity and are therefore of great interest to various industries. The most dominant source of microbially derived bioactive compounds is a group of bacteria known to have relatively large genomes and constitutes one of the main phyla within the *Prokaryotes*: The class *Actinobacteria* (Ludwig and Klenk, 2001). The class *Actinobacteria* play important roles in the environment, e.g., nutrient cycling, but also include major plant, animal and human pathogens (Embley and Stackebrandt, 1994), well known examples are the causative agents of leprosy and tuberculosis. Baltz (2008) assumed 5–10% of their genome coding capacity to be used for the production of secondary metabolites and indeed more than 35% of all known bioactive microbial metabolites and more than 63% of all known prokaryotic bioactive metabolites arise from actinobacteria (Bérdy, 2012). Most secondary metabolite producers described belong to families of the *Actinomycetales*, but it is estimated that only ∼1% of them are culturable (Bérdy, 2012). Many of these actinobacterial secondary metabolites exhibit antibacterial, antifungal, antitumor, anticancer and/or cytotoxic properties (Manivasagan et al., 2013). Antibiotics, with around 10,000 compounds described (Bérdy, 2012) is by far the largest group of metabolites isolated from actinobacteria. Depending on their chemical nature, the huge number of antibiotic compounds can roughly be classified into *peptides*, *aminoglycosides*, *polyketides*, *alkaloids*, fatty acids, and *terpenes* (Manivasagan et al., 2013; Abdelmohsen et al., 2014). Besides antibiotics, other actinobacterial compounds described are bioactive compounds with pharmacological activity (pheromones, toxins, enzyme inhibitors, receptors and immunological modulators), with agricultural activity (pesticides, herbicides and insecticides) and other industrially relevant properties (pigments and surfactants). Most compounds are derived from members of the genus *Streptomyces,* however, other so-called "rare" actinomycetes are increasingly playing a more important role in the production of biocompounds (Berdy, 2005; Kurtboke, 2010).

To fully understand the taxonomic distribution of the actinobacterial strains identified to produce biosurfactants and bioemulsifying compounds, taxonomic data of the class *Actinobacteria* was evaluated. Information were retrieved from the taxonomy browser of the National Center for Biotechnology Information[1] considering 16S rRNA gene sequence based reclassifications according to Zhi et al. (2009) and Goodfellow and Fiedler (2010). The order *Thermoleophilales* that has been reclassified into a new class (Euzéby, 2013) has been excluded and the recently identified order *Gaiellales* has been included (Euzéby, 2012). Overall, the class *Actinobacteria* contains five subclasses and nine orders with a total of 54 families (**Figure 1**). The largest order, *Actinomycetales*, is divided into 14 suborders and contains by far the highest diversity within the class *Actinobacteria*. It is therefore not surprising that biosurfactants reported in literature focuses on members of this order. The next few paragraphs will go into more detail around the different types of biosurfactants that have been identified to be produced by actinobacterial strains, their production, purification and structural elucidation, as well as the clear influence of the environment the producer organism is found in and their ability to produce biosurfactants.

[1]National Center for Biotechnology Information (NCBI) Taxonomy Browser. Available online at: http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Undef&id=201174&lvl=5&lin (accessed 01.07.2014 - 07.01.2015).

Trehalose-Comprising Glycolipids

The best described biosurfactants amongst the actinobacteria are glucose-based glycolipids, most of which have a hydrophilic backbone consisting of two α,α-1,1 glycosidic linked glucose units forming a trehalose moiety. Different types of trehalose-containing glycolipids and their producers have been extensively reviewed (Asselineau and Asselineau, 1978; Asselineau and Lanéelle, 1998; Franzetti et al., 2010; Kuyukina and Ivshina, 2010; Shao, 2011; Khan et al., 2012). Those of the class *Actinobacteria* are mainly found within the genera *Rhodococcus, Mycobacterium*, *Nocardia, Arthrobacter* and *Corynebacterium*, and less frequently within the genera *Tsukamurella, Brevibacterium,* and *Micrococcus* (**Tables 1**, **2**). Different structures of trehalose lipid comprising amphiphilic molecules have been reported: Acyl chains with glycosidic linkages to glucose or trehalose units have been reported to vary in number of occurrence, length and type, as well as the position (and number) of their linkage to the sugar rings and exhibit different cellular functions.

For the hydrophobic moiety of trehalose-comprising glycolipids, the structures of two main types of trehalose lipids have been elucidated: those carrying a mycolic fatty acid ester and those carrying a fatty acid ester.

The smallest hydrophilic backbone in glycolipids constitutes glucose, the building block of the sugar dimer trehalose. Complete structures of acylglucoses carrying mycolic acid esters have been elucidated and reported to be produced by isolates belonging to the genera *Corynebacterium* and *Mycobacterium* (Brennan et al., 1970) (**Table 1**), whereas acylglucoses carrying fatty acid esters have been described for *Brevibacterium* spp. (Okazaki et al., 1969) (**Table 2**).

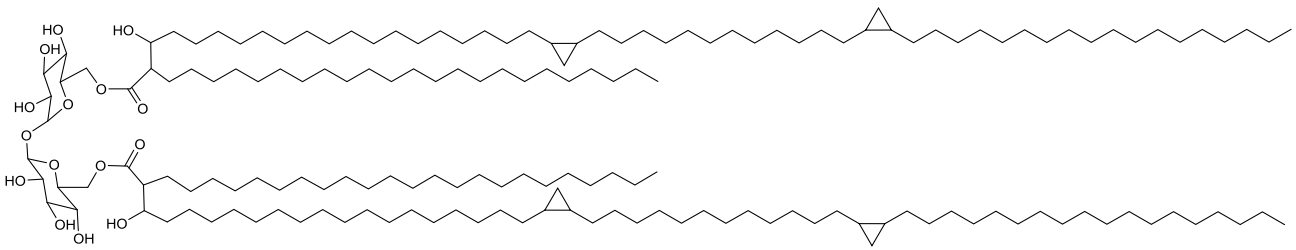
Trehalose Lipid Mycolic Acid Esters

Mycolic acids are long-chain fatty acids and a major component of the cell wall in various actinobacteria. Species-dependent, its lengths varies from 22 to 92 carbon atoms; they possess long β-hydroxy-α-branched acyl chains, including cyclopropane patterns and oxygenic groups. The synthesis of mycolic acids includes condensation reactions, and they are also referred to as eumycolic acid, corynemycolic acid and nocardio-mycolic acid, depending on their presence in *Mycobacterium* spp., *Corynebacterium* spp., and *Nocardia* spp., respectively (Asselineau and Lanéelle, 1998).

TABLE 1 | Mycolic and corynemycolic containing trehalose lipids that are of actinobacterial origin.

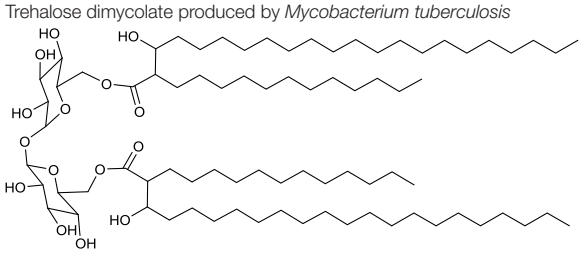
Species	Strain	TL mycolic acid ester	References
Arthrobacter paraffineus	KY 4303	TL mycolic (C32–C36)	Suzuki et al., 1969
Brevibacterium sp.	KY 4304/4305	TL mycolic (C32–36)	Suzuki et al., 1969
Brevibacterium vitarumen	12143	TL dimycolic (C28–C38)	Lanéelle and Asselineau, 1977
Corynebacterium diphtheriae	n.a.	Glucose mycolic (C32)	Brennan et al., 1970
Corynebacterium spp. *(fasciens, pseudodiphtheriae)*	KY 3543 KY 3541	TL mycolic (C32–36)	Suzuki et al., 1969
Corynebacterium matruchotii	ATCC 14266	TL dimycolic (C28–C38)	Datta and Takayama, 1993
Mycobacterium spp. *(smegmatis, tuberculosis)*	BCG, n.a.	Glucose mycolic (C32)	Brennan et al., 1970
Mycobacterium spp.* (*bovis, fortuitum, kansaii, malmoense, phlei, tuberculosis, smegmatis, szulgai,* etc.)	Various	TL mycolic, dimycolic,	Reviewed in: Asselineau and Asselineau, 1978; Gautier et al., 1992; Asselineau and Lanéelle, 1998; Vergne and Daffé, 1998; Dembitsky, 2004; Ishikawa et al., 2009; Shao, 2011
Nocardia spp.	n.a.	TL mycolic (C32–36)	Suzuki et al., 1969
Rhodococcus spp.* (*erythropolis, opacus, ruber,* etc.)	Various	TL mycolic,dimycolic,	Reviewed in: Asselineau and Asselineau, 1978; Lang and Philp, 1998; Kuyukina and Ivshina, 2010; Shao, 2011; Khan et al., 2012

EXAMPLES OF MYCOLIC ACID CONTAINING TREHALOSE LIPIDS



1

Trehalose dimycolate produced by *Mycobacterium tuberculosis*



2

Trehalose dicorynemycolate produced by *Rhodococcus erythropolis*

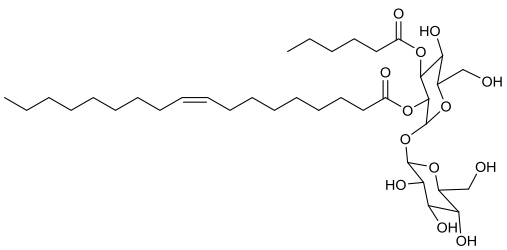
*Several producing species are reported; TL, trehalose lipid; n.a., information not available.

Mycolic acid comprising trehalose lipids (**Table 1**) can be distinguished into two different types, the trehalose mycolic lipids and the trehalose corynemycolic lipids. These mycobacterial trehalose mycolates or dimycolates are by far the most hydrophobic glycolipids. Linked to C6 (and C6′) of the sugar rings, they vary among species in length and branching. They are shaped to form bilayers, implemented in the outer cell wall and usually not found on the bacterial cell surface (Vergne and Daffé, 1998). Trehalose dimycolates (**1**, **Table 1**), also referred to as "cord factor," serve a particular function for the cell. They act as virulence factors and have immuno-modulating activity (Shao, 2011). They may further be important to maintain a hydrophobic cell wall of the organism hence facilitating the uptake of hydrophobic carbon sources. The other type, trehalose lipids containing corynemycolic acid also carry β-hydroxy-α-branched fatty acid moieties and have been described to occur within the genus *Rhodococcus* (**2**, **Table 1**), carrying 30–56 carbon atoms and within the genus *Corynebacterium*, carrying 22–36 carbon atoms. They are also described to occur in mycobacteria (Brennan et al., 1970) and found in trehalose lipids of *Brevibacterium vitarumen* (Lanéelle and Asselineau, 1977), *Arthrobacter paraffineus* and a *Nocardia* sp. (Suzuki et al., 1969). Corynemycolic acids are much shorter than their mycobacterial counterparts: they lack functional groups and are often unsaturated. Within virulent strains

TABLE 2 | Trehalose lipid ester of actinobacterial origin.

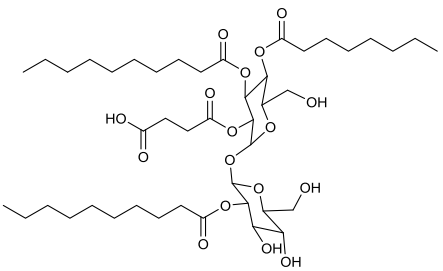
Species	Strain	TL ester	References
Arthrobacter sp.	EK 1	TL tetraester (C12–C18)	Passeri et al., 1990
Brevibacterium thiogenitalis	No. 653	Glucose diester (C18)	Okazaki et al., 1969
Micrococcus luteus	BN56	TL tetraester (C9–C14)	Tuleva et al., 2009
Mycobacterium spp.* (*africanum, bovis, fortuitum, tuberculosis*, etc.)	Various	TL ester	Reviewed in: Vergne and Daffé, 1998; Dembitsky, 2004; Shao, 2011
Mycobacterium tuberculosis	H37Rv	TL sulfolipid	Goren, 1970; Gilleron et al., 2004
Nocardia farcinica	BN26	TL succinic tetraester (C7-12)	Christova et al., 2014
Rhodococcus spp.* (*erythropolis, longus, wratislavensis*, etc.)	Various	TL ester, TL succinic ester	Reviewed in: Asselineau and Asselineau, 1978; Lang and Philp, 1998; Kuyukina and Ivshina, 2010; Shao, 2011; Khan et al., 2012
Tsukamurella pulmonis	PCM 2578T	TL diester (C18–20/C4–5)	Pasciak et al., 2010a
Tsukamurella spumae *Tsukamurella pseudospumae*	DSM 44113, DSM 44114 DSM 44117	TL diester (C16–18/C4–6)	Kügler et al., 2014
Tsukamurella tyrosinosolvens	DSM 44370	TL diester (C16–18/C2–6)	Vollbrecht et al., 1998

EXAMPLES OF TREHALOSE LIPID ESTERS



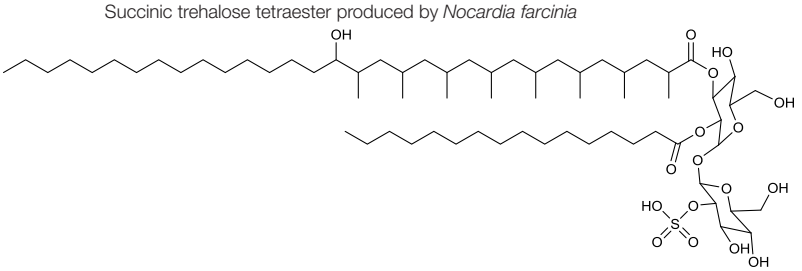
3

Trehalose diester produced by *Tsukamurella spumae*



4

Succinic trehalose tetraester produced by *Nocardia farcinia*



5

Diacetylated trehalose sulfolipid produced by *Mycobacterium tuberculosis*

*Several producing species are reported; TL, trehalose lipid.

of mycobacteria, five different sulfonated forms of trehalose esters have been found, varying in their acylation pattern (Khan et al., 2012).

Trehalose Lipid Esters

Actinobacterial trehalose lipid esters are mainly acylated at C6/C6′ or at C2/C3 and are summarized in **Table 2**. The amount of hydrophobic chains linked to the trehalose unit varies from one to four, forming trehalose mono-, di-, tri- and tetraesters, but also octaesters (Singer et al., 1990) (**3**, **Table 2**). The acyl chains varies in lengths from C8 to C20, show an unsaturated pattern or form short succinoyl acids, giving the trehalose lipid an anionic character (Lang and Philp, 1998; Tokumoto et al., 2009) (**4**, **Table 2**). They are reported to be linked to the chain length present in hydrophobic carbon source fed to the producing strain. These glycolipid-linked medium chain length fatty acids are found within the following actinobacterial genera: *Arthrobacter*, *Brevibacterium, Caseobacter, Micrococcus, Mycobacterium, Nocardia, Rhodococcus*, and *Tsukamurella* (**Table 2**).

An exception among the trehalose lipid esters described, is sulfolipid 1 (Goren, 1970) (**5**, **Table 2**), a sulfonated and acylated trehalose lipid carrying phtio- and hydroxyphtioceranic compartments. They are known to contribute to the pathogenesis and virulence of *Mycobacterium tuberculosis*, the causative agent of tuberculosis. Diacyltrehalose sulfate, the biosynthetic precursor for sulfolipid 1, has recently been isolated from *M. tuberculosis* (Domenech et al., 2004) and has been used as a target for T-cell mediated recognition and elimination of *M. tuberculosis* infected cells (Gilleron et al., 2004).

Oligosaccharide Lipids

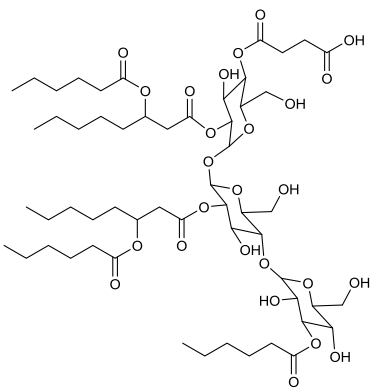
A glycosylated backbone of trehalose is found in oligosaccharide lipids (**Table 3**) carrying two to five sugar units. Trisaccharide lipids that have been reported for the class *Actinobacteria* all differ with respect to the acylation pattern of the third glucose unit. One sugar of the 1-1′ linked di-glucose backbone is further linked to a third sugar unit at C2 in the hydrophilic moeity of oligosaccharides produced by *Mycobacterium leprae* (Brennan, 1989) and *Tsukamurella tyrosinosolvens* (Vollbrecht et al., 1998). The third sugar unit is linked at C3 in a terrestrial actinomycete reported by Esch et al. (1999) and at C4 in a *Rhodococcus* sp. (Konishi et al., 2014) (**6**, **Table 3**). They also differ with respect to their hydrophobic nature. The latter two are acylated at all three sugar units, both carrying a C6 fatty acid moiety at the third sugar unit and succinic acid at the first sugar unit. Something that is rather exceptional is the acylation pattern at the trehalose backbone that, in its hydrophobic moieties, carries at each unit an acyloxyacyl structure in the *O*-ester linkage to the carbohydrate where the 3-hydroxy C8 or C10 fatty acid moiety is further acylated with a C6 fatty acid (**6**, **Table 3**). The *Tsukamurella* sp. trisaccharide lipids are acylated at two sugar units, each carrying two ordinary C8–C10 fatty acid units. Furthermore, a tetrasaccharide lipid form of this glycolipid has also been found to occur (Vollbrecht et al., 1998) (**7**, **Table 3**).

Non-trehalose based oligosaccharide lipids are found within phenol-phtiocerol glycosides in various mycobacteria. These

TABLE 3 | Actinobacterial oligosaccharide lipids.

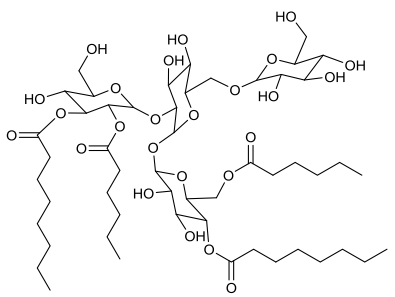
Species	Strain	Oligosaccharid lipids	References
Mycobacterium spp.* (avium, *kansaii, leprae, linda, malmoense, smegmatis, szulgai, tuberculosis*)	Various	oligosaccharide ester, phenolic glycolipids	Reviewed in: Saadat and Ballou, 1983; Brennan, 1989; Dembitsky, 2005b
Nocardia corynebacteroides	SM1	Pentasaccharide succinic octaester (C2–C8)	Powalla et al., 1989
Rhodococcus sp.	NBRC 1097287	Trisaccharid succinic tetraester (C8-*O*-C6/C6)	Konishi et al., 2014
Rhodococcus fascians	NBRC 12155		
Tsukamurella tyrosinosolvens	DSM 44370	Tri/tetrasaccharide ester (C8-10)	Vollbrecht et al., 1998

EXAMPLES OF OLIGOSACCHARIDE LIPIDS



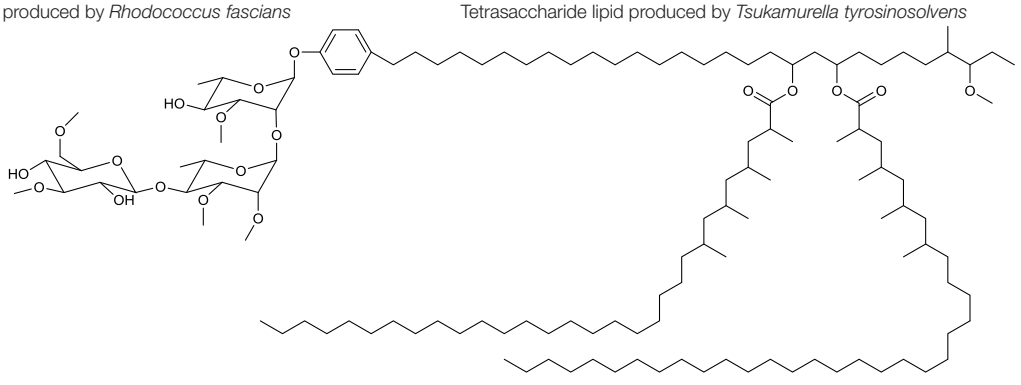
6

Succinic trisaccharide lipid produced by *Rhodococcus fascians*



7

Tetrasaccharide lipid produced by *Tsukamurella tyrosinosolvens*



8

Methylated dirhamnose/glucose phenol phtiocerol named phenolic glycolipid I of *Mycobacterium leprae* Brennan, 1989

*Several producing strains are reported.

oligosaccharide lipids, also termed phenolic glycolipids, contain tri- and tetraglycosyl units composed of various methylated sugars that are mainly based on rhamnose and partly on fucose, glucose and arabinose (Brennan, 1989). The rarely described phenolic acylation pattern is bound to dimycocerosyl phtiocerol acyl groups. The phenolic glycolipid I of *M. leprae* carries three mycocerosyl acyl groups each in length of C30–C34 (Brennan, 1989) (**8**, **Table 3**).

In industrial and environmental processes the potential of trehalose lipids could become valuable as they have shown interesting properties in several studies that focus on the remediation of hydrocarbon contaminated soils, the removal of suspended solids from wastewater (Franzetti et al., 2010) and in enhanced oil recovery (Christofi and Ivshina, 2002). However, most research are centered around the bio-activity of trehalose lipid molecules that exhibit biomedical properties such as antimicrobial, antiviral (Azuma et al., 1987; Watanabe et al., 1999; Shao, 2011) and antitumor activities (Sudo et al., 2000; Franzetti et al., 2010; Gudiña et al., 2013). Due to their functions in cell membrane interactions they can act as therapeutic agents (Zaragoza et al., 2009; Shao, 2011) or have an impact on the pathogenesis of causative agents of infections, such as those caused by pathogenic *M. tuberculosis, Corynebacterium diphteriae*, and the opportunistic pathogens, *Mycobacterium avium, Mycobacterium intracellulare, Nocardia asteroides, Corynebacterium matruchotii*, and *Corynebacterium xerosis* (Kuyukina and Ivshina, 2010). Trehalose lipids can be excreted into the cultivation supernatant or can be produced as non-covalently linked lipids bound to the cell wall or they can be cell wall integrated thus posing limits to quantities produced by the organisms, a disadvantage for its potential exploitation in large scale production processes.

Non-Trehalose Glyolipids

Hexose-Comprising Glycolipids

Besides the trehalose-containing biosurfactants and its congeners, several glycolipids have been elucidated that are produced by actinobacteria and share other hydrophilic moieties. By simply varying the carbon source in the growth media from n-alkanes to either sucrose or fructose, the hydrophilic part of the surfactant produced was reported to be switched from trehalose to fructose by members of the genus *Arthrobacter*, *Corynebacterium*, *Nocardia*, *Brevibacterium*, and *Mycobacterium* (Itoh and Suzuki, 1974) and sucrose in the case of the same genera except *Mycobacterium* (Suzuki et al., 1974). Compounds for which structures have been elucidated are listed in **Table 4**.

Besides the rhamnose-containing phenolic glycolipids mentioned in the oligosaccharide lipid section, the occurrence of other rhamnose-based lipids have recently been detected in a deep sea isolate identified as *Dietzia maris* (Wang et al., 2014) and has been identified as a C10:C10 di-rhamnolipid. This represents a unique occurrence within the class *Actinobacteria*. Other rhamnolipid producing actinobacteria are admittedly declared as producing strains in literature, however the surface active compounds produced have either not been elucidated or identified as rhamnolipids with debatable structural characterizations (*Rhodoccocus fascians* Gesheva et al., 2010, *Renibacterium salonarium* Christova et al., 2004, and a *Nocardioides* sp. Vasileva-Tonkova and Gesheva, 2005) (**Table 11**).

A different group of glycolipids are lipidic structures based on dimannose. Typically they are linked via a glycerol unit to different numbers of fatty acid chains. They have been reviewed in Shaw (1970) and structures have been identified for compounds produced by species belonging to the actinobacterial genera *Micrococcus* (Lennarz and Talamo, 1966), *Curtobacterium* (Mordarska et al., 1992), *Saccharopolyspora* (Gamian et al., 1996), *Rothia* (Pasciak et al., 2002, 2004), *Nocardiopsis* (Pasciak et al., 2004), *Arthrobacter* (Pasciak et al., 2010b) as well as the strain *Sinomonas atrocyaneus* (Niepel et al., 1997), formerly classified as *Arthrobacter atrocyaneus*. These dimannose based glycolipids are composed of hydrophilic α-D-mannopyranose dimers linked with two C14–C16 *iso* or ante*iso* fatty acid chains. One chain is directly esterified to the C6 hydroxyl group of one sugar unit, while the second fatty acid chain is linked via a glycerol moiety to the C3 of the same sugar unit. The glycerol moiety is monoacylated at either the primary or secondary methylene position (**9**, **Table 4**) and its acylation site can be used to distinguish taxonomic properties of the different producer strains. These compounds have been isolated intracellularly and they act as precursors and cell membrane anchors for the synthesis of lipoarabinomannan, a polymeric surfactant and actinobacterial cell wall component (Pakkiri and Waechter, 2005) (see section on polymeric biosurfactants).

The coexistence of galactosyl diglycerides (**10**, **Table 4**) in *Arthrobacter scleromae* and *Arthrobacter globiformis* (Pasciak et al., 2010b) have been described and can be used as a glycomarker to distinguish these strains from the opportunistic pathogens, *Rothia mucilaginosa* and *Rothia dentocariosa* (Pasciak et al., 2002, 2004).

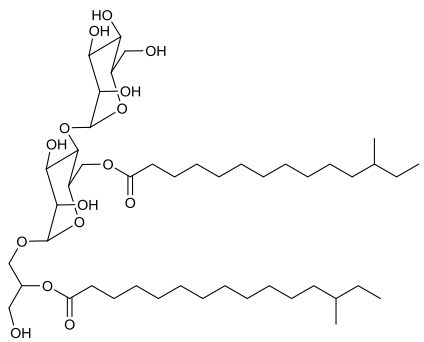
Macrocyclic Glycosides

Among the biosurfactants produced by actinobacteria, macrocyclic glycosides (**Table 5**) and macrocylcic dilactones (**Table 6**) can be distinguished and are often known to exhibit bio-activity against a range of organisms. The aliphatic macrolide antibiotic, brasilinolide, is produced by *Nocardia brasiliensis* and exhibits both antifungal and antibacterial activity. Three different variants have been described by Tanaka et al. (1997), Mikami et al. (2000) and Komatsu et al. (2004). All consist of a C32-membered macrolide with a sugar moiety but differ with regards to the acylation site of a malonic acid ester side chain (**11**, **Table 5**). The C16-membered dimeric macrolide elaiophylin and its variants have been isolated from various *Streptomyces* spp. including high producer strains. It exhibits bio-active properties against intestinal worms as well as antimicrobial, antitumor and immunosuppressant activities. A putative 95 kbp biosynthetic gene cluster of elaiophylin has been proposed (Haydock et al., 2004). Dembitsky (2005a,c) reviewed the different types of C14-membered lactam rings that are attached to an aminosugar (**12**, **Table 5**). Fluvirucin has been isolated from various *Actinomadura* spp., *Streptomyces* spp., *Microtetraspora* spp., and *Saccharotrix mutabilis*. The different fluvirucins share a common lactam ring unit but differ in terms of glycosylation. All of them act as potent antifungal

TABLE 4 | Non-trehalose comprising glycolipids produced by actinobacteria.

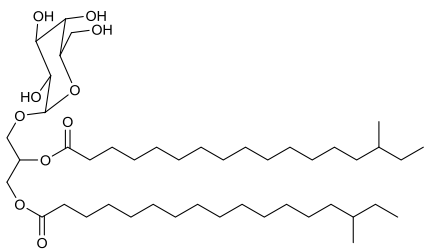
Species	Strain	Hexose lipids	References
Arthrobacter paraffineus	KY 4303	Sucrose mycolic (C32–C36)*	Suzuki et al., 1974
Arthrobacter paraffineus	KY 4303	Fructose coryne- and dicorynemycolic*	Itoh and Suzuki, 1974
Arthrobacter spp. *(globiformis, scleromae)*	ATCC 8010^T YH 2001^T	Dimannosylacyl (C15–C17) monoglyceride (C15–C17) Galactosyl diglyceride(C15–C17)	Pasciak et al., 2010b
Brevibacterium butanicum	KY 4332	Fructose coryne- and dicorynemycolic*	Itoh and Suzuki, 1974
Brevibacterium spp.	n.a.	Sucrose mycolic (C32–C36)*	Suzuki et al., 1974
Corynebacterium spp.	n.a.	Sucrose mycolic (C32–C36)*	Suzuki et al., 1974
Corynebacterium spp.	n.a.	Fructose coryne- and dicorynemycolic*	Itoh and Suzuki, 1974
Curtobacterium flaccumfaciens	ATCC 13437	Di- and trimannosylglyceride (C18–C19 cyclopropane)	Mordarska et al., 1992
Dietzia maris	MCCC 1A00160	Rhamnolipid (C10/C10)	Wang et al., 2014
Micrococcus lysodeikticus	ATCC 4698	Dimannosylglyceride (C14)	Lennarz and Talamo, 1966
Mycobacterium avium	KY 3844	Fructose coryne- and dicorynemycolic*	Itoh and Suzuki, 1974
Mycobacterium koda	KY 3852		
Nocardia butanica	KY 4333	Sucrose mycolic (C32–C36)*	Suzuki et al., 1974
Nocardia convulutus	KY 3907		
Nocardia rubra	KY 3844	Fructose coryne- and dicorynemycolic*	Itoh and Suzuki, 1974
Nocardia butanica	KY 4333		
Nocardia convulutus	KY 3907		
Nocardiopsis dassonvillei	PCM 2492T (ATCC 23218)	Dimannosylacyl (C15) monoglyceride (C16)	Pasciak et al., 2004
Rothia dentocariosa	PCM 2249^T (ATCC 17931)	Dimannosylacyl monoglyceride (C16–C19)	Mordarska et al., 1992; Pasciak et al., 2002
Rothia mucilaginosa	PCM 2415^T (ATCC 25296^T)	Dimannosylacyl (C15) monoglyceride (C16)	Pasciak et al., 2004
Saccharopolyspora spp. *(erythraea, hirsuta, rectivirgula,* sp.*)*	ATCC 27875^T ATCC 11635^T IMRU1258 LL-100-46)	Dimannosylacyl (C15–C16) monoglyceride (C16)	Gamian et al., 1996; Pasciak et al., 2002, 2004
Sinomonas artrocyaneus	LMG 3814^T	Dimannoseylacyl (C14) monoglyceride (C16)	Niepel et al., 1997

EXAMPLES OF NON-TREHALOSE COMPRISING HEXOSELIPIDS



9

Dimannosylacyl monoglyceride produced by *Rothia mucilaginosa*



10

Galactosyl diglyceride produced by *Arthorbacter globiformis* and *Arthrobacter scleromae*

*n.a., no information available; *Sucrose and fructose based surfactants are variants of trehalose lipids.*

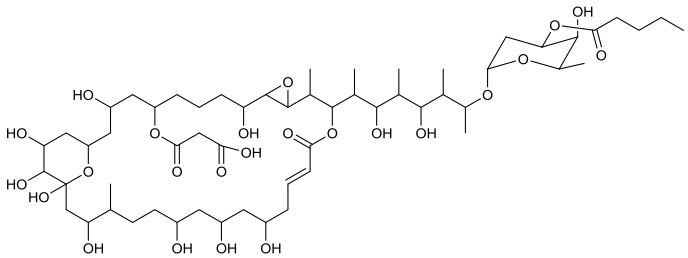
agents against *Candida* spp. and show antiviral properties against influenza A virus (Dembitsky, 2005c).

Among the macrocyclic dilactones, glucolypsin, an acylglucose dimer has been isolated from *Streptomyces purpurogeniscleroticus* and *Nocardia vaccinii* by Qian-Cutrone et al. (1999). This extraordinary glycolipid is formed out of two glucose units linked to identical iso-branched C18 acyl chains that each carry a methyl group at C2 and a hydroxyl group at C3 of the acyl chain. By connecting the C6′ of the glucose molecule to the carboxy-C1 of the fatty acid chain, a rotationally symmetric dimer is formed (**13**, **Table 6**). Glucolypsin variants with C18 and C17 fatty acid chains of the same type also occur. Glycolypsin is reported to increase the activity of glucokinases by relieving its inhibition via long chain fatty acyl CoA esters (Qian-Cutrone et al., 1999). Derivates of glucolypsin that share a common backbone, have been shown to exhibit antiviral and antibiotic properties. In contrast to glucolypsin, the acylglucose dimer of fattiviracins (C24/C26) and cycloviracins (C24/C33) are built up out of trihydroxy fatty acids, each of them glycosidic linked to a further glucose unit at the third hydroxyl group. Cycloviracins are characterized by

TABLE 5 | Macrocyclic glycosides produced by actinobacteria.

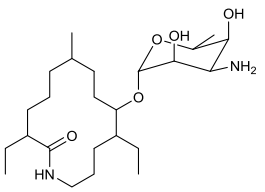
Species	Strain	Macrocyclic glycoside	References
Actinomadura spp.* *(roseorufa roseorura, vulgaris, yumaensis)*	Various	Fluvirucin (14C macrolide)	Reviewed in: Dembitsky, 2005a,c
Microtetraspora pusilla	R359-5	Fluvirucin B1 (14C macrolide)	Dembitsky, 2005a
Microtetraspora tyrrheni	Q464-31	Fluvirucins (14C macrolide)	Dembitsky, 2005a,c
Nocardia brasiliensis	IFM 0406	Brasilinolide A, B, C (32C macrolide)	Tanaka et al., 1997; Mikami et al., 2000; Komatsu et al., 2004
Saccharothrix mutabilis	R869-9	Fluvirucin A2 (14C macrolide)	Dembitsky, 2005a
Streptomyces spp.* (*antibioticus, erythreus, felleus, hygroscopicus, melanosporus, narbonensis, spinichromogenes, violaceoniger*)	various	Elaiophylin and derivates (16C macrolide) Fluvirucin (14C macrolide)	Reviewed in: Dembitsky, 2005a

EXAMPLES OF MACROCYCLIC GLYCOSIDES



11

Brasilinoide A produced by *Nocardia brasiliensis*



12

Fluvirucin B1 produced by *Actinomadura vulgaris* subsp. *lanata*

*Several producing strains are reported.

a fifth glucose unit bound to the C26 fatty acid chain, the three non-cyclic sugar units are methoxylated at C2, and the methyl branches at C2 of the fatty acid moieties are missing. Congeners of fattiviracin are divided into five families according to the length of their fatty acid moiety with each family showing similar antiviral activity against herpes, influenza and human immunodeficiency viruses (Uyeda, 2003). No alterations in the fatty acid chain length of cycloviracins have been reported. Fattiviracins (**14**, **Table 6**) have been shown to be produced by *Streptomyces microflavus* (Uyeda et al., 1998) and cycloviracins (**15**, **Table 6**) by *Kibdelosporangium albatum* (Tsunakawa et al., 1992b).

Terpenoids and Terpene Glycosides

Actinobacterial terpenoid and terpene glycosides are summarized in **Table 7**. Vancoresmycin is a C65 highly oxygenated terpenoid glycoside produced by an *Amycolatopsis* sp. It contains a tetramic acid unit and is glycosidic linked to a methylated carbohydrate moiety containing one amino group (**16**, **Table 7**). Antimicrobial effect against various bacteria was reported by Hopmann et al. (2002), most notably against species resistant to the antibiotic vancomycin (often considered to be the antibiotic of last resort for the treatment of resistant bacteria). Besides the terpenoid glycoside, several different types of terpene glycosides are produced by actinobacterial strains. They are surfactants that mostly carry terminal hydrophilic groups linked by a hydrophobic carotenoid moiety.

Terpene glycosides have been elucidated as products obtained from members of the following genera: *Corynebacterium* (Weeks and Andrewes, 1970), *Arthrobacter* (Arpin et al., 1972), *Rhodococcus* (Takaichi et al., 1997), and *Micrococcus* (Osawa et al., 2010) (**Table 7**). Most of them share a backbone of a C50 atom carotenoid. They can either be linked to one or two hydroxyl groups at the terminal ends (decaprenoxanthin and sarcinaxanthin) or one hydroxyl group and one glycosidic moiety (corynexanthin, decaprenoxanthin monoglycoside and sarcinaxanthin monoglycoside). Di-glycosylated forms are found within *Arthrobacter* and *Micrococcus* (decaprenoxanthin diglucoside and sarcinaxanthin diglucoside) (**17**, **Table 7**) and further exist as an acetylated form at all hydroxyl groups. The terpene glycosides produced by *Rhodococcus rhodochrous,* differ from the one mentioned above, as they contain a monocyclic carotenoid backbone linked to a glucopyranosyl residue at the non-cyclic end (**18**, **Table 7**). The glucose unit is further acylated at C6 to a C36–C50 mycolic acid moiety leading to carotenoid glucoside mycolic acid esters. These terpene glycosides are mainly found in pigmented bacteria and it is hypothesized that they act as antioxidants to protect organisms from injuries caused by free radicals (Osawa et al., 2010).

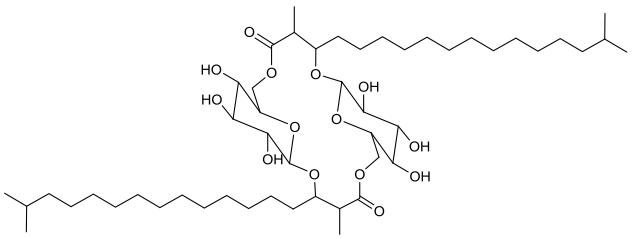
Polymeric Biosurfactants

The most common polymeric surfactants produced by actinobacteria are macro-amphiphilic lipoglycans such as lipoarabinomannan and its precursors, lipomannan and phosphatidylinositol mannosides. In contrast to the core of the actinobacterial cell wall, arabinogalactan and peptidoglycan, these polymeric lipoglycans are non-covalently attached to the cell membrane although phosphatidylinositol mannides are structurally related to lipomannan and lipoarabinomannan anchor units. These polymeric glycolipids have been isolated from *Mycobacterium* spp., *Gordonia* spp., *Rhodococcus* spp.,

TABLE 6 | Macrocyclic dilactones produced by actinobacteria.

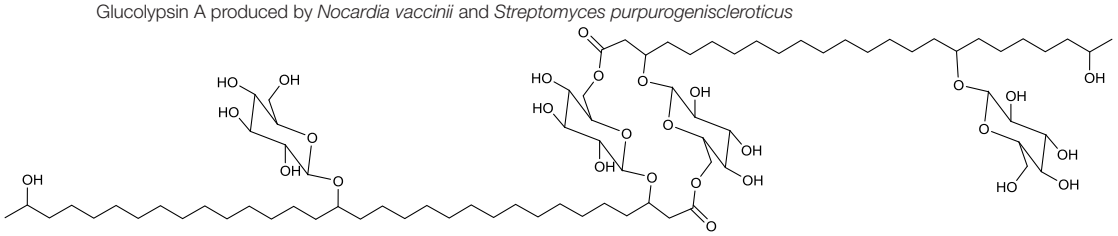
Species	Strain	Macrocyclic dilactones	References
Kibdelosporangium albatum	*ATCC 55061*	Cycloviracin B1 and B2 (C23/C26)	Tsunakawa et al., 1992a,b
Nocardia vaccinii	WC65712	Glucolypsin A and B (C19/C19)	Qian-Cutrone et al., 1999
Streptomyces microflavus	No.2445	Fattiviracin a1 (C22–28/C22–24)	Uyeda et al., 1998; Yokomizo et al., 1998
Streptomyces purpurogeniscleroticus	WC71634	Glucolypsin A and B (C19/C19)	Qian-Cutrone et al., 1999

EXAMPLES OF MACROCYCLIC DILACTONES



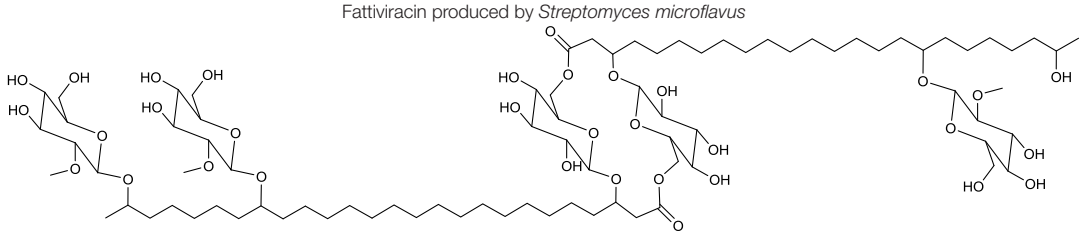
13

Glucolypsin A produced by *Nocardia vaccinii* and *Streptomyces purpurogeniscleroticus*



14

Fattiviracin produced by *Streptomyces microflavus*



15

Cycloviracin B_1 produced by *Kibdelosporangium albatum*

Dietzia maris, Tsukamurella paurometabolus, Turicella otitidis, and *Amycolatopsis sulphurea* (**Table 8**). Except for *A. sulphurea*, all of these strains belong to the suborder *Corynebacteridae* that are known to contain mycolic acids in their cell wall. It comprises the presence of mycolic acids and contain lipid rich cell envelope structures (Sutcliffe, 1997) forming an extremely robust and impermeable cell envelope (Berg et al., 2007). Lipoarabinomannans are well known to cause immunorepressive functions in diseases such as tuberculosis and leprosy that are caused by the pathogenic mycobacterial strains *M. tuberculosis* and *M. leprae*. However, non-pathogenic species have also been shown to produce lipoarabinomannans and are reported to have an opposite effect thus stimulating pro-inflammatory responses (Briken et al., 2004). The mannan core of lipoarabinomannan and the number of branching units is species dependent. Further differences in its structure is traced back to capping motifs present at the non-reducing termini of the arabinosyl side chains. Mannan caps are mainly present in pathogenic strains, whereas inositol phosphate caps are present in non-pathogenic mycobacteria (Briken et al., 2004). Lipoarabinomannans show structural similarity to its precursors lipomannan and phosphatidylinositol mannoside and consist of an α-1,6 linked mannan core with frequent α-1,2 mannose branches leading to a mannan backbone of approximately 20–25 mannose residues substituted with arabinofuran residues that carry terminal extension motifs, which vary among the producer species (Berg et al., 2007). The lipophilic part consists mainly of C16 glycerides that are linked to the mannan core by a phosphate group (**19**, **Table 8**).

Lipopeptides

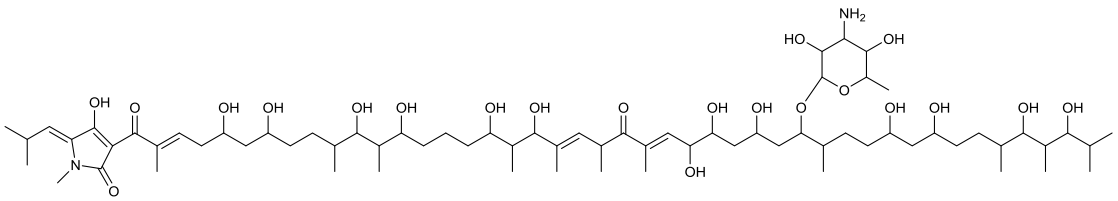
Cyclic and linear lipopeptides are produced by various actinobacterial strains and are summarized in **Table 9**.

Cyclic Lipopeptides

Cyclic lipopeptides are the most common type of lipopeptides and consist of a peptide chain of various types and numbers of amino acids circularized and linked to mainly one fatty acid

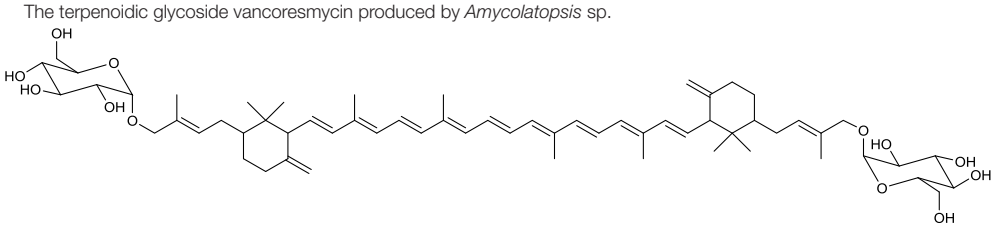
TABLE 7 | Terpenoid and terpene-containing biosurfactants produced by actinobacteria.

Species	Strain	Terpenoids and terpenes	References
Amycolatopsis sp.	DSM 12216	Vancoresmycin (65C terpenoid)	Hopmann et al., 2002
Arthrobacter sp.	M3	Corynexanthin mono- and diglycosides (C50 terpene)	Arpin et al., 1972; Dembitsky, 2005b
Corynebacterium sp.	CMB 8	Corynexanthin (C50 terpene)	Weeks and Andrewes, 1970
Micrococcus yunnanensis	AOY-1	Sarcinaxanthin, sarcinaxanthin mono- and diglucosides (C50 terpene)	Osawa et al., 2010
Rhodococcus rhodochrous	RNMS1	Carotenoid (C40 terpene) glycoside (C36–C50 mycolic)	Takaichi et al., 1997
EXAMPLES OF TERPENE AND TERPENOID GLYCOSIDES			



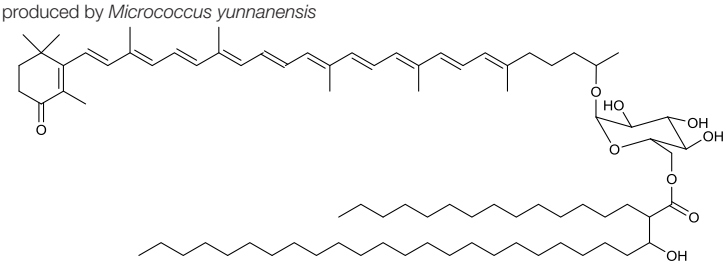
16

The terpenoidic glycoside vancoresmycin produced by *Amycolatopsis* sp.



17

Sarcinaxanthin diglycoside produced by *Micrococcus yunnanensis*



18

Carotenoid glycoside esterified with a rhodococcus type mycolic acid produced by *Rhodococcus rhodochrous*

chain. A surfactant often falsely cited to be produced by an actinobacterium but not of actinobacterial nature, is the eleven amino acid cyclic lipopeptide arthrofactin. It was initially postulated to be produced by an *Arthrobacter* sp. (Morikawa et al., 1993) but later corrected to originate from a *Pseudomonas* strain (Roongsawang et al., 2003).

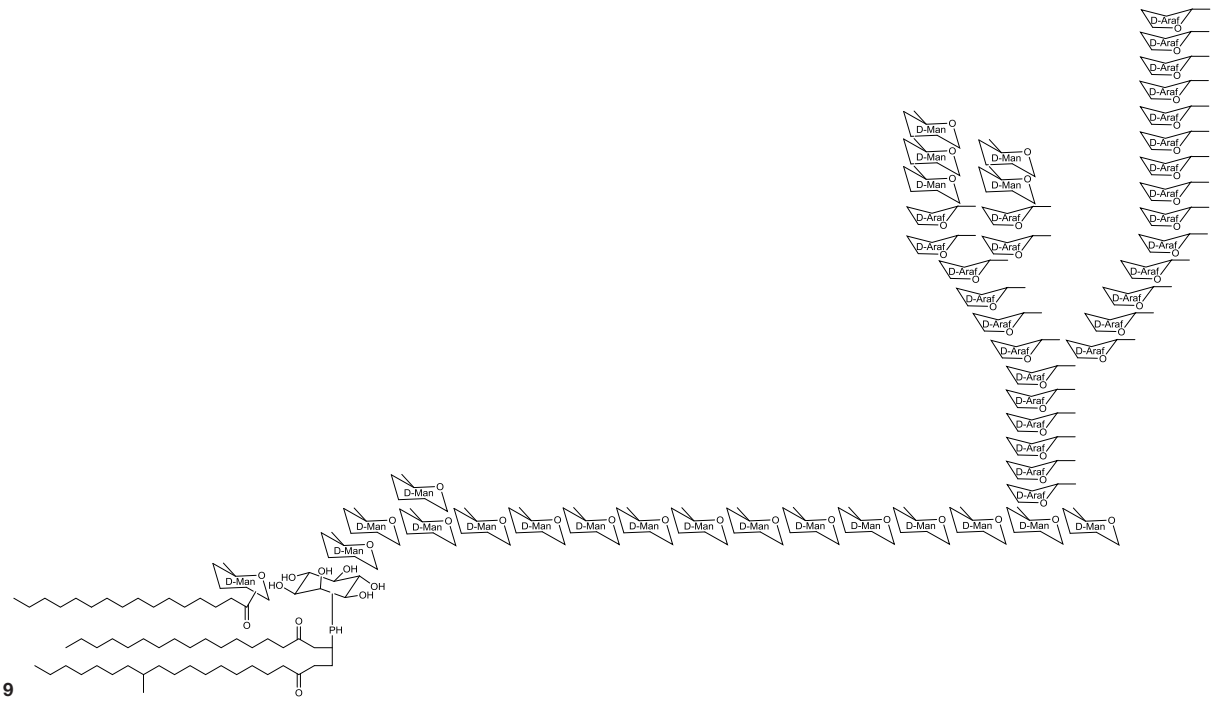
Cyclic lipopeptides that have been reported within the class *Actinobacteria* are the six amino acid containing cystargamide produced by *Kitasatospora cystarginea* (Gill et al., 2014) (**20**, **Table 9**), the thirteen amino acid containing daptomycin produced by *Streptomyces roseosporus* (Debono et al., 1987) (**21**, **Table 9**) and the depsipeptide ramoplanin, containing 16 amino acids, and which is produced by an *Actinoplanes* sp. (Ciabatti et al., 1989) (**22**, **Table 9**). All of them are cyclic due to an ester linkage between the carboxyl terminus and a hydroxyl group of either a threonine or hydroxyl-asparagine.

In cystargamide, the smallest cyclic lipopeptide, an uncommon 2,3 epoxy fatty acid chain (C10) is linked to the threonine amine. Besides proteinogenic amino acids, cystargamide further contains rare 5′-hydroxy-trypthophan and 4′-hydroxyphenylglycine (**20**, **Table 9**). No antimicrobial activity of cystargamide could be demonstrated (Gill et al., 2014).

An outstanding example of successful screening for a surfactant with bioactive properties are A21978C complexes, known as precursors of daptomycin. They were structurally elucidated in 1987 (Debono et al., 1987) and A21978C comprises thirteen different amino acids, 10 of them in the cyclic part of the structure and three in the extension of the hydrophobic tail (**21**, **Table 9**). Three different lipophilic tails are known, C10 ante*iso*, C11 *iso* branched and C12 ante*iso*. The most bioactive form of A21978C is daptomycin and has been generated by enzymatic deacylation of the mixture of lipophilic tails and chemical reacylation with a decanoyl fatty acid moiety. It was approved by the U.S. Food and Drug Association (FDA) in 2003 as the first antibiotic of its kind, and commercialized as cubicin®. It is active against various gram positive bacteria including the methicilin-resistant pathogen *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae* and vancomycin resistant enterococci (Miao

TABLE 8 | Polymeric glycolipids of actinobacterial origin.

Species	Strain	Polymeric glycolipid	References
Corynebacterium matruchotii	NCTC 10207	Lipoarabinomannan	Sutcliffe, 1995
Turicella otitidis	DSM 8821	Lipoarabinomannan	Gilleron et al., 2005
Dietzia maris	N1015	Lipoarabinomannan	Sutcliffe, 2000
Mycobacterium spp.* *(avium, bovis, chelonae, fortuitum, kansaii, leprae, smegmatis, tuberculosis, etc.)*	Various	Lipoarabinomannan and lipomannan	Reviewed in: Chatterjee and Khoo, 1998; Brennan, 2003; Nigou et al., 2003; Briken et al., 2004
Gordonia bronchialis	N654^T	Lipoarabinomannan, phosphatidylinositol mannoside	Garton and Sutcliffe, 2006
Gordonia rubripertincta	ATCC 25689	Lipoarabinomannan, phosphatidylinositol mannoside	Flaherty and Sutcliffe, 1999
Rhodococcus spp.* (*equi, rhodnii, ruber,* etc)	Various	Lipoarabinomannan	Reviewed in: Sutcliffe, 1997
Tsukamurella paurometabola	DSM 20162	Lipoarabinomannan	Gibson et al., 2004
Amycolatopsis sulphurea	DSM 46092	Lipoarabinomannan	Gibson et al., 2003
EXAMPLE OF LIPOARABINOMANNAN			



Simplified structure of lipoarabinomannan produced by *Mycobacterium tuberculosis* with only one arabinofuran branch shown. Modified from Berg et al. (2007)

*Several producing strains are reported.

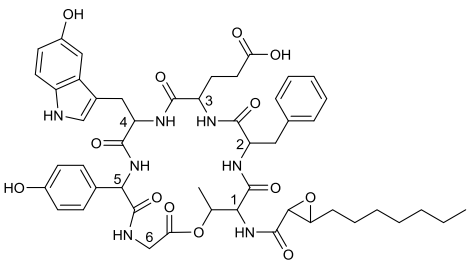
et al., 2005). Its ability to act as an antimicrobial requires the presence of calcium. The cyclic lipopeptide oligomerizes and uses its C10 hydrophobic tail to interact with the bacterial membrane creating a membrane perforation and cell death. This displays a novel mode of action among antimicrobial agents. Daptomycin shows high activity and a resistance to its mechanism is more difficult to generate compared to conventional antibiotics (Vilhena and Bettencourt, 2012). It is produced by a non-ribosomal peptide synthetase (NRPS) in *S. roseosporus*. The NRPS contains three subunits whose main genes have recently been identified in a 128 kb cluster as *dpt*A, *dpt*BC, and *dpt*D (Miao et al., 2005) with several other genes necessary to synthesize an active form of daptomycin. Its production yield of approximately 0.5 g l^{-1}, is relatively low compared to industrial production of other microbial products. Current attempts for a heterologous production not only target novel congeners of daptomycin but also the search for high producing strains. Similar production yields compared to the wild type strain have been reported for heterologous production which was developed using a combination of metabolic flux analysis and genetic modifications (Huang et al., 2012).

Antimicrobial activity against gram positive bacteria has also been detected for ramoplanin produced by an *Actinoplanes* sp. It contains 17 amino acids, 16 of which are part of the cyclic section of the compound. It is further glycosylated at a hydroxyphenylglycine with either di-mannose (Ciabatti et al., 1989) or mannose (Gastaldo et al., 1992), thus its classification as a glycolipopeptide. Besides its glycosylation pattern, members of ramoplanin can be differentiated by their acyl amides that consist of different di-unsaturated fatty acids linked to the distal hydroxyl-asparagine. The fatty acid chain varies in

TABLE 9 | Lipopeptides produced by actinobacterial strains.

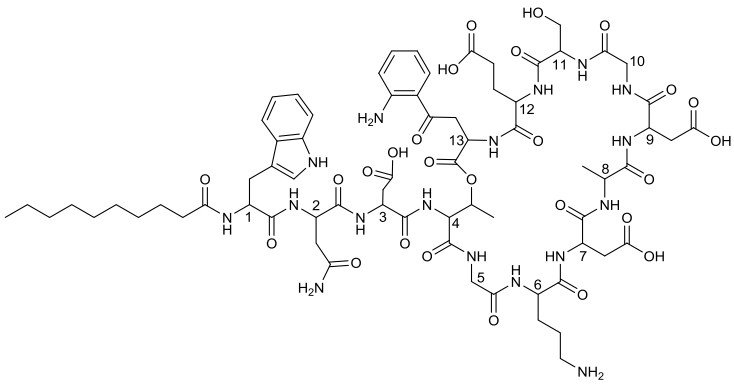
Species	Strain	Lipopeptide	References
Actinoplanes sp.	ATCC 33076	Ramoplanin (glycosylated 17aa, C8–C10)	Ciabatti et al., 1989; Gastaldo et al., 1992
Kitasatospora cystarginea	NRRL-B16505	Cystargamide (6aa, 2′-3′epoxy-C10)	Gill et al., 2014
Rhodococcus sp.	MCCC 1A00197	rhodocfactin	Peng et al., 2008
Streptomyces roseosporus	NRRL 11379	A21978C (daptomycin) (13aa, C10–12)	Debono et al., 1987
Streptomyces tendae	Tü 901/8c	Streptofactin	Richter et al., 1998
Streptosporangium amethystogenes subsp. *fukuiense*	AL-23456	TAN-1511 A, B, C	Takizawa et al., 1995

EXAMPLES OF LIPOPEPTIDES



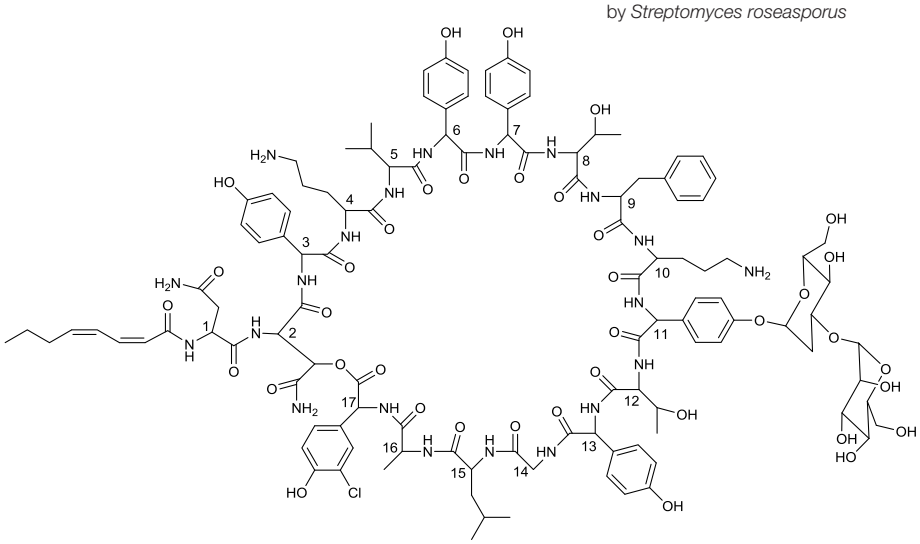
20

Cystargamide produced by *Kitasatospora cystarginea*



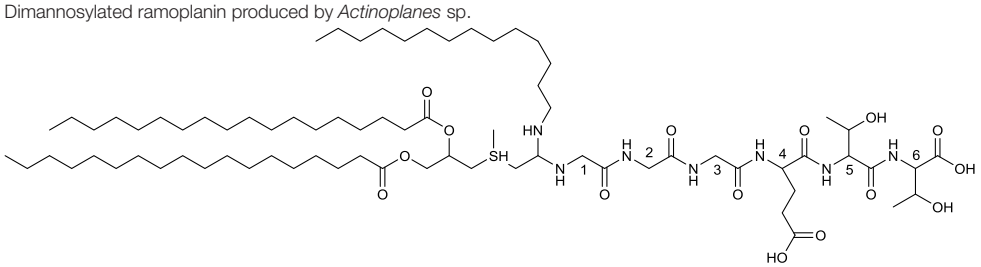
21

Daptomycin reacylated with decanoic acid from the core complex A21978C, produced by *Streptomyces roseasporus*



22

Dimannosylated ramoplanin produced by *Actinoplanes* sp.



23

Linear TAN-1511 A produced by *Streptosporangium amethystogenes* subsp. *fukuiense*

aa, amino acid.

length between C8 and terminal branched C9 and C10 (**22, Table 9**).

A peptide-based surfactant produced by *Streptomyces tendae*, streptofactin, was found to contain hydrophobic amino acids, but lacked fatty acid chains (Richter et al., 1998).

Linear Lipopeptides

Linear lipopeptides have been found in *Streptosporangium amethystogenes* (Takizawa et al., 1995). They are reported to protect against infections in patients with leucopenia caused by cancer therapies by stimulating bone marrow cells. Different structures of these compounds are described, all share a 4′-thio C7 fatty acid chain with two ester linked C16–C19 fatty acid chains and one amide linked C13–C15 fatty acid chain. Three glycine amino acids are linked at the amide bond of the thio fatty acid with three to four proceeding amino acids varying in type (**23**, **Table 9**).

Other Actinobacterial Biosurfactants

Phenazine Ester

Phenazines are a rare class of alkaloid esters. A marine *Streptomyces* sp. has been described to produce a phenazine ester that contain the desoxy pyranose quinovose esterified at either C3 or C4 to the carboxyl end of the phenazine. This phenazine-quinovose ester has been shown to exhibit antimicrobial activity. Several different types of the compound have been characterized also varying in hydroxylation and acetylation pattern at the desoxyglucose unit (Pathirana et al., 1992) (**24**, **Table 10**).

Amide Glycosides

Various surfactants with nucleoside fatty amide glycoside structure are produced by actinobacteria. A group of amide glucosides is based on the uracil and disaccharide-containing tunicamycin, a glycoprotein with antibacterial properties (Dembitsky, 2005c). In this glycoprotein, two saturated or unsaturated partly branched fatty acid chains varying in length are linked via an amide to the galactosamine/glucosamine disaccharide. Besides tunicamycin, produced by *Streptomyces* spp., the tunicamycin-based surfactants streptovirudin (containing dihydrouracil) and corynetoxin (**25**, **Table 10**) have been reported. The latter is produced by *Corynebacterium rathayi*, a pathogen of rye grass. The organism multiplies within the galls of sheep spreading the toxic metabolite (Frahn et al., 1984). In addition, the inhibitors of bacterial peptidoglycan synthesis, liposidomycin A, B, and C, have been reported to be produced by *Streptomyces griseosporus*. Liposidomycin A contains the so far uniquely described fatty acid composition of 3′-hydroxy-7,10-hexadecanoic acid (Dembitsky, 2005c) (**26**, **Table 10**).

Not Yet Elucidated Surfactants and their Producing Strains

Surface or emulsifying activity has been observed to occur from secondary metabolites of other members of the class *Actinobacteria*. **Table 11** gives an overview of strains that are described to produce surface active compounds. Only some of the structures of these compounds have been partially elucidated.

Partly characterized surface active flocculants consisting of lipids, fatty acids and corynemycolic fatty acids of *Corynebacterium lepus* have been described by Cooper et al. (1979b). In addition, eleven different glycolipids that consist of hexoses and pentoses linked to diverse fatty acid moieties that vary in length of C10–C18 have also been described.

Besides *D. maris* (see glycolipid section), three other putative rhamnolipid-producing actinobacteria have been described. Vasileva-Tonkova and Gesheva (2005) and Gesheva et al. (2010) detected thin layer retention values equal to L-rhamnose after acid hydrolysis of a biosurfactant produced by a *Nocardioides* sp. and *Rhodococcus fascians*. The putative rhamnolipid was not further examined in terms of the hydrophilic moiety or fatty acid compositions. Christova et al. (2004) reported the production of rhamnolipid by *Renibacterium solmonarium* in comparison to commercial rhamnolipids in thin layer chromatography and infrared spectroscopy. The infrared spectra showed homologies to ester and carboxylic groups; thin layer chromatographic data were not shown in the study. In all cases the detection of rhamnolipids were putative and further structural analyses remains necessary for confirmation.

Other surface active compounds were only putatively classified based on the component analysis of the crude extract toward lipid, peptide and carbohydrate compositions. Based on this limited information, it was concluded that the production of either glycolipids or lipopeptides took place (**Table 11**).

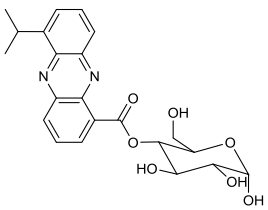
Mass spectroscopic analysis greatly assisted to partly characterize the putative wax esters produced by *D. maris* (Nakano et al., 2011). In addition, Kiran et al. (2010a,b, 2014) described the production of furan-containing glycolipids in *Brachybacterium* spp., *Brevibacterium* spp., and *Nocardiopsis* spp. By analyzing hydrophilic and hydrophobic moieties after acid hydrolization, database comparison of gas chromatography-mass spectroscopic plots were used. ^{1}H NMR evaluation of compounds from the two latter strains were described to approve the resulting structure, however relative data were not shown.

Similar results have been observed for surface active extracts with a majority of peptidic compounds in the hydrophilic part in *Brevibacterium aurum* (Kiran et al., 2010c) where fractions of the biosurfactant showed molecular weights of C9–C29 methyl esters and a mass that putatively confers to a proline-leucine-glycine-glycine amino acid chain. However, mass spectroscopic database comparisons remains putative. *Leucobacter komagate* is described to produce surfactin or a surfactin-like lipopeptide. This was concluded from mass spectroscopy, ^{1}H NMR and infrared spectral data by Saimmai et al. (2012b), but the full elucidation of the structures could not be achieved.

The long list of non-elucidated actinobacterial surface active compounds underlines the extraordinary potential of finding novel biosurfactants in actinobacteria and displays the great need for structure elucidation to allow for a better understanding of the novelty and biodiversity of the compounds produced.

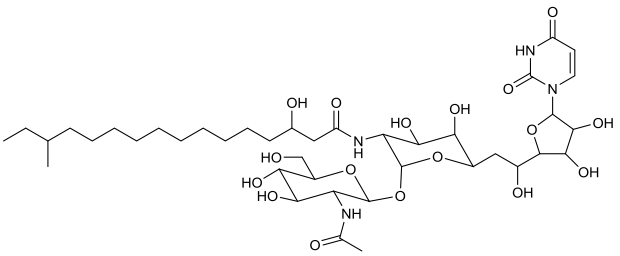
TABLE 10 | Other biosurfactants produced by actinobacteria.

Species	Strain	Compound	References
Streptomyces sp.	CNB-253	Phenazine-quinovose	Pathirana et al., 1992
Streptomyces spp.* (*griseoflavus, griseosporus, halstedii, lysosuperficus, nursei, vinausdrappus*)	various	Fatty acid amide glycoside (Tunicamycin, Streptovirudin, Liposidomycins)	Reviewed in: Dembitsky, 2005c
Corynebacterium rathayi	n.a.	Corynetoxin	Frahn et al., 1984
EXAMPLES			



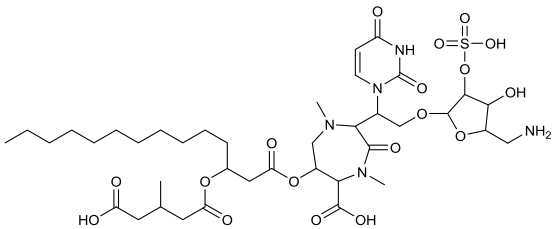
24

Phenanzine-quinovose ester produced by *Streptomyces* sp.



25

Corynetoxin produced by *Corynebacterium rathayi*



26

Liposidomycin A produced by *Streptomyces* sp.

*Several producing strains are reported.

Structural Elucidations of Actinobacterial Surfactants

Various factors have been shown to influence the production, extraction, purification and structure elucidation of novel biosurfactants produced by actinobacterial strains. Due to their phenotypic growth characteristics, distinct membrane compositions and their function within the utilization of hydrocarbons, the surfactants produced are often membrane integrated, membrane associated, extracellular or a mixture of the above, and is always dependent on their particular function within the producing strains. Commonly the compounds produced exhibit antimicrobial properties, on the one hand proposing wide ranging applications, on the other resulting in opposing challenges during the production process. Special considerations are necessary when aiming for the extraction of the compound in an adequate amount and purity for structural elucidation as well as surfactant characterization. This section gives an overview of the most common techniques used to achieve successful structural elucidations.

Detection

Novel surfactant producing strains can be detected through the use of screening assays that determine a surfactant's activity either from liquid culture (cell-free supernatant or culture broth) or from solid agar plates. Various detection methods have been described, but they mostly focus on changes observed in surface tension or the solubilization and emulsification of hydrocarbons. High throughput compatible assays can be distinct from more precise assays that need several milliliters of the compound to be tested. The latter often are also applied to characterize the activity of a purified biosurfactant. Good reviews on screening techniques have been summarized by Walter et al. (2010) and Satpute et al. (2010).

Production

The manufacturing capacity of biosurfactants by a bacterial culture is limited. Wild type producing strains of the best described microbial surfactants, cultured with optimized process methods in suitable media and culture vessels reach production quantities of up to 422 g l^{-1} for sophorose lipids (Daniel et al., 1998), 112 g l^{-1} for rhamnose lipids (Giani et al., 1996), 110 g l^{-1} for spiculisporic acids (Tabuchi et al., 1977), 106 g l^{-1} for mannosylerythritol lipids (Morita et al., 2008) and 3,6 g l^{-1} for surfactin (Yeh et al., 2005). These are rare exceptions within the typical amounts produced by microorganisms, which usually do not exceed milligram amounts. The production level is strongly influenced by non-favorable growth and production conditions due to a lack

TABLE 11 | Actinobacterial strains identified to produce surface active compounds for which no structures have been elucidated.

Species	Strain	Compound	References
Actinopolyspora sp.	A18	n.d. GLP	Doshi et al., 2010
Amycolatopsis tucumanensis	DSM 45259	n.d. (bioemulsifier)	Colin et al., 2013
Brachybacterium paraconglomeratum	MSA21	n.d. GL (putative furan lipid/C12)	Kiran et al., 2014
Brevibacterium aureum	MSA13	n.d. LP (putative brevifactin/C18)	Kiran et al., 2010c
Brevibacterium casei	MSA19	n.d. GL (putative furan lipid/C18)	Kiran et al., 2010a
Corynebacterium hydrocarboclastus	n.a.	n.d. polymer	Zajic et al., 1997
Corynebacterium lepus	n.a.	n.d. LP	Cooper et al., 1979a
Corynebacterium lepus	n.a.	n.d. GL	Cooper et al., 1979a
Corynebacterium lepus	n.a.	p.d. (lipid, fatty acid, mycolic acid)	Cooper et al., 1979b
Corynebacterium xerosis	n.a.	n.d. LP	Margaritis et al., 1979
Dietzia maris	WR-3	p.d. (putative wax-ester)	Nakano et al., 2011
Dietzia sp.	S-JS-1	n.d. LP	Liu et al., 2009
Frankia sp.	CpI1	n.d. GL	Tunlid et al., 1989
Gordonia amarae	SC1	n.d. (extracellular with high molecular weight)	Iwahori et al., 2001
Gordonia rubripertincta	DSM 46038	n.d.	Pizzul et al., 2006
Gordonia sp.	ADP	n.d.	Pizzul et al., 2006
Gordonia sp.	BS29	n.d. GL	Franzetti et al., 2010
Gordonia sp.	JE-1058	n.d. (extracellular)	Saeki et al., 2009
Kocuria marina	BS-15	n.d. LP	Sarafin et al., 2014
Leucobacter komagatae	183	p.d. LP	Saimmai et al., 2012b
Microlunatus sp.	NA2	n.d.	Saimmai et al., 2012a
Nocardia erythropolis	ATCC 4277	n.d. GL, PL	Macdonald et al., 1981
Nocardioides sp.	A-8	n.d. GL (putative Rhamnolipid)	Vasileva-Tonkova and Gesheva, 2005
Nocardiopsis alba	MSA10	n.d. LP	Gandhimathi et al., 2009
Nocardiopsis lucentensis	MSA04	n.d. GL (putative furan lipid/C9)	Kiran et al., 2010b
Oerskovia xanthineolytica	CIP 104849	p.d. GL (hexose, pentose C10–C18)	Arino et al., 1998
Pseudonocardia sp.	BSNC30C	n.d.	Ruggeri et al., 2009
Renibacterium salmoninarum	27BN	n.d. GL (putative Rhamnolipid)	Christova et al., 2004
Rhodococcus fascians	A-3	n.d. GL (putative Rhamnolipid)	Gesheva et al., 2010
Streptomyces sp.	n.a.	n.d. GL	Khopade et al., 2011

GL, Glycolipid; GLP, Glycolipiopeptide; LP, Lipopeptide; PL, Phospholipid; n.a., information not available; n.d., not determined; p.d., partly determined.

of knowledge about the organism used and compound produced when initially screening for novel surfactants or novel producer strains.

With a few exceptions (Qian-Cutrone et al., 1999; Kügler et al., 2014), the average minimum volume for successful structure elucidation of an actinobacterial biosurfactant, is typically 20 l. Harvesting of the surfactants is type dependent and either whole cell broth (intracellular or membrane associated surfactants) or cell free supernatant is used as a starting point.

Glycolipids

A typical method for the extraction of surfactants from culture broth or supernatant is the use of two phase extractions. In a first step, if appropriate, non-polar solvents (e.g., n-hexane) are used to remove residual hydrocarbons from the cultivation broth. If extraction is carried out from whole cell broth or wet cell mass, glycolipids are either captured by direct cell extraction or by cell treatment (e.g., sonication) prior to the extraction.

In a second step, the surfactant is removed by repeated agitation with a medium polar solvent or solvent mixture. Most commonly, combinations of chloroform and methanol or polar aprotic solvents such as ethyl acetate or methyl-*tert*-butyl ether are used. A frequency solvent distribution for the extraction of glycolipids from "rare" actinobacteria is shown in **Figure 2**, comprising data of 47 two-phase extraction methods used to enrich surfactants produced from either cell-free supernatant or the culture broth. Depending on the chemical characteristics of the glycolipid, an acidification step (pH2–pH3) with subsequent incubation (4°C) prior to the extraction process could result in enhanced product recoveries (Passeri et al., 1990; Konishi et al., 2014). Often, after dehumification, further washing steps are applied, either of a hydrophilic (e.g., ultrapure water) or a hydrophobic (e.g., n-hexane) nature. For the polymeric glycolipid lipoarabinomannan and related structures, a hot-phenol water method is almost exclusively used (Sutcliffe, 2000).

The glycolipids produced, mainly present in mixtures of different forms, need to be separated for structural analysis.

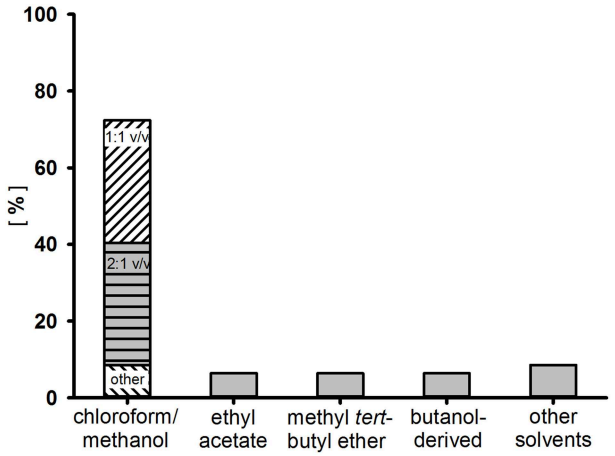


FIGURE 2 | Frequency distribution of solvents used for the enrichment of surfactants by two-phase extraction from the culture broth or cell free supernatant of 47 "rare" actinobacteria.

This procedure is usually performed by combinations of chromatographic steps using either gradient columns or preparative medium- and high pressure chromatography. In addition, preparative planar chromatographies are reported as an additional purification step for the isolation of pure compounds (Powalla et al., 1989; Pasciak et al., 2002, 2004). Rarely applied is the use of absorbers within the cultivation process. The number one choice for chromatography is the use of hydrophobicity affiliated separations with silicic acids as an absorbing material. In approximately 80% of structure reports from "rare" actinobacteria, silicic acid is used with various elution gradients of nonpolar and polar solvents. Separated compounds are often further purified by repetitive silica chromatography using different gradients or by subsequent (or preceding) steps with different column material. Therefore, either reverse-phase C18 chromatography or cellulose-based ionic interaction chromatography are widely used.

Lipopeptides

The diversity of different peptide-based surface active compounds produced by actinobacterial strains is much smaller than that of reported glycolipids. Depending on the lipopeptide produced, two different approaches for the concentration of the surfactants are used. Either the lipopeptide can be precipitated from the liquid culture/supernatant by either using cold acetone, methanol, salt concentrations, acidic environments, or a direct extraction by medium polar solvents similar to those used for glycolipids have been reported. Besides the chromatographic purification steps used for glycolipids, gel filtration has been successfully used as an additional step (Takizawa et al., 1995).

Structural Elucidation

Once a compound is purified to a sufficient extent, component analysis, specific staining methods and mass spectroscopic examinations are widely used to get a first hint about the type of surfactant produced. A more detailed schematic of the surfactant can be deduced from mass spectroscopy fragmentation studies, often revealing mass abundances of separated hydrophilic and hydrophobic parts of the glycolipid. However, complete structure examinations (of complete compounds or hydrolyzed components) rely on multi-dimensional nuclear magnetic resonance spectroscopy.

Natural Habitats of Biosurfactant-Producing Actinobacteria

With the exception of a few strains, the great majority of surfactant-producing actinobacteria have been isolated from three different environments. These are: (1) Hydrocarbon contaminated soils, (2) infections caused by the actinobacterium itself, and (3) marine-derived samples. Obviously, this must not reflect the distribution of surfactant-producing actinobacteria in nature, but it is clear that there is a link between the type of environment and the ability of actinobacteria to produce biosurfactants and can be considered to be environmentally-driven.

Hydrocarbon Contaminated Soil

The formation of various actinobacterial surfactants is mainly observed during growth in a range of different hydrophobic carbon sources such as n-paraffin, n-hexadecane or vegetable oils. Occurrences of surfactant-producing microorganisms seems to correlate to environments in which hydrophobic carbon sources are present, no matter if these are oil contaminated or oil enriched (Powalla et al., 1989; Arino et al., 1998; Christova et al., 2004, 2014; Pizzul et al., 2006; Liu et al., 2009; Ruggeri et al., 2009). Evoked by their hydrophobic cell wall due to incorporation and association of various lipoglycosides, actinobacteria preferably grow in hydrophobic droplets that are dispersed in the aqueous phase when cultured in cultivation devices. The surfactants produced facilitate the uptake of these difficult–to-access carbon sources by dispersing it into small droplets that can easily be pre-digested by extracellular enzymes.

Infections

A second feature of surfactants is the antimicrobial property exhibited by most of these compounds. Endowed with nutritional and growth advantages toward surrounding organisms, surfactant producers can become rampant, and are often less affected by substances present during its growth, e.g., antimicrobial drugs. They have been found in patients that suffer from infections/diseases caused by human deficiency viruses (Guérardel et al., 2003), patients with lung infections and infections of the oral cavity (Datta and Takayama, 1993; Sutcliffe, 1995; Tanaka et al., 1997). In addition, biosurfactant-producing actinobacterial strains have also been isolated from infected plant tissue (Frahn et al., 1984).

Marine Habitat

Many actinobacteria are specialists in survival and native to a wide range of extreme environments. Surfactant-producing genera have been isolated from various marine-associated habitats (Passeri et al., 1990; Khopade et al., 2011; Nakano et al., 2011).

Several of these environments exhibit rather extreme conditions, amongst which are deep sea sediments or hydrothermal fields (Peng et al., 2008; Konishi et al., 2014; Wang et al., 2014), ornithogenic exposed soil (Vasileva-Tonkova and Gesheva, 2005) as well as actinobacteria isolated from sponges (Gandhimathi et al., 2009; Kiran et al., 2010a,b,c, 2014) and hard corals (Osawa et al., 2010). An antimicrobial effect of surfactants produced in a highly procaryotic populated sponge tissue is apparent. However, the reason for the frequent occurrence of surfactant producers within the other marine habitats, still remains to be understood.

Summary and Conclusion

A wide range of unique and diverse surfactants produced by actinobacteria have been reported. Various glycolipids, lipopeptides and other surfactant types are produced by numerous species, all belonging to the order *Actinomycetales*. Taking into account the fact that only a minority of actinobacteria is culturable and the given list of surfactant producing strains without structurally elucidated compounds (**Table 11**), the sheer magnitude of actinobacterial surfactants that still remain undetermined is evident. The ability of actinobacteria to produce biosurfactants seems to be influenced by their natural habitat. From the three main sources of surfactant producing actinobacteria it can be concluded that the compounds produced mainly serve for either gaining access to hydrophobic carbon sources or as a bioactive agent against competing strains.

In order to pave the way toward biotechnological applications of actinobacterial surfactants, emphasis should be placed on (1) structural elucidation of described, but not identified biosurfactants, (2) the identification of novel actinobacterial surfactants by the implementation of next generation screening methods; (3) the production of sufficient amounts of surfactants for application based studies; and (4) production processes that result in high yields and that would cut down on the production costs.

(1) Actinobacterial strains with a surface active culture broth or supernatant often are declared as "novel" biosurfactant producing strains, without elucidation of the surface active compound(s) produced and a list of producing strains is given in this article whose surfactant structures remain to be identified (**Table 11**). For a successful structural identification of the compound, sufficient quantities of the isolated surface active compound at an adequate purity is necessary in order to apply the various analytical methods necessary. This aspect was reviewed in the structural elucidation of actinobacterial compounds section. Quite a few of the studies cited lacked sufficient strain information and further research can only be ensured if the strains reported have designated strain numbers and thus are available for other researchers to pursue the production of these potentially novel biosurfactants.

(2) Approaches for the identification of novel biosurfactants mainly remain traditional by the detection of interesting producing strains and subsequent isolation and characterization of the compound produced. To further expand the variety of actinobaterial surfactants, alternative screening methodologies that are already known to be used for the detection of novel lead molecules in the pharmaceutical industry could be applied. Genome-based information technology to reveal pathways that can be implemented into artificial surfactant synthesis cascades are currently being investigated. These attempts would allow for access to both undetected and cryptic pathways present in actinobacteria. By direct sequencing of metagenomic derived DNA, enzyme information acquired could be expanded to information gained from non-culturable and slow growing species.

(3) Many of the surface active compounds produced by actinobacteria potentially show interesting properties as biotechnological products or additives. Often, as is the case for many of the compounds summarized in this article, an application based study is lacking. This is most probably due to low availability of the product and can be traced back to the use of low quantity producing strains. Focus on a novel actinobacterial surfactant, along with progress in the development toward novel biotechnology-based products, will only be made possible if enough substance for initial studies on bioactivity or other interesting applications can be acquired. If an adequate amount of substance is not achievable by standard bioprocess engineering attempts, metabolomic approaches and flux analysis could lead the way. Furthermore, the identification of enzymes involved in the synthesis and their genetic regulation can give an important input into the improvement of fermentation processes. An implementation of the surfactant's synthesis through adequate heterologous production strains could lead to higher quantities of the different surfactants produced. Potential applications of a novel compound is a guarantee of success in white biotechnology and negates the efforts made with regards to its production, purification and elucidation.

(4) Currently, comparatively high production costs combined with low production yields restrict the development of compounds as valuable products, and are mainly limited to high purity applications, e.g., the drug industry. Several examples in the past have shown that once a potential application for a specific compound is foreseen, intensive research is set in motion to facilitate production and purification processes, cutting costs, enhancing yields and, although research often lasts for decades, compounds might end in industrial scale production and application.

One example of an actinobacterial surfactant that successfully underwent the process from detection to application is the antimicrobial agent daptomycin. It was initially produced semi-synthetically in a three step procedure, but later a direct synthesis of daptomycin was achieved by feeding toxic decanoic acid to a carbon-limited production culture (Huber et al., 1988). Production rates were further increased by 10–30% by using a mixture of less toxic decanal and a solvent to solubilize the hydrophobic

carbon feed (Bertetti et al., 2012). Mutagenesis approaches (Yu et al., 2011; Li et al., 2013), genome shuffling (Yu et al., 2014) and directed overexpression (Huang et al., 2012), have recently led to further increases in production yields. Other examples of success stories, are non-actinobacterial surfactants that have been pushed to application: sophorolipids, mannosyl erythritol lipids and the lipopeptide surfactin have found application in cosmetic industries (Fracchia et al., 2014). Sophorolipids are even applied in low cost cleaning products.

Actinobacteria clearly represents a unique and vast untapped resource for the discovery of novel and potentially useful biosurfactants. The surfactants produced by members of the class *Actinobacteria* are a highly interesting group of products that could be of great importance in the future in both the area of basic research and application-oriented industrial research.

Author Contributions

JK has designed, conceived, and written this review, it's figures and tables as well as acquired and interpreted the relevant data used. All authors have fruitfully discussed content and structure of the review. In particular, ML has given substantial contributions related to actinobacteria and CS and RH have given substantial contributions related to biosurfactants.

Acknowledgments

We acknowledge support by Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of Karlsruhe Institute of Technology, Germany.

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Conflict of Interest Statement: 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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 17 April 2015
doi: 10.3389/fmicb.2015.00292

The anionic biosurfactant rhamnolipid does not denature industrial enzymes

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Biosurfactants (BS) are surface-active molecules produced by microorganisms. Their combination of useful properties and sustainable production make them promising industrial alternatives to petrochemical and oleochemical surfactants. Here we compare the impact of the anionic BS rhamnolipid (RL) and the conventional/synthetic anionic surfactant sodium dodecyl sulfate (SDS) on the structure and stability of three different commercially used enzymes, namely the cellulase Carezyme® (CZ), the phospholipase Lecitase Ultra® (LT) and the α-amylase Stainzyme® (SZ). Our data reveal a fundamental difference in their mode of interaction. SDS shows great diversity of interaction toward the different enzymes. It efficiently unfolds both LT and CZ, but LT is unfolded by SDS through formation of SDS clusters on the enzyme well below the cmc, while CZ is only unfolded by bulk micelles and on average binds significantly less SDS than LT. SDS binds with even lower stoichiometry to SZ and leads to an increase in thermal stability. In contrast, RL does not affect the tertiary or secondary structure of any enzyme at room temperature, has little impact on thermal stability and only binds detectably (but at low stoichiometries) to SZ. Furthermore, all enzymes maintain activity at both monomeric and micellar concentrations of RL. We conclude that RL, despite its anionic charge, is a surfactant that does not compromise the structural integrity of industrially relevant enzymes. This makes RL a promising alternative to current synthetic anionic surfactants in a wide range of commercial applications.

Keywords: biosurfactant, detergent, enzyme, rhamnolipid, SDS, surfactant

OPEN ACCESS

Edited by:
Pattanathu K. S. M. Rahman, Teesside University/TeeGene Biotech, USA

Reviewed by:
Rudolf Hausmann, University of Hohenheim, Germany
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Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *27 January 2015*
Accepted: *24 March 2015*
Published: *17 April 2015*

Citation:
Madsen JK, Pihl R, Møller AH, Madsen AT, Otzen DE and Andersen KK (2015) The anionic biosurfactant rhamnolipid does not denature industrial enzymes. Front. Microbiol. 6:292. doi: 10.3389/fmicb.2015.00292

Introduction

Washing detergents are complex formulations that among many other components include surfactants and enzymes. Both surfactants and enzymes have key roles in the cleaning process. Surfactants are surface active agents that serve several roles, including reduction of surface tension, solubilization of stains and preventing redeposit. Enzymes catalyze the breakdown of difficult stains but can also act directly on fabric. The addition of enzymes to detergent formulation has made it possible to reduce washing temperatures dramatically and thereby reduce energy costs. During washing processes (and during storage in liquid detergent formulations), surfactants not only

Abbreviations: BS, Biosurfactants; CD, circular dichroism; cmc, critical micelle concentration; CZ, Carezyme; ITC, isothermal titration calorimetry; IP, inflection point; LT, Lecitase; RL, rhamnolipid; SDS, sodium dodecyl sulfate; SZ, Stainzyme.

interact with stains but also with detergent enzymes. Such interaction can lead to enzyme denaturation and inactivation, which can impair washing performance. Not all enzymes are however denatured and inactivated by anionic surfactants. E.g., enzymes such as papain and pepsin (Nelson, 1971), glucose oxidase (Jones et al., 1982a) and bacterial catalase (Jones et al., 1982b) can maintain enzyme activity in the presence of anionic surfactants such as SDS. Some enzymes are even activated by surfactants as seen for lipases (Martinelle et al., 1995; Mogensen et al., 2005). In general the interactions between proteins and surfactants are many-faceted and depend on protein structure, protein surface potential, surfactant structure and charge (Otzen, 2011). Nevertheless, for optimal performance, detergents need to be formulated to maintain enzyme activity during washing.

The use of surfactants in detergents is currently dominated by surfactants produced from either non-renewable petrochemical resources or renewable plant-based resources. They are manufactured by complex chemical processes such as distillation, fractionation and hydrogenation, and are therefore considered synthetic. Continued use is however restricted by toxicity, low biodegradability, allergenicity, poor skin compatibility and strict pollution/health regulations (Lima et al., 2011). Focus is shifting to green alternatives based on sustainable production from renewable resources. Of particular interest are the so-called second generation biosurfactants (BS), i.e., surface-active compounds produced mainly by microorganisms. BS show low or no toxicity, high biodegradability and excellent surface activity at extreme pH and temperature (Edwards et al., 2003; Patel, 2003). Substitution of chemical surfactants with BS can give a 37% reduction in life-cycle CO_2 emission, corresponding to 0.02–0.09% of total CO_2 emission (Patel, 2003). Unlike the first generation BS produced by chemical synthesis from different sugars and lipids, second generation BS are economically increasingly attractive alternatives, in terms of cost-to-performance ratio, due to rapidly decreasing BS production costs based on production from renewable resources (Daniel et al., 1998; Daverey and Pakshirajan, 2010), yields up to 400 g/L (Franzetti et al., 2010) and rising oil prices.

One of the most promising BS is the glycolipid biosurfactant rhamnolipid (RL) from *Pseudomonas aeruginosa*. It is relevant to compare its protein interactions with that of SDS, an intensely studied model surfactant that is known to have high protein denaturation potency because of its strong binding affinity and highly charged sulfate head group (Otzen, 2011). This makes it useful in applications such as SDS-PAGE. While both SDS and RL are anionic, there are large differences in the molecular structure. SDS has a molecular weight of 265 Da (without Na counter ions) and a volume of 331.3 $Å^3$ (Smith et al., 2000). In contrast mono-rhamnolipid (RL1) and di-rhamnolipid (RL2) have molecular weights of 504 and 605 Da, and volumes of 813 and 1052 $Å^3$ (Chen et al., 2010), respectively. Furthermore, the anionic group of SDS is a sulfate group while that of RL is a carboxylate.

Few studies have addressed the interactions of RL with proteins. BSA has been found to bind 1–2 RL molecules which lead to increased thermal stability (Sanchez et al., 2008). At concentrations above the critical micelle concentration (cmc), RL can stabilize and facilitate folding of outer membrane proteins (Andersen and Otzen, 2014a). RL denatures both α-lactalbumin (αLA) and bovine myoglobin (Mb) (Andersen and Otzen, 2014b) but is also claimed to stabilize xylanase and—to a smaller extent—cellulases (Liu et al., 2011).

Here we systematically compare SDS and RL in their interactions with three commercial enzymes namely the cellulase Carezyme® (CZ), the phospholipase Lecitase Ultra® (LT) and the α-amylase Stainzyme® (SZ). Both CZ and SZ are commonly used in detergents. We include LT to expand the collection of industrially relevant enzymes. LT is currently not used in detergents as such, but rather for vegetable oil degumming, egg-yolk modification and lecithin hydrolysis (Bojsen et al., 2000). However, Lecitases substrates are amphiphilic just like surfactant molecules. Furthermore, during hydrolysis of substrate, LT produces and interacts with anionic free fatty acids and lysolecithin, thus making it relevant to study how LT interacts with anionic molecules and surfactants.

The 37 kDa CZ consists of a catalytic 218-residue active core (CAD) and a 38-residue cellulose binding domain (CBD). The two domains are connected by a 33 aa linker region containing 22 O-glycosylated serines and threonines and a number of prolines (Schülein, 1997), which provides great flexibility between the CAD and the CBD. While crystallization of the full enzyme has not been successful, the structure of the CAD of endoglucanase V has been solved to reveal 7 barrel-forming β-sheets and 3 α-helices, as well as a groove with two catalytically active Asp residues (Davies et al., 1993). 339-residue LT is a hybrid enzyme with aa 1–284 from the *Thermomyces lanuginosus* lipase gene and aa 285–339 from the structurally homologous *Fusarium oxysporum* phospholipase gene (Wang et al., 2011). This has led to an enzyme with the high stability of the *Thermomyces Lanuginosus* enzyme and the high activity of the *Fusarium oxysporum* enzyme (Wang et al., 2011). The α-amylase SZ originates from a *Bacillus* species.

Using spectroscopic and calorimetric approaches we show that all three enzymes interact with SDS. LT is denatured by SDS monomers, CZ is only denatured by SDS micelles, and SZ is thermally stabilized by SDS well below the cmc. However, none of the enzymes are denatured by RL and all enzymes maintain activity in the presence of both monomeric and micellar concentrations of RL. Weak interaction between RL and the enzymes LT and CZ lead to a slight thermal destabilization while SZ is thermally stabilized. This makes RL highly compatible with industrial enzymes and promising substitutions for chemical surfactants in a wide range of commercial applications.

Materials and Methods

Materials

Tris was from AppliChem (Darmstadt, Germany), and sodium dodecyl sulfate (SDS), 4-nitrophenyl-α-D-maltohexaoside and 4-nitrophenyl butyrate was from Sigma-Aldrich (St. Louis, MO, USA). Azo-CM-Cellulose were from Megazyme International (Ireland). JBR515 rhamnolipid (RL) was provided by Jeneil Biosurfactant Company (Saukville, WI, USA) as a liquid solution consisting of 15% RL of the highest grade. JBR515 is a 1:0.35 mixture of mono-rhamnolipid (RL1) and di-rhamnolipid

(RL2) with molecular weights of 504 and 650 Da, respectively. Stainzyme® plus 12 L, Carezyme® and Lecitase Ultra® were generously provided by Novozymes A/S (Bagsvaerd, Denmark) as liquid formulations. The enzymes were extensively dialyzed before experiments. LT was dialyzed against MilliQ water and SZ against 50 mM Tris pH 8. CZ required additional purification and was purified by ion-exchange chromatography on HiTrap Q sepharose FF 5 mL column (GE Healthcare, Pittsburgh, PA, USA). CZ was added to the column in 20 mM Tris pH 8.0 and washed in 20 mM Tris pH 8.0 until UV_{280} stabilized. Elution was achieved with a gradient from 0 to 500 mM NaCl in 20 mM Tris pH 8.0. Fractions with CZ were pooled and extensively dialyzed against 50 mM Tris pH 8.0. The following extinction coefficients (ε_{280}) were used to determine enzyme concentration: SZ: 154.050 M^{-1} cm^{-1} (provided by Novozymes); CZ: 61.300 M^{-1} cm^{-1} and LT: 56.830 M^{-1} cm^{-1} (ε_{280} for CZ and LT calculated from the sequence).

Determination of the Critical Micelle Concentration and Hemi Micelles by Pyrene Fluorescence

The cmc of SDS and RL in buffer was determined by pyrene fluorescence as described in Andersen and Otzen (2009). Pyrene's fluorescence is sensitive to the environment and the ratio between the intensities of two emission peaks at 372.5 (I_1) and 383.5 nm (I_3) changes as pyrene partitions into surfactant micelles, making I_1/I_3 a good probe for the polarity of pyrene's environment (Kalyanasundaram and Thomas, 1977). Briefly, different concentrations of surfactant in buffer were prepared. After equilibration for 30 min, pyrene was added from a 100 μM stock in ethanol to a final concentration of 1 μM. Fluorescence scans were performed on a LS-55 luminescence spectrometer (Perkin-Elmer Instruments, UK), using an excitation wavelength of 335 nm, emission from 360 to 410 nm and excitation/emission slits of 5/2.5 nm. Possible complexes formed between surfactants and enzymes at concentration below the cmc were investigated by incubation of 2 μM enzyme with different concentrations of SDS or RL for 60 min before pyrene addition.

Circular Dichroism

Spectra were recorded on a JASCO J-810 spectropolarimeter (Jasco Spectroscopic Co. Ltd., Japan) equipped with a Jasco PTC-423S temperature control unit. Far-UV CD scans were recorded in the wavelength range 200–250 nm, with a bandwidth of 2 nm, a scanning speed of 50 nm/min and a response of 2 s. Measurements were conducted in a 0.1 mm quartz cuvette. Six accumulations were averaged and buffer background contributions were subtracted. Near-UV CD scans were recorded in the wavelength range 320–260 nm, with a bandwidth of 2 nm, a scanning speed of 50 nm/min and a response of 2 s. Measurements were conducted in a 1 cm quartz cuvette. Six accumulations were averaged and buffer background contributions were subtracted. Thermal scans were carried out by monitoring ellipticity at 222 nm using a temperature scan speed of 90°C/h and a data pitch collection of 0.1 nm. Measurements were conducted in a sealed 1 mm quartz cuvette. LT and SZ were measured at enzyme concentrations of 0.2 mg/mL, whereas CZ was measured at 0.4 mg/mL.

Determination of Enzyme Activity

CZ: The activity of CZ was determined using Azo-CM-Cellulose as substrate. 0.2 μM CZ was incubated with 0–10 mM surfactant in 50 mM Tris pH 8.0. 2% (w/v) unbuffered substrate was mixed 1:1 (v/v) with CZ samples and mixed thoroughly. After incubation for 20 min at room temperature, 2.5 x volume of a precipitation buffer was added (300 mM sodium acetate and 20 mM zinc acetate in 75% ethanol, pH 5). After incubation for 10 min, samples were centrifuged at 2500 g in a bench top centrifuge for 10 min. Absorbance of the released product in the supernatant was measured at 590 nm with a Varioscan Platereader (Thermo Scientific, USA). Activity was normalized to the activity of CZ in buffer.

LT: The activity of LT was determined using 4-nitrophenyl butyrate (pNPB) as substrate. 0.25 μM LT was incubated with 0–10 mM surfactant in 50 mM Tris pH 8. 25 mM pNPB in 96% ethanol was mixed 1:100 (v/v) with LT samples, after which absorbance was immediately followed for several minutes at 405 nm on a Shimadzu UV-1700 PharmaSpec UV-VIS Spectrophotometer (Shimadzu Corp., Japan). Activity was determined as the slope by linear regression and normalized to the activity of LT in buffer.

SZ: The activity of SZ was determined using 4-nitrophenyl-α-D-maltohexaoside (pNPM) as substrate. 2 μM SZ was incubated with 0–10 mM surfactant in 50 mM Tris pH 8. 10 mM pNPM in 50 mM Tris pH 8 was mixed 1:10 (v/v) with SZ samples and absorbance at 405 nm was followed for several minutes using a Varioscan Platereader (Thermo Scientific, USA). Activity was determined as the slope by linear regression and normalized to the activity of SZ in buffer.

Isothermal Titration Calorimetry

ITC measurements were conducted on a VP-ITC calorimeter (MicroCal, Inc., Northampton, MA). All experiments were carried out in 50 mM Tris pH 8 at 25°C, except for CZ-SDS where 23°C was used. Initial titration of CZ with SDS indicated slow denaturation kinetics around the cmc (data not shown). CZ titration parameters were therefore optimized with regards to temperature and spacing time between injections. A temperature of 23°C and a spacing of 900 s gave satisfactory and reproducible results. The reference cell was filled with water and the ITC parameters adjusted to optimize the different experiments to account for kinetics. CZ was investigated in a concentration range of 0–3 mg/mL, LT in a range of 0–4 mg/mL and SZ in a range of 0–4 mg/mL. SDS injections were performed with different conditions for optimized SDS concentration, spacing and temperature. SDS-LT experiments were performed with 100 mM SDS and a spacing of 600 s, SDS-CZ with 40 mM SDS at a spacing of 900 s and SDS-SZ with 4 mM SDS at a spacing of 1000 s. In RL experiments, parameters were varied to a lesser degree, all being performed with 25 mM RL injectant with a spacing of 300 s for both LT and SZ and 450 s for CZ. The heat signals were integrated using the Origin software supplied by MicroCal, Inc. To calculate the binding stoichiometry, enzyme dilution during ITC analysis was taken into account.

Results

The Enzymes Vary in Their Level of Interaction with SDS and RL below the cmc

Proteins may interact with both monomeric or micellar surfactant. We therefore determined the exact critical micelle concentration of SDS and RL in buffer using the hydrophobic probe pyrene whose fluorescence is sensitive to environmental factors.

The cmc of SDS is around 7 mM in water but reduces with increasing ionic strength (Jönsson et al., 1998). Incubation of pyrene with increasing concentrations of SDS reveals a systematic development in the I_3/I_1 fluorescence ratio with increasing SDS concentration: the ratio is stable around 0.6 between 0 and ∼2 mM SDS where after it increases to reach a plateau of 0.9 at ∼3 mM SDS (**Figure 1A**). This indicates that micelles are formed in solution around 2–3 mM SDS. The three enzymes all behaved in different ways in the pyrene model system. CZ did not change the titration pattern (**Figure 1A**), indicating that SDS does not form micellar structures on CZ below the cmc. In contrast, both LT and SZ lead to change in pyrene fluorescence below the cmc. For SZ the I_3/I_1 ratio increased from 0.6 to a plateau around 0.7 already at 0.25 mM SDS, indicating interactions at very low SDS concentrations. The ratio then merged with the protein-free SDS curve around the cmc. For LT the ratio was stable at 0.6 until ∼0.75 mM SDS where after the I_3/I_1 ratio steadily increased with increasing SDS concentration, but only merged with the protein-free sample at a ratio of ∼0.8, in the middle of the transition region.

RL has a much lower cmc than SDS. In buffer the I_3/I_1 ratio remains stable at 0.6 until 0.1 mM RL, after which it increases to ∼1.05 at 1 mM RL (**Figure 1B**). This concentration range is in good agreement with other studies which report cmc values of rhamnolipid mixtures between 0.1 and 1 mM (Sanchez et al., 2007; Chen et al., 2010). Neither CZ nor LT affected the pyrene fluorescence pattern, indicating that the enzymes do not aid RL micelle formation below the cmc. However, for SZ the I_3/I_1 ratio already rises abruptly from ∼0.05 mM RL, and only merges with the protein-free sample around 0.6 mM RL. This indicates that SZ interacts with monomeric RL, leading to micelle-like structures on the surface of SZ.

Investigation of Enzyme Secondary- and Tertiary Structure by far-UV and Near-UV CD

To investigate whether surfactant clustering on the enzymes below the cmc is accompanied by denaturation, we used far- and near-UV circular dichroism to analyze how the surfactants affected enzyme secondary and tertiary structure.

Far-UV CD spectra of CZ in buffer show a local minimum and maximum at 230 and 220 nm, respectively (**Figure 2A**). Titration with RL did not lead to any change in the spectra indicating that neither monomeric or micellar RL denature CZ. In contrast, super-cmc SDS concentrations led to large spectral change; plotting ellipticity at 220 nm as a function of SDS show that the change in secondary structure occurs around the cmc (**Figure 2B**). These conclusions were reinforced by near-UV CD spectra (**Figure 2C**). Titration of CZ with RL did not lead to any change in spectra while titration with SDS led to disappearance of the two local maxima at ∼285 and 295 nm around the cmc region (**Figure 2D**). Thus, CZ is only denatured by SDS micelles formed in the bulk phase.

In the case of LT, the enzyme preserves native tertiary and secondary structure in the presence of RL monomers and micelles, while SDS leads to large changes in both secondary and tertiary structure (**Figures 3A,C**). The change in both secondary and tertiary structure is induced already around 1 mM SDS and the transition is complete around 2 mM SDS (**Figures 3B,D**). This indicates that LT is denatured below the cmc and therefore by SDS monomers.

Titrations of SZ with RL or SDS did not lead to any changes in either far-UV or near-UV CD spectra (**Figure 4**). This indicates

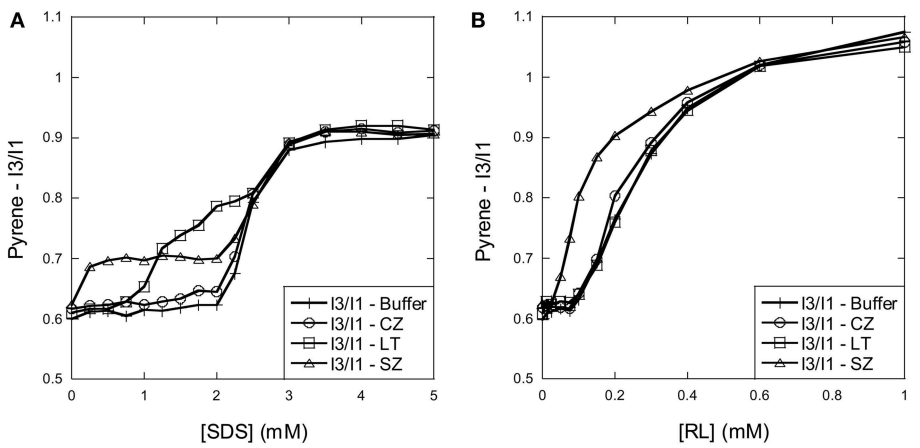

FIGURE 1 | Using pyrene fluorescence to determine SDS and RL cmc and cluster formation in the presence of the three enzymes. (A) SDS: The I3/I1 ratio of pyrene changes around 2–3 mM SDS in the absence of enzymes. In the presence of SZ and LT the I_3/I_1 ratio already starts to rise at 0.25 and 1 mM SDS, respectively, indicating formation of SDS micellar clusters on the enzyme surface below the cmc. **(B)** RL: The I3/I1 ratio of pyrene changes around 0.1–1 mM RL in the absence of enzymes. The increase of the I_3/I_1 ratio increases already at 0.05 mM in the presence of SZ suggests that RL forms micellar clusters on the surface of SZ below the cmc.

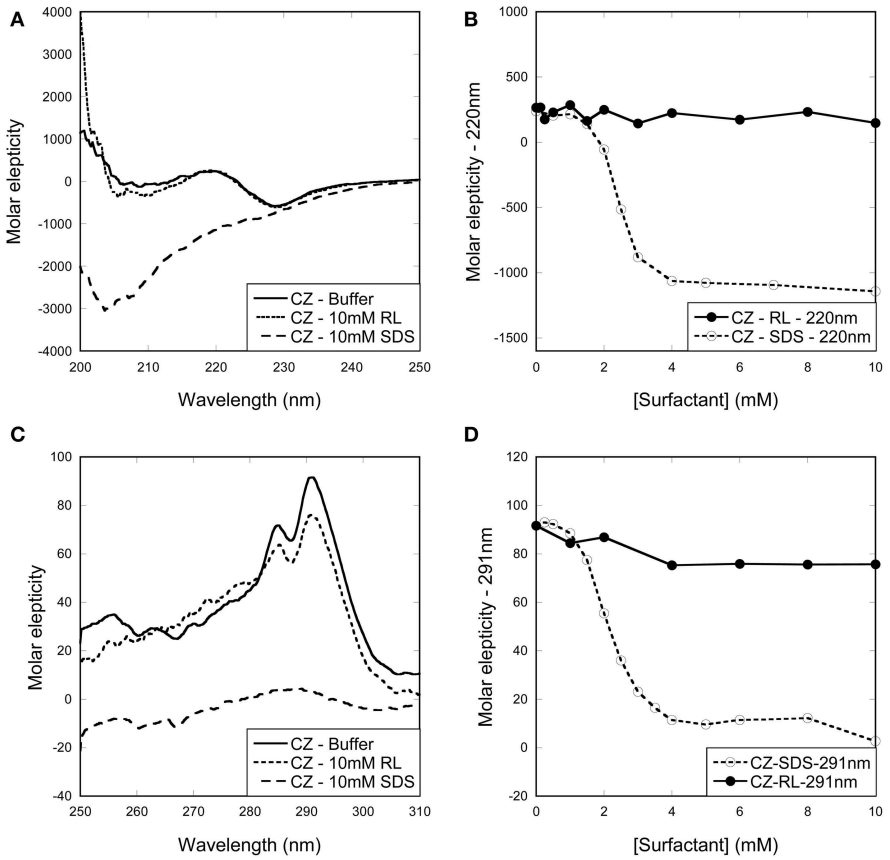

FIGURE 2 | Change in (A,B) secondary and (C,D) tertiary structure of CZ with increasing surfactant concentration. (A) Far-UV and **(C)** near-UV CD spectra of CZ in buffer and in the presence of surfactants. Spectra of CZ in buffer and with RL are essentially identical, while SDS induces changes in both secondary and tertiary structure. Changes in the ellipticity at **(B)** 220 nm and **(D)** 291 nm reveal a structural change around 2 mM SDS. This coincides with the formation of micelles in the bulk phase.

that SZ is a stable enzyme that preserves its native structure in the presence of both SDS and RL.

Thermal Stability of All Enzymes Are Affected Less by RL than by SDS

Pyrene fluorescence and CD indicate that at room temperature, none of the enzymes are denatured by RL, while LT is denatured by SDS monomers and CZ by SDS micelles. Pyrene investigations indicate that SZ interact with both RL and SDS monomers, but the interactions do not lead to enzyme denaturation. To determine how SDS and RL influenced enzyme stability at elevated temperatures, we subjected all 3 enzymes to thermal scans monitored by far-UV CD at 222 nm. In 50 mM Tris pH 8 and in the absence of surfactant, melting temperatures were 86, 74, and 60°C for SZ, CZ, and LT, respectively (**Figures 5A–C**). As summarized in **Figure 5D**, both SDS and RL shifted SZ's unfolding curve to higher temperatures, indicating that the surfactants bind to the native state of SZ and actually stabilize it against denaturation. This is in excellent agreement with the observation that SZ interact with both monomeric SDS and RL but is not denatured by either surfactant. SZ's high intrinsic thermal stability made it difficult to determine melting temperatures (t_m) in the presence of surfactants, since unfolding was incomplete at 95°C (the temperature limit in the experiment). Therefore it is not possible to determine the exact t_m at concentrations above 1 mM surfactant.

1 mM RL (a concentration where RL micelles are present as the majority species) reduced LT's t_m by about 7°C, but higher concentrations did not lower the t_m further. In contrast, SDS continuously lowered t_m without reaching a plateau t_m-value; 1 mM SDS reduced t_m to 48°C and at higher concentration no thermal unfolding could be observed. We conclude that >1 mM SDS, LT is already denatured at room temperature, which is consistent with investigations of the enzymes' secondary and tertiary structure.

A similar pattern was seen for CZ: RL lowered t_m only slightly from 74°C in the absence of surfactant to ∼70°C at 1 mM RL and above. With SDS, the thermal transition decreased steadily from 74 to ∼45°C at 2 mM SDS and no thermal transition was seen at higher SDS concentrations.

Enzyme Activity Is Correlated to Surfactant Induced Structural Change

To further consolidate our understanding of the difference in how the two anionic surfactants interact with enzymes, we

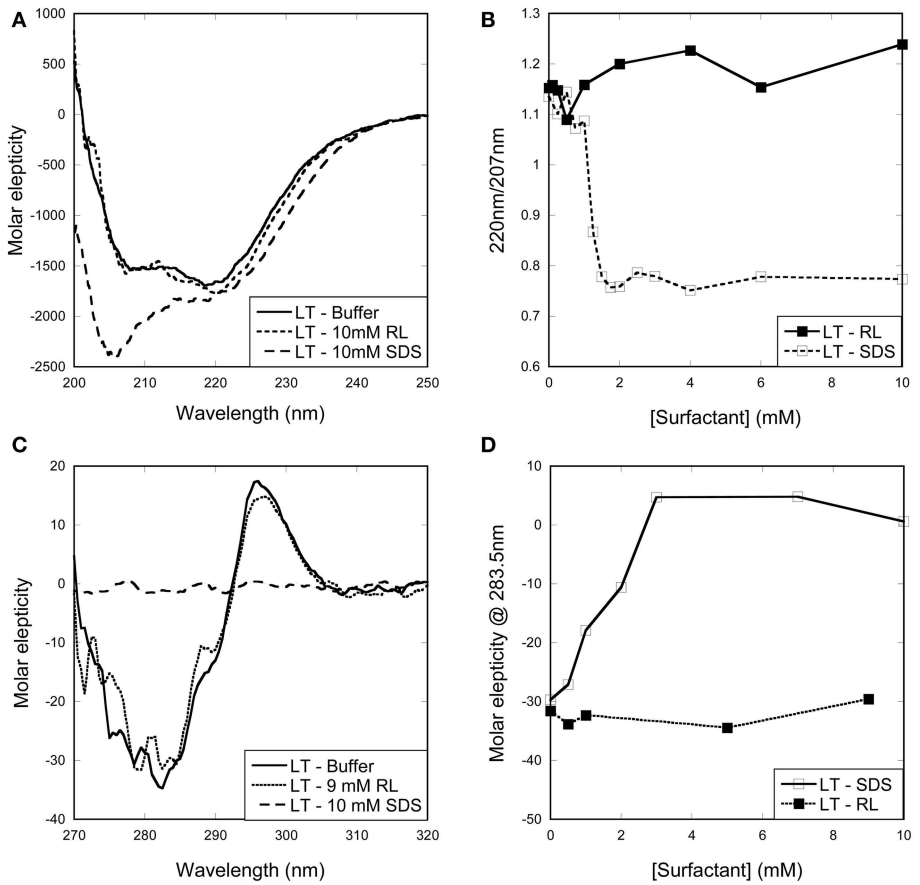

FIGURE 3 | Change in (A,B) secondary and (C,D) tertiary structure of LT with increasing surfactant concentration. (A) Far-UV and **(C)** near-UV CD spectra of LT in buffer and in the presence of surfactants. Spectra of LT in buffer and with RL are essentially identical, while SDS induces changes in both secondary and tertiary structure. **(B)** Changes in the ellipticity ratio 220/207 nm reveal a structural change at 1 mM, i.e., below the cmc. **(D)** Changes in the ellipticity at 283.5 nm confirm that a structural change is induced by SDS below its cmc.

monitored the enzymatic stabilities of all three enzymes as a function of surfactant concentration. Our results (**Figures 6A–C**) nicely corroborate the stability data. The activity of CZ in SDS increases slightly at low SDS concentrations, but then starts to decline steeply around 2 mM SDS (**Figure 6A**), exactly the same concentration range where our CD data indicate onset of unfolding. Activity is retained in RL at all concentrations, consistent with our CD data.

LT activity increases at low SDS concentrations but is reduced to 0.1–0.4% between 1 and 2 mM SDS (**Figure 6B**). This correlates well with CD determined unfolding which occurs between 1 and 1.75 mM SDS. RL shows a more complex effect on LT activity; a 40% reduction in activity at very low RL concentrations (50–100 μM) is followed by an increase in activity to 150–200% at concentrations above the cmc (1–10 mM). We have no simple explanation for this reduction and subsequent recovery in activity though it may be related to competition with the hydrophobic substrate for the active site or other interactions between substrate and RL. However, we note that RL has no adverse effect on activity above 0.1 mM, consistent with its lack of effect on LT structure.

Finally, SZ clearly retains activity at all tested SDS and RL concentrations (**Figure 6C**), which is completely consistent with the lack of unfolding in either SDS or RL.

ITC Reveals Major Differences in the Binding Stoichiometry of Surfactants to Enzymes

As a further aid to explain the (de)stabilizing effects of surfactants on enzymes, we used ITC to resolve the binding stoichiometry. ITC provides valuable information about the thermodynamics and stoichiometry of binding as shown in several studies (Nielsen et al., 2005, 2007; Bagger et al., 2007; Andersen et al., 2008, 2009; Otzen et al., 2009). Different protein concentrations of the three enzymes were therefore subjected to titrations with SDS and RL and the recorded heat flow was accordingly analyzed.

Dilution of micellar SDS into buffer resulted in an endothermic signal at low SDS concentrations as a result of the dissociation of SDS micelles (**Figure 7A**). Above 2 mM SDS there is a decrease in the endothermic signal which levels out from around 3 mM SDS, indicating that no demicellization occurs. Thus, ITC concurs with pyrene fluorescence in establishing SDS's cmc to be around 2–3 mM in our buffer system.

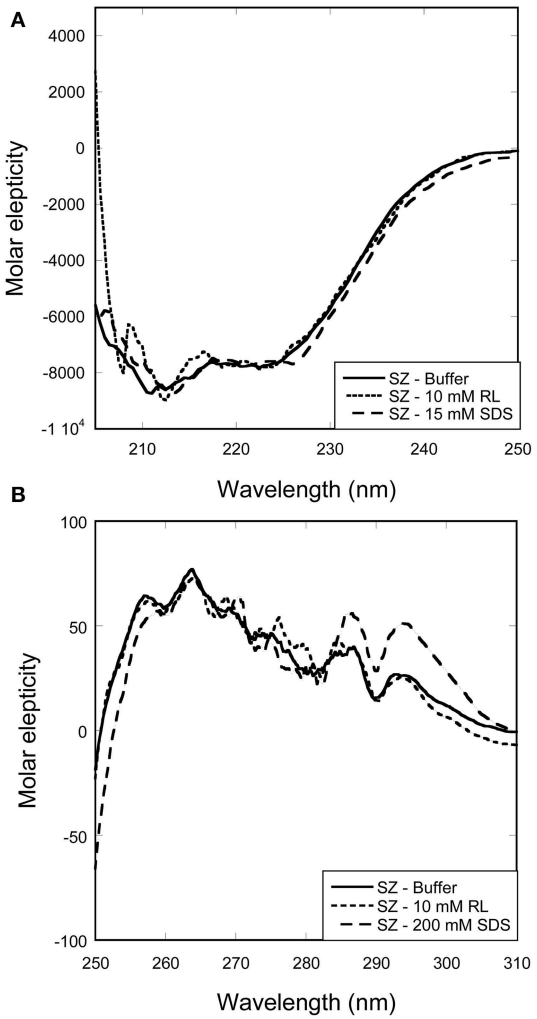

FIGURE 4 | Change in secondary and tertiary structure of SZ with increasing surfactant concentration. Overlapping spectra shows that neither RL nor SDS induces changes in the **(A)** secondary or **(B)** tertiary structure of SZ.

Titration of SDS and RL into LT

LT titrations with SDS at 25°C results in a number of reproducible transitions that shift to higher SDS concentrations with increasing LT concentrations (**Figure 7A**). Unlike SDS titrations into buffer, these titrations show an exothermic minimum (although the net signal is overall endothermic) <2 mM SDS, showing (like pyrene data) that LT interacts with monomeric SDS. Above 2 mM SDS, further interactions between SDS and LT result in a second and much larger exothermic minimum, after which a steady plateau region is reached between 5 and 10 mM SDS increasing with increasing protein concentration. Finally the signal merges with the protein-free signal from > ∼10 mM SDS, indicating that no more interaction between SDS and LT occur. To obtain the stoichiometry of binding, we define five characteristic inflection points (IP) in the LT/SDS enthalpogram which systematically increase with protein concentration (**Figure 7B**), and plot the SDS concentration at the different inflection points as a function of LT concentration (**Figure 7C**). The binding stoichiometry may be derived using the following mass balance:

$$[S]_{Total} = [S]_{Free} + N[Protein]$$

where N is the number of surfactant molecules bound per protein and $[S]_{free}$ is the concentration of surfactant that is not bound to protein. Results are summarized in **Table 1**.

The position of IP1 was determined by fitting the points around the minimum to a 2nd order polynomial and deriving the position of the minimum from the fitting parameters, leading to a satisfactory linear fit, with a binding number of ∼4 and a free [SDS] of 0.93 mM. Clearly LT binds significant amount of SDS well below the cmc. These numbers rise as we progress through the different IPs, until at we reach IP5, where LT is fully saturated with SDS, and the 339-residue enzyme binds 143 ± 5 SDS molecules. This corresponds to one SDS molecule pr 2.4 amino acids, i.e., 1.16 g SDS/g LT. Globular proteins typically bind 1.4 g SDS/g protein (Reynolds and Tanford, 1970), though values of 1.5–2 g SDS/g protein have also been reported (Tanford, 1980). The presence of disulfide bonds can reduce this binding ratio by up to a factor of 2 (Pitt-Rivers and Impiombato, 1968), which is certainly compatible with our data in view of LT's 3 intact disulfide bonds.

Importantly, titration of RL into LT showed no difference compared to RL titration into buffer (**Figure 7D**). We conclude that in contrast to SDS, we are not able to detect significant levels of interaction of RL with LT at room temperature. The minor reduction in LT thermal stability by RL (**Figure 5**) may reflect a small degree of binding to the denatured state at elevated temperatures, displacing the equilibrium toward the denatured state.

Titration of SDS and RL into SZ

The SZ/SDS enthalpogram showed an exotermic signal at very low SDS concentrations (**Figure 8A**) with a minimum region that widened with increasing SZ concentrations (**Figure 8A**). It was not possible to increase the SZ concentration beyond 1 mg/mL because of visible precipitation in the presence of SDS, possibly due to neutralization of exposed positively charged residues by SDS. Subsequently the signal merged with the signal for SDS titration into buffer at concentrations (0.2–0.4 mM) well below the cmc. Within error, SZ did not change the enthalpic signals occurring around the cmc (data not shown). The position of IP1 (**Figure 8B**) increased linearly with [SZ] to yield a binding number of 10 SDS per protein (**Figure 8C** and **Table 1**), consistent with the thermal stabilization at very low SDS concentrations (**Figure 5**). This very low binding level, corresponding to one SDS molecule per 46 amino acids, is also consistent with CD investigations that showed that SZ native and tertiary structure is preserved even in the presence of micellar concentrations of SDS. The low binding number also explains the very weak effect of SZ on the enthalpogram of SDS around the cmc.

SZ titrations with RL resulted in enthalpograms which only differed slightly from RL titration into buffer (**Figure 8D**). There was however a small but systematic increase toward higher RL

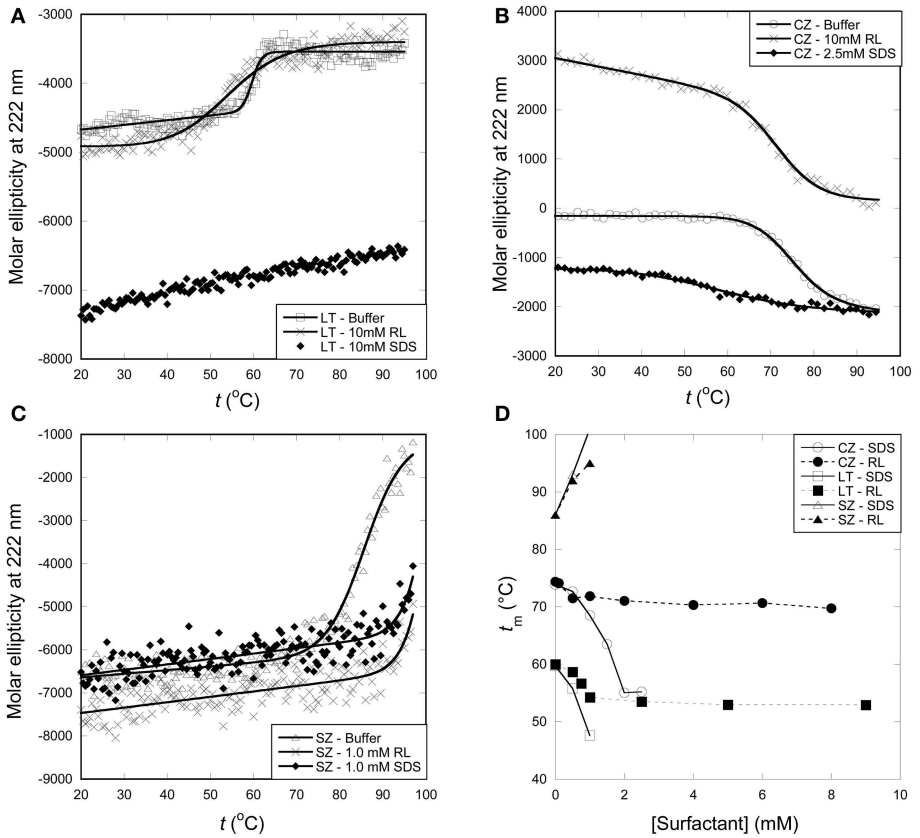

FIGURE 5 | Thermal stability of enzymes with surfactants monitored by far-UV CD thermal scans at 222 nm. (A) t_m of LT is reduced from 60°C in buffer to ∼54°C around the cmc of RL. Higher concentrations of RL do not reduce t_m any further, while SDS progressively lowered the t_m and no thermal transition was observed above ∼1 mM SDS. **(B)** t_m of CZ is lowered by RL by a few degrees while SDS lowers the t_m from 74°C to ∼58°C at 2.5 mM SDS. A thermal transition was not observed >2.5 mM SDS. **(C)** t_m of SZ was increased by both RL and SDS. **(D)** Change in enzyme thermal stability with increasing concentrations of surfactants.

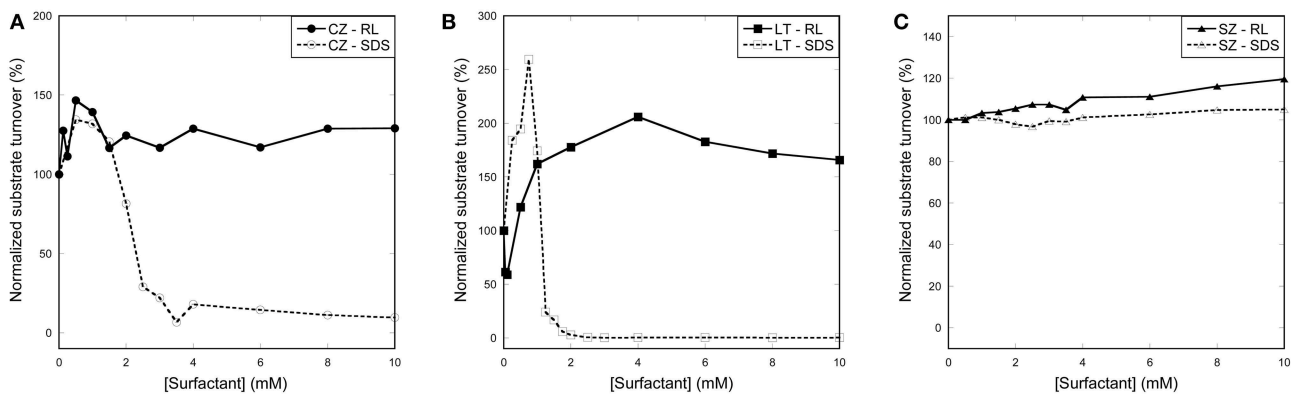

FIGURE 6 | Activity of enzymes with increasing concentration of surfactant. (A) CZ activity declines steeply around 2 mM SDS while RL has little effect on activity. **(B)** LT activity is increased between 0 and 1 mM SDS where after it declines to ∼0% between 1 and 2 mM SDS. LT activity is decreased at low RL concentration but increases at concentrations above the cmc. **(C)** SZ activity is only slightly affected by both SDS and RL.

concentrations with increasing SZ concentration. We define IP1 as the concentration where the signal levels off (**Figure 8E**); IP1 scales linearly with [SZ], leading to a binding number of 10 RL molecules per SZ (**Figure 8F**), identical to the binding number of SDS. The higher molecular weight of RL means that SZ binds almost twice as much RL by weight as SDS. However, at IP1, $[RL]_{free}$ is 0.27 mM, which is much larger than $[SDS]_{free} = 0.07$ mM, indicating weaker binding overall (since a

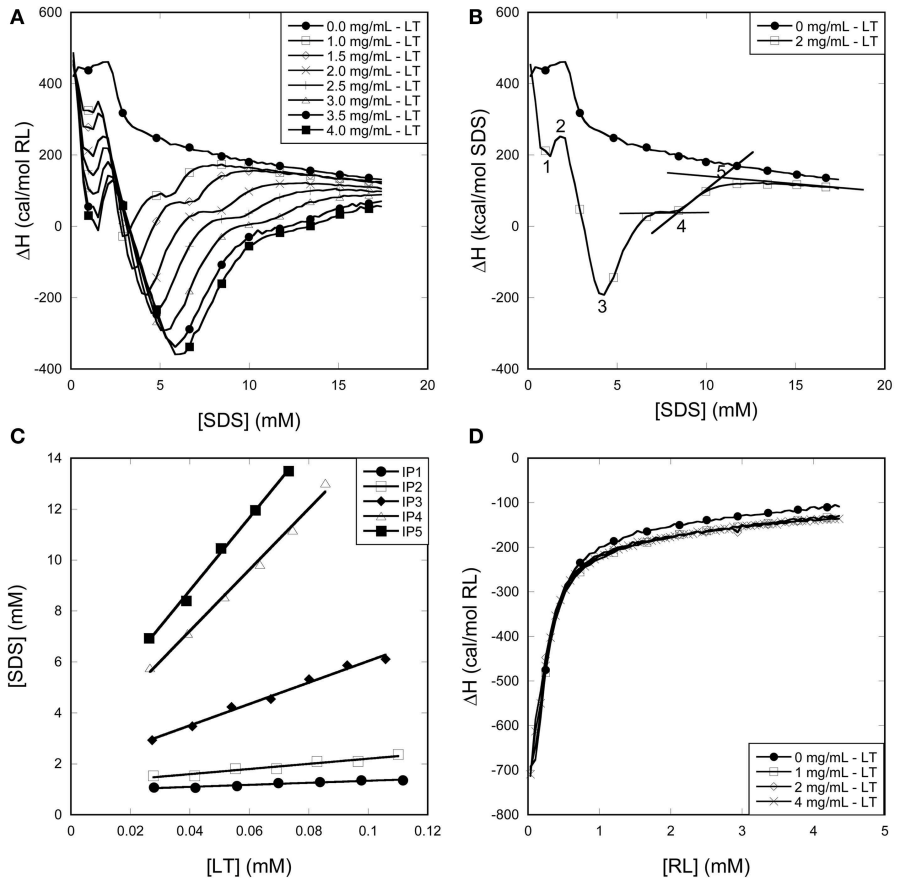

FIGURE 7 | Using ITC to determine the binding stoichiometry of surfactants to LT. (A) Enthalpograms for the titration of SDS into LT. **(B)** Representative enthalpograms which illustrate inflection points used to calculate binding numbers. **(C)** SDS inflection points plotted as a function of LT concentration. The linear fit to Equation (1) provides binding numbers (see text). **(D)** Titration of RL into LT did not show any significant effect of the presence of the enzyme.

TABLE 1 | Binding parameters derived from ITC data[a].

Enzyme	Inflection point	$[Surf]_{free}$ (mM)[b]	Surf. pr. Enzyme[b]	Amino acids per Surf.	g Surf/g Enzyme[c]
LT	LT-SDS-1	0.93 ± 0.03	4.0 ± 0.4	85.5	0.03 ± 0.00
	LT-SDS-2	1.19 ± 0.08	10.1 ± 1.1	33.5	0.08 ± 0.01
	LT-SDS-3	1.82 ± 0.15	42.1 ± 2.0	8.0	0.34 ± 0.01
	LT-SDS-4	2.42 ± 0.29	120.0 ± 4.9	2.8	0.98 ± 0.02
	LT-SDS-5	3.10 ± 0.25	142.6 ± 4.7	2.4	1.16 ± 0.02
SZ	SZ-SDS-1	0.07 ± 0.00	10.5 ± 0.3	46	0.05 ± 0.00
	SZ-RL-1	0.27 ± 0.03	11.1 ± 0.6	44	0.11 ± 0.01
CZ	CZ-SDS-1	2.03 ± 0.06	22.9 ± 1.8	12.6	0.18 ± 0.01[d] 0.22 ± 0.02[e]
	CZ-SDS-2	2.52 ± 0.03	34.2 ± 0.9	8.5	0.26 ± 0.01[d] 0.33 ± 0.01[e]

[a] *All experiments done in 50 mM Tris pH 8 at 25°C, except CZ-SDS where 23°C was optimal.*
[b] *Data based on fits in* ***Figures 7C, 8C,F, 9C****.*
[c] *Errors calculated based on errors in RL:protein stoichiometry (column 4).*
[d] *Based on the mass of glycosylated CZ (~37 kDa).*
[e] *Based on the mass of non-glycosylated CZ (~30 kDa).*

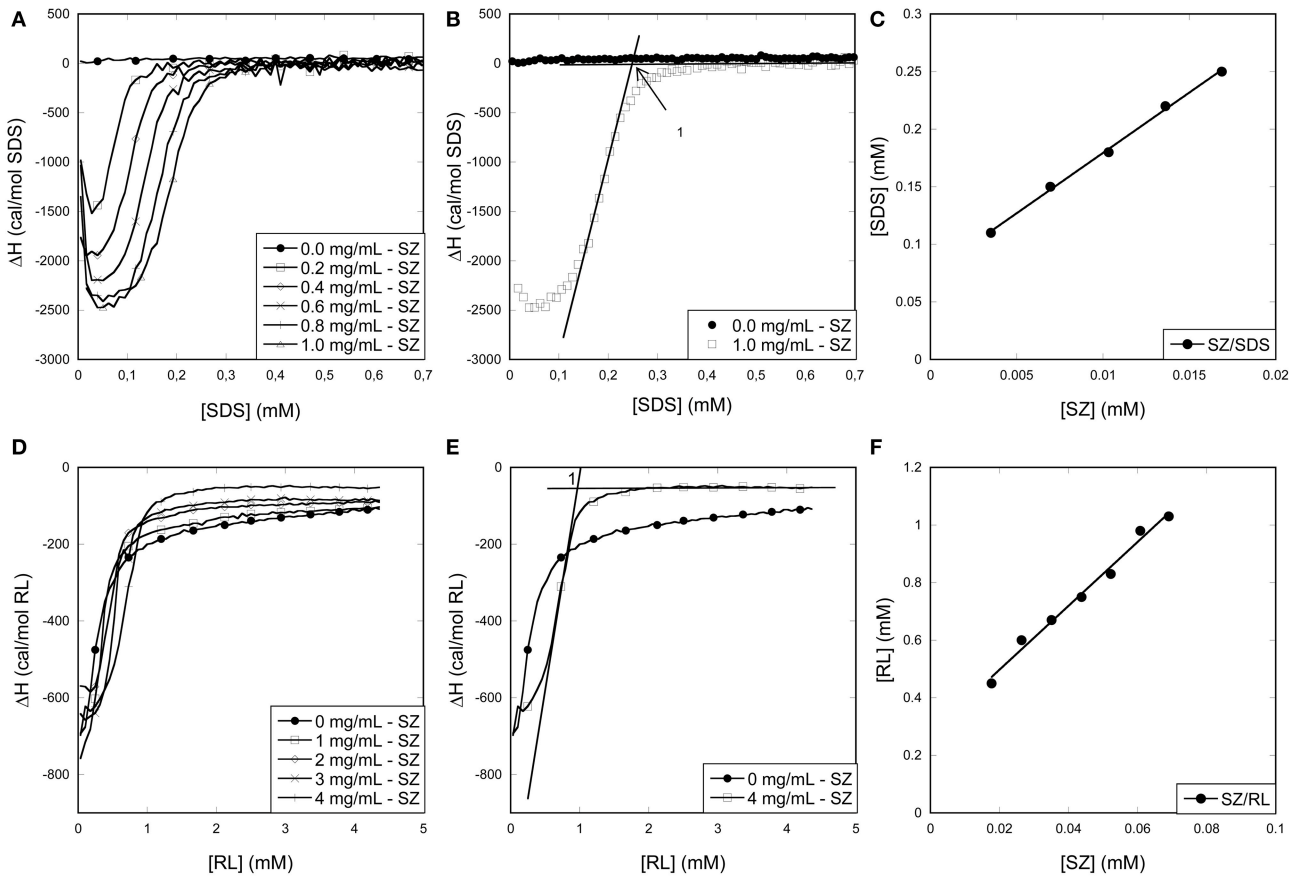

FIGURE 8 | Using ITC to determine the binding stoichiometry of surfactants to SZ. Data for titration of **(A–C)** SDS or **(D–F)** RL into SZ. **(A,D)**: Raw enthalpograms of titration. **(B,E)**: Representative enthalpograms highlighting inflection points used to calculate binding numbers. **(C,F)**: Inflection points plotted as a function of SZ concentration. The linear fit provides binding numbers (see text).

higher concentration of RL is required to reach a binding number of 10).

Titration of SDS and RL into CZ

All CZ titrations with SDS overlapped with SDS titrations into buffer between 0 and 2 mM SDS, indicating that CZ does not interact with monomeric SDS (**Figure 9A**). This correlates well with fluorescence and CD experiments which indicated that no micelles are formed on the surface of CZ and that denaturation only occur when SDS micelles are formed in the bulk phase. At concentrations above 2 mM SDS, an exotermic signal was observed which eventually merged with the buffer signal. The exothermic signal increased with increasing concentration of CZ and the concentration of SDS where the titration merged with the buffer titration increased as well. We define two inflection points: CZ-SDS-1 (the minimum of the exothermic signal) and CZ-SDS-2 (where the titration of CZ merges with the titration of SDS into buffer) (**Figure 9B**). CZ binds 22.9 ± 1.8 and 34.2 ± 0.9 SDS molecules at the two inflection points (**Figure 9C**), equivalent to one SDS molecule pr. 12.6 and 8.5 amino acid residues, respectively. The binding of 1 SDS molecule pr. 8.5 amino acid residues at saturation is very low compared to LT and other proteins denatured by SDS. The low binding number may however be due to a combination of several disulfide bonds and the heavy glycosylation of the linker. Glycosylation has previously been shown to decrease the amount of SDS which binds to enzymes (Bagger et al., 2007). In contrast to SDS, CZ titrations with RL overlapped with RL titrations into buffer (**Figure 9D**). We conclude that CZ does not interact with RL at room temperature.

Discussion

We undertook this study to compare the impact of the synthetic surfactant SDS and the microbially produced biosurfactant RL on the structure, stability and enzymatic activity of 3 widely used industrial enzymes. Importantly, all three sets of data corroborate each other and demonstrate that SDS displays a great deal of versatility in its type of interaction with the enzymes. One enzyme (SZ) is actually stabilized by SDS while two others are destabilized and denatured but in different ways, LT by SDS clusters formed on the protein below the cmc and CZ by binding of bulk micelles at low stoichiometries. This diversity of binding and unfolding reflects how the enzymes can make specific interactions between protein and SDS. Binding is

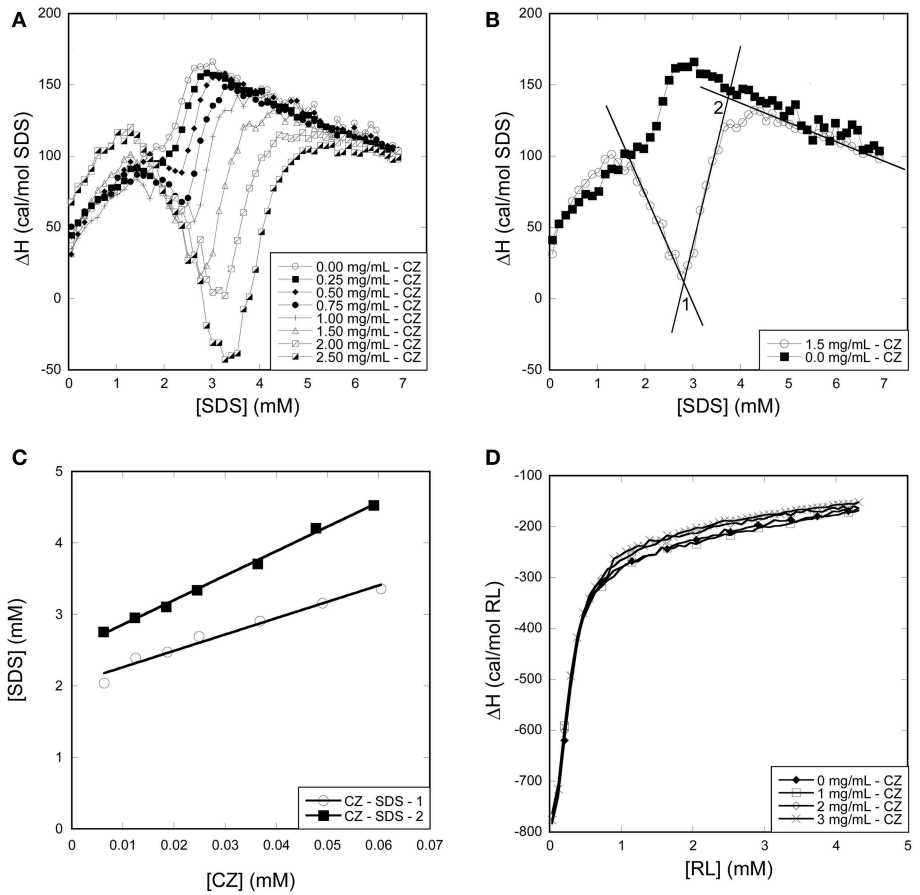

FIGURE 9 | Using ITC to determine the binding stoichiometry of surfactants to CZ. (A) Enthalpograms for the titration of SDS into CZ. **(B)** Representative enthalpograms which illustrate inflection points used to calculate binding numbers. **(C)** SDS Inflection points plotted as a function of CZ concentration. The linear fit provides binding numbers (see text). **(D)** Titration of RL into CZ did not show any significant effect of the presence of the enzyme.

mediated by a highly concentrated negative charge on the sulfate headgroup in combination with a long and unbranched alkyl chain, promoting binding at multiple different places on the protein surface depending on electrostatic and hydrophobic binding opportunities (Otzen, 2011). For example, two structurally related β-sheet proteins were denatured at sub- and super-cmc SDS concentrations, respectively (Yonath et al., 1977a,b; Nielsen et al., 2007), and we have attributed this to differences in potential electrostatic binding sites. Thus, sub-cmc unfolding may require the presence of cationic hot-spots to attract a multiple number of SDS monomers in a small region of the protein, promoting subsequent cluster formation by association of the adjoining alkyl chains. While we cannot make such simple comparative conclusions for LT vs. CZ as they represent very different structures, we consider it likely that similar mechanisms are at play here. The stabilization of SZ by SDS must reflect the binding of a small number of SDS monomers to one or a few sites on SZ which are found in the native state; simple mass-action then dictates that binding will stabilize the native state rather than denaturing it, just as observed for BSA (Khan et al., 2013).

In contrast to SDS, RL shows a very weak level of interaction with the enzymes, in no case inducing structural changes, at most changing the melting temperature by a few degrees upward or downward and generally having little effect on enzyme activity. For all three enzymes, RL:protein binding stoichiometries are low or undetectable. This does not imply that RL is completely unable to perturb protein structure; we have recently reported that sub-cmc RL is able to denature the notoriously unstable apo-form of α-lactalbumin while super-cmc concentrations are required to denature the disulfide-free protein myoglobin (Andersen and Otzen, 2014b). However, even when denaturing these relatively unstable proteins, the denaturation process is slow and does not involve many binding steps unlike SDS, where efficient denaturation is likely achieved by an accumulation of different binding and denaturation steps. We have attributed the weak binding of RL to a weakly acidic carboxylic head group and a branched hydrophobic chain, both of which promote micelle formation at the expense of (extensive) protein binding (Andersen and Otzen, 2014b). Larger proteins evolved for microbial extracellular secretion are often stabilized against, e.g., proteolytic attack to ensure their ability to persist

in an exposed and competitive extracellular environment (Kirk et al., 2002), and this increased stability likely tips the balance against denaturation by RL. Current enzymes used in the detergent industry are typically engineered to withstand denaturation by relatively aggressive mixtures of anionic and non-ionic surfactants (Otzen et al., 1999). Thus, RL is likely to be compatible with all the industrial enzymes currently in use in detergents, and may even allow the introduction of enzymes that are sensitive to the present harsh synthetic anionic surfactants. Biosurfactants have already been shown to emulgate vegetable oils efficiently and to be compatible with commercial laundry detergents (Mukherjee, 2007). Thus, there are definitely bright prospects for the inclusion of biosurfactants in future commercial applications.

Acknowledgments

KA is funded by a grant from the Danish Research Council | Technology and Production. JM is financed by a PhD grant co-financed by the Danish Ministry of Science and Innovation and Zealand Pharma A/S. We are grateful to Kim Borch (Novozymes A/S) for generously providing enzymes and to Dr. Lise Giehm (Zealand Pharma A/S) for ongoing discussions about protein-surfactant interactions.

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Conflict of Interest Statement: 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.

frontiers in
MICROBIOLOGY

OPINION ARTICLE
published: 02 September 2014
doi: 10.3389/fmicb.2014.00454

Rhamnolipid biosurfactants—past, present, and future scenario of global market

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Keywords: biosurfactants, rhamnolipids, market Access, surfactants, sustainability

RHAMNOLIPIDS—BRIEF OUTLINE

Biosurfactants, widely known as surface-active agents of biological origin, have carved a niche for themselves in the market due to their unique environment-friendly properties. They have come a long way since first biosurfactant "surfactin" was purified and characterized by Arima et al. (1968). Biosurfactants have been researched thoroughly and satisfactorily since then by many research groups across the world yet there are aspects that elude our understanding. There are five major categories of biosurfactants viz. glycolipids, phospholipids and fatty acids, lipopeptides and lipoproteins, polymeric biosurfactants and particulate biosurfactants that have found applications in agricultural, pharmaceutical, food, cosmetics, and detergent industries. Data reveals there are more than 250 patents obtained on these wonder biodegradable molecules so far (Shete et al., 2006; Rahman and Gakpe, 2008). It has also been observed that microbial biosurfactants are advantageous over plant-based surfactants because of the scale-up capacity, rapid production, and multi-functional properties. Several plant-based biosurfactants for example saponins, lecithins, and soy proteins have excellent emulsification properties but are expensive to produce at industrial scale and have other debatable issues such as solubility and hydrophobicity (Xu et al., 2011).

Among the various categories of biosurfactants the glycolipid biosurfactants "rhamnolipids" stand apart. Rhamnolipid, primarily a crystalline acid, is composed of β-hydroxy fatty acid connected by the carboxyl end to a rhamnose sugar molecule. Rhamnolipids are predominantly produced by *Pseudomonas aeruginosa* and classified as: mono and di-rhamnolipids. Other *Pseudomonas* species that have been reported to produce rhamnolipids are *P. chlororaphis*, *P. plantarii*, *P. putida*, and *P. fluorescens*. Some bacteria are known to produce only mono-rhamnolipids while some produce both. The ratio of mono and di-rhamnolipid can also be controlled in the production method. There are enzymes available that can convert mono-rhamnolipids into di-rhamnolipids. In 1984, the first patent for the production of rhamnolipids was filed by Kaeppeli and Guerra-Santos (US 4628030) and obtained in 1986 for their work on *Pseudomonas aeruginosa* DSM 2659 (Kaeppeli and Guerra-Santos, 1986). Subsequently, Wagner et al. filed a patent (US 4814272) in 1985 for the biotechnical production of rhamnolipids from *Pseudomonas sp.* DSM 2874 and obtained the same in 1989 (Wagner et al., 1989). In the past close to three decades, there has been a great body of research work carried on rhamnolipids revealing many of their astonishing applications and making them reach the pinnacle of popularity among all the categories of biosurfactants in the global market. The reason behind the current global interest in rhamnolipid production owes to their broad range of applications in various industries along with many spectacular "eco-friendly" properties.

The current critique articulates to present opinion on rhamnolipid research and is an attempt to retrospect what brings rhamnolipids in the forefront. This article is a bird's-eye view on a timeline of rhamnolipids story so far and also a critical analysis on why despite so many patents and research work rhamnolipids still do not rule the global biosurfactant market.

INIMITABLE APPLICATIONS OF RHAMNOLIPIDS

Over the years rhamnolipids are becoming broadly pertinent in various industries and are posing a serious threat to the synthetic surfactants. Before venturing into the current production economics of rhamnolipids it is imperative to evaluate the major applications of rhamnolipids that make them noticeable among other biosurfactants. A list of five major applications of rhamnolipids that cater to the wide range of industrial demands includes:

(1) *Bioremediation and enhanced oil recovery (EOR)*: Rhamnolipids show excellent emulsification properties, efficiently remove crude oil from contaminated soil and facilitate bioremediation of oil spills (Rahman et al., 2003; Costa et al., 2010).
(2) *Pharmaceuticals and therapeutics*: Rhamnolipids show low toxicity, surface active properties and antimicrobial activities against several microbes (*Bacillus cereus, Micrococcus luteus, Staphylococcus aureus, Listeria monocytogenes*) thereby showing promising applications in pharmaceuticals

Table 1 | Biosurfactant producing companies around the globe.

S. No.	Company	Location(s)	Product(s)	Focus on
1	TeeGene Biotech	UK	Rhamnolipids and Lipopeptides	Pharmaceuticals, cosmetics, antimicrobials and anti-cancer ingredients
2	AGAE Technologies LLC	USA	Rhamnolipids (R95, an HPLC/MS grade rhamnolipid)	Pharmaceutical, cosmeceutical, cosmetics, personal care, bioremediation (*in situ* & *ex situ*), Enhanced oil recovery (EOR)
3	Jeneil Biosurfactant Co. LLC	USA	Rhamnolipids (ZONIX, a bio-fungicide and RECO, a rhamnolipid used in cleaning and recovering oil from storage tanks)	Cleaning products, EOR
4	Paradigm Biomedical Inc.	USA	Rhamnolipids	Pharmaceutical applications
5	Rhamnolipid Companies, Inc.	USA	Rhamnolipids	Agriculture, cosmetics, EOR, bioremediation, food products, pharmaceutical
6	Fraunhofer IGB	Germany	Glycolipids, Cellobiose lipids, MELs	Cleansing products, shower gels, shampoos, washing-up liquids, pharmaceutical (bioactive properties)
7	Cognis Care Chemicals	China, Germany, USA	Alkyl polyglucoside APG®, Plantacare 1200 GLY (green surfactant for use in oral-dental formulations), Rheocare TTA (for cleansing formulations)	Used in formulations for household cleaners, bath/shower gels, dish washing, laundry detergents and in agrochemicals
8	Saraya Co. Ltd.	Japan	Sophorolipids (Sophoron, a low-foam dishwasher detergent)	Cleaning products, hygiene products
9	Ecover Belgium	Belgium	Sophorolipids	Cleaning products, cosmetics, bioremediation, pest control, pharmaceuticals
10	Groupe Soliance	France	Sophorolipids	Cosmetics
11	MG Intobio Co. Ltd.	South Korea	Sophorolipids (Sopholine—functional soap with Sophorolipids secreted by yeasts)	Beauty and personal care, bath supplies e.g., soaps with new functions
12	Synthezyme LLC	USA	Sophorolipids	Cleaning products, cosmetics, food products, fungicides, crude oil emulsification
13	Allied Carbon Solutions (ACS) Ltd	Japan	Sophorolipids (ACS-Sophor—first bio-based surfactant from Indian mahua oil)	Agricultural products, ecological research
14	Henkel	Germany	Sophorolipids, Rhamnolipids, Mammoslyerthritol lipids	Glass cleaning products, laundry, beauty products
15	Lion Corporation	Japan	Methyl ester sulfonates (MES)	Detergents formulations, cleaning products
16	Lipo Chemicals	USA	Lipomulse Luxe (high-temperature resistance emulsifier)	Skin care, sun-lotions hair care formulations, thickening polymers, rheological modifiers, natural gums
17	Kaneka Co.	Japan	Sophorose lipids	Cosmetics and toiletry products

and therapeutics (Magalhaes and Nitschke, 2013).

(3) *Cosmetics*: Rhamnolipid as an active ingredient is found to be effective for several skin treatments i.e., wound healing with reduced fibrosis, cure of burn shock, treatment of wrinkles hence are in demand in the health and beauty industry (Piljac and Piljac, 2007).

(4) *Detergents and cleaners*: Rhamnolipids are natural emulsifiers and surface active agents leading to their wide spread usage in detergent compositions, laundry products, shampoos and soaps (Parry et al., 2013).

(5) *Agriculture*: Rhamnolipids are already used for soil remediation for improving soil quality and are now further getting explored for plant pathogen elimination, for aiding the absorption of fertilizers and nutrients through roots and as biopesticides (Sachdev and Cameotra, 2013).

BIOSURFACTANT PRODUCING COMPANIES—WITH FOCUS ON RHAMNOLIPIDS

Rhamnolipids are highly applicable in various activities with some researchers advancing the technology from laboratory to higher scale. However, there still are very limited companies in the field which are producing biosurfactants at a marketable scale. We tried to compile a list of biosurfactant producing companies around the globe (**Table 1**). The compilation evidently defines which biosurfactants are mostly researched and produced at higher scale.

AVAILABILITY OF FEEDSTOCK AND ITS IMPACT ON BIOSURFACTANTS

Biodiesel is produced by the transesterification of vegetable oils and fats with methanol in the presence of a catalyst. Glycerol is received as a by-product from this reaction. The production of 1 ton biodiesel generates about 100 kg of glycerol. Hence, the European biodiesel industry might release about 600 Kiloton glycerol per year with an increasing tendency in Europe and worldwide. Oversupply of glycerol, essentially due to increasing biodiesel production, leads to decreasing prices and weak markets.

The price of pure glycerol varied from $0.50 to $1.50/lb and crude glycerol from $0.04/kg to $0.33/kg over the past few years. The price of glycerol in the market will continue to drop in such an over saturated market. Currently, the main supply of glycerol coming into the market is from the rapidly growing biodiesel industry. Estimated production of glycerol would reach 5.8 billion pounds in 2020. This is due to demand of biodiesel that is projected at 8 billion gallons in 2020 (Ayoub and Abdullah, 2012). Hence new outlets for glycerol are urgently needed, particularly in the case of crude glycerol released by the biodiesel processes. As glycerol is a nontoxic, edible, biodegradable compound, it will provide important environmental benefits to the new platform products.

In case of biosurfactant production, dramatically rising in biodegradable, nontoxic and eco-friendly alternative for chemical surfactants and the re-discovered opportunity of biosurfactants that gave rise to invention and investment ahead of the typical rigors of techno-economic modeling for the use of glycerol as a feed stock, leading typically to unmet expectations. Bacteria produce biosurfactants if grown on carbon sources such as glucose, glycerol, and various vegetable oils. Our research on biosurfactant production by bacteria indicates that glycerol can be used efficiently for biosurfactant production (Rahman et al., 2002).

The considerable interest in biosurfactants in the recent years is also due to their low toxicity, biodegradable nature and specificity, which would be very suitable to meet the European Surfactant Directive. Regulation EC No.: 648/2004 requires clear and precise description of the biodegradability of the surfactant and test methods to give assurance of its aerobic biodegradability. This regulation establishes rules designed to achieve the free movement of detergents and surfactants for detergents in the internal market while, at the same time, ensuring a high degree of protection of the environment and human health.

Surfactants constitute an important class of industrial chemicals and are widely used in almost every sector of modern industry. Most of the commercially available surfactants are chemical surfactants mainly, petroleum-derived. However, rapid advances in biotechnology and increased environmental awareness among consumers combined with expected new environmental legislation has provided further impetus for serious consideration of biological surfactants as possible alternatives to existing products.

BIOSURFACTANT'S ECONOMIC FEASIBILITY—WHAT IT TAKES TO BECOME A MARKET LEADER?

As described in the previous section, there is enormous awareness among the consumers these days with regard to sustainability and global warming. The demand for bio-based technologies is ever increasing and "green solutions" are sought for every process. Rhamnolipids have promising properties and fulfill the eco-friendly criteria, one of the main

Table 2 | Cost of biosurfactant per liter of solution (diluted and the CMC based cost calculation carried out by Connolly et al., 2010).

Biosurfactant	Origin	Supplier	ST mN/m	CMC (%)	Cost (£/L)
BioFuture	Bacterial rhamnolipid	BioFuture Ltd. Dublin	28	0.08	0.02
Citrasolv	Orange peel	Cleveland Biotech Ltd., Teesside	30	0.9	0.01
EC601	Bacterial rhamnolipid	Ecochem Ltd., Canada	29	0.2	0.23
EC1800	Bacterial consortium	Ecochem Ltd., Canada	28	0.04	0.01
Petrosolv	Bacterial unknown	Enzyme Technologies Inc., USA	34	0.2	0.01
Saponin	Plant bark	Sigma UK	45	0.1	0.50

The table also gives the origin of biosurfactant along with surface tension (ST) and critical micelle concentration (CMC) values.

Table 3 | A timeline and the major patents and grants obtained on rhamnolipids so far.

S. No.	Patent or Application No.	Filed	Issued	Title	Inventors
1	4628030	Aug 1984	Dec 1986	Process for the production of rhamnolipids	Kaeppeli and Guerra-Santos
2	4814272	Feb 1985	March 1989	Process for the biotechnical production of rhamnolipids including rhamnolipids with only one. Beta.-hydroxydecanoic acid residue in the molecule	Wagner et al.
3	4933281	March 1987	June 1990	Method for producing rhamnose	Daniels et al.
4	4902512	Jan 1988	Feb 1990	Rhamnolipid liposomes	Ishigami et al.
5	5417879	Sep 1993	May 1995	Synergistic dual-surfactant detergent composition containing sophoroselipid	Hall et al.
6	5455232	April 1994	Oct 1995	Pharmaceutical preparation based on rhamnolipid	Piljac and Piljac
7	5550227	May 1994	Aug 1996	Method for the preparation of rhamnose monohydrate from rhamnolipids	Mixich et al.
8	5466675	July 1994	Nov 1995	Immunological activity of rhamnolipids	Piljac and Piljac
9	5520839	Jan 1995	May 1996	Laundry detergent composition containing synergistic combination of sophorose lipid and non-ionic surfactant	Hall et al.
10	5501966	Jan 1995	March 1996	*Pseudomonas aeruginosa* and its use in a process for the biotechnological preparation of L-rhamnose	Giani et al.
11	5658793	June 1995	Aug 1997	*Pseudomonas aeruginosa* and its use in a process for the biotechnological preparation of L-rhamnose	Giani et al.
12	5514661	Aug 1995	May 1996	Immunological activity of rhamnolipids	Piljac and Piljac
13	5767090	Jan 1996	June 1998	Microbially produced rhamnolipids (biosurfactants) for the control of plant pathogenic zoosporic fungi	Stanghellini et al.
14	7129218	Aug 2000	Oct 2006	Use of rhamnolipids in wound healing, treatment and prevention of gum disease and periodontal regeneration	Stipcevic et al.
15	7262171	Aug 2000	Aug 2007	Use of rhamnolipids in wound healing, treating burn shock, atherosclerosis, organ transplants, depression, schizophrenia and cosmetics	Piljac and Piljac
16	20040224905	May 2002	Nov 2004	Use of rhamnolipids in wound healing, treatment and prevention of gum disease and periodontal regeneration	Stipcevic et al.
17	20060233935	Nov 2003	Oct 2006	Rhamnolipids in bakery products	Haesendonck and Vanzeveren
18	7202063	Aug 2005	April 2007	Processes for the production of rhamnolipids	Gunther et al.
19	20070191292	Feb 2006	Aug 2007	Antimycotic rhamnolipid compositions and related methods of use	Gandhi et al.
20	20070155678	Feb 2007	July 2007	Use of rhamnolipids in wound healing, treating burn shock, atherosclerosis, organ transplants, depression, schizophrenia and cosmetics	Piljac and Piljac
21	20070207930	Feb 2007	Sep 2007	Rhamnolipid compositions and related methods of use	Gandhi et al.
22	7968499	Feb 2007	June 2011	Rhamnolipid compositions and related methods of use	Gandhi and Skebba
23	20080213194	July 2007	Sep 2008	Rhamnolipid-based formulations	Keith DeSanto

(Continued)

Table 3 | Continued

S. No.	Patent or Application No.	Filed	Issued	Title	Inventors
24	7985722	July 2007	July 2011	Rhamnolipid-based formulations	Keith DeSanto
25	20100249058	Oct 2007	Sep 2010	Feed additive and feed	Ito et al.
26	20090126948	Nov 2007	May 2009	Use of rhamnolipid based formulations for fire suppression and chemical and biological hazards	Keith DeSanto
27	20080261891	Feb 2008	Oct 2008	Compositions and methods for using syringopeptin 25A and rhamnolipids	Bart C. Weimer
28	20090220603	May 2009	Sep 2009	Use of rhamnolipids in wound healing, treating burn shock, atherosclerosis, organ transplants, depression, schizophrenia and cosmetics	Piljac and Piljac
29	20110123623	Nov 2010	May 2011	Rhamnolipid mechanism	Keith DeSanto
30	20120322751	Feb 2011	Dec 2012	Use of rhamnolipids as a drug of choice in the case of nuclear disasters in the treatment of the combination radiation injuries and illnesses in humans and animals	Goran Piljac
31	20110257115	June 2011	Oct 2011	Method for treating rhinitis and sinusitis by rhamnolipids	Anton Leighton
32	20110306569	June 2011	Dec 2011	Rhamnolipid biosurfactant from *Pseudomonas aeruginosa* strain NY3 and methods of use	Yin et al.
33	8592381	June 2011	Nov 2013	Method for treating rhinitis and sinusitis by rhamnolipids	Anton Leighton
34	20110270207	July 2011	Nov 2011	Rhamnolipid based formulations	Keith DeSanto
35	8183198	July 2011	May 2012	Rhamnolipid-based formulations	Keith DeSanto
36	20130130319	July 2011	May 2013	Cells and methods for producing rhamnolipids	Schaffer et al.
37	20120255918	April 2012	Oct 2012	Use of rhamnolipids in the water treatment industry	DeSanto and Keer
38	20130296461	May 2013	Nov 2013	Aqueous coatings and paints incorporating one or more antimicrobial biosurfactants and methods for using same	Lakshmi Sadasivan
39	20130310330	July 2013	Nov 2013	Method for treating obesity	Anton Leighton
40	8765694	July 2013	July 2014	Method for treating obesity	Anton Leighton
41	20140080771	Nov 2013	March 2014	Method for treating rhinitis and sinusitis by rhamnolipids	Anton Leighton
42	20140148588	Nov 2013	May 2014	Process for the isolation of rhamnolipids	Schilling et al.

drivers, but are still struggling to become market leaders. The economics of production is a major bottleneck in the outburst of commercialization of rhamnolipids and other biosurfactants (**Table 2**). There is still no downstream technology economical and convincing enough to recover and purify rhamnolipids at industrial scale. In case of biosurfactant production the downstream processing accounts for 70–80% of the entire production costs.

It is a no-brainer that in order to gain higher profit at commercial scale it requires access to very cheap feedstock. There are some other key parameters that need thorough consideration in order to make any product economically feasible. Technological fit and process optimization are among the main drivers. Fermentation time is another key to success. Fermentation performance and scale impact process economy as it is directly related to the yield, titer, and productivity. High cost of production especially because of the expensive substrates and down-stream processes makes it difficult

to bring down the price of these environment friendly biomolecules. In order to compete with the synthetic detergents or surfactants the cost of production must be brought down to £1.70 per liter which is in itself a challenging task. As there are many barriers in the commercialization of biosurfactants, there seems no dearth of opportunities in this field. Cost comparison of various technologies viz. enzymatic, continuous, shake flask, batch, and fed-batch used for biosurfactant production pinpoint the requirement of innovative methods wherein rhamnolipids can be produced in static conditions to reduce the fermentation cost. The operating costs can be brought down by robust wild-type strains or recombinant mutant strains. Testing the possibility of co-products and/or enzymes is another attractive solution to surge the net profit—for example: esterases released during the production of lipopeptides by *Bacillus* strain and its recombinants (Sekhon et al., 2011, 2012). Co-products and by-products are value drivers and increase the economic viability of any business. The search of cheap and easily accessible raw material or substrate for biosurfactants production has been going on for years. The utilization of by-products, even if from a different process could be another smart solution—for example: glycerol, which is a by-product of biodiesel production, is available in surplus amount in the global market (Albarelli et al., 2011) which might be a cheap alternative for biosurfactant production.

Rhamnolipids are well-characterized and scientifically proven biosurfactants which are slowly and steadily becoming highly sought after biomolecules. Among other biosurfactants rhamnolipids have the highest number of patents (**Table 3**) and research publications. However, cost-competitiveness is one of the major factors that is holding rhamnolipids back from becoming the champions of their field. Research needs to be focused on suitable vigorous production strains, cheap substrates and nominal bioreactor technology. The current market price of rhamnolipid (R-95, 95%) is $227/10 mg (Sigma-aldrich) and $200/10 mg (AGAE technologies, USA) calling for strenuous research. Rhamnolipids have favorable applications in various sectors and if made economically sustainable nothing can stop these biomolecules to rule the surface-active compounds market.

CONCLUDING REMARKS

As the Health and Safety in the bioprocessing become paramount for large scale production there are significant interests in the search for novel non-pathogenic rhamnolipid producers. The numbers of cultured organisms from the environmental samples are only a tiny fraction (0.001%) of the actual microbial diversity. There are significant number of microbial isolates that needs to be explored and exploited for rhamnolipid and other bioproduct manufacturing. Biosurfactant producing probiotic organisms will play a key role in the future of biosurfactant market. Edible emulsifiers from these processes would be applicable to many applications including food, cosmetic, environmental clean-up, biomedical and natural therapy. Rhamnolipid could be a potential alternative for the synthetic surfactant molecules and an important platform chemical cluster with the market value of $2.8 billion in 2023 (Grand View Research Inc., 2014). There is a significant need for the discovery of novel non-pathogenic rhamnolipid producers with enhanced production capacity and efforts to scale up through bioprocess engineering are important to meet the future predictions of biosurfactants market.

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Conflict of Interest Statement: 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.

Received: 23 June 2014; accepted: 11 August 2014; published online: 02 September 2014.
*Citation: Sekhon Randhawa KK and Rahman PKSM (2014) Rhamnolipid biosurfactants—past, present, and future scenario of global market. Front. Microbiol. **5**:454. doi: 10.3389/fmicb.2014.00454*
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 16 February 2015
doi: 10.3389/fmicb.2015.00088

Rhamnolipids: solution against *Aedes aegypti*?

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Aedes aegypti mosquitoes are the primary transmitters of dengue fever, urban yellow fever, and chikungunya viruses. This mosquito has developed resistance to the insecticides currently used to control their populations. These chemical insecticides are harmful to the environment and can have negative effects on human health. Rhamnolipids are environmentally compatible biological surfactants, but their insecticidal activity has not been extensively studied. The present study evaluated the potential larvicidal, insecticidal, and repellent activities of rhamnolipids against *A. aegypti*. At concentrations of 800, 900, and 1000 mg/L, rhamnolipids eliminated all mosquito larvae in 18 h and killed 100% of adults at 1000 mg/L. According to the results it may be conclude that rhamnolipids should be applied to control larvae and mosquitos besides present the repellency activity against *A. aegypti*.

Keywords: biosurfactant, entomology, *Pseudomonas aeruginosa*, tropical diseases, repellent

INTRODUCTION

Rhamnolipids are biological surfactants that exhibit low toxicity and high biodegradability with few adverse effects on the environment. Environmental compatibility associated with the use of renewable carbon sources and production by microorganisms makes these compounds excellent substitutes for chemical surfactants given that both have similar physico-chemical properties (Desai and Banat, 1997; Banat et al., 2000; Rahman et al., 2010; Sekhon Randhawa and Rahman, 2014). Due to their capacity to reduce surface and interfacial tension, application of these microbial compounds in a broad range of industrial sectors has been proven and new applications have been evaluated (Rahman and Gakpe, 2008; Vatsa et al., 2010).

Kim et al. (2011) demonstrated the potential of rhamnolipids produced by *Pseudomonas aeruginosa* as an insecticide against aphids. These microbial metabolites are also efficient against *Rhyzopertha dominica*, a beetle species that attacks stored grains (Kamal et al., 2012). Biosurfactants produced by microbes can be used to control pests (Awada et al., 2005).

Substitution of insecticides by compounds with low toxicities such as rhamnolipids may contribute to reduced environmental and social impacts compared to chemicals. Furthermore, these compounds are an alternative to resistance developed by a significant number of arbovirus vectors (arthropod-borne viruses).

Aedes aegypti is anthropophilic and endophilic mosquito species. The larvae use tracheal breathing, and their respiratory siphon is located at the posterior of their body. For gas exchange, they must remain in an angle close to 90° with the surface of the water (±20°) depending on larval stage (Christophers, 1960).

This culicid species is of significant medical importance because it is the primary vector of urban yellow fever, dengue fever and chikungunya (Eldridge, 2005; Vontas et al., 2012).

According to Tsai et al. (2013), 40% of the worldwide population is at risk of contracting dengue fever, rendering this disease one of the main arboviruses transmitted from mosquitoes to humans. An estimated 50–100 million cases of dengue fever occur worldwide per year (Kroeger and Nathan, 2006; Kourí et al., 2007; Garelli et al., 2013). These viruses are responsible for 30,000 deaths per year (World Health Organization, 2009).

Due to globalization, rampant population growth and climate change, it is likely that areas previously not know for dengue fever may become suitable sites for disease occurrence. After more than a half-century without a dengue outbreak in the United States, there have been recent outbreaks in Texas (2004–2005) and Florida (2009–2011) (Eisen and Moore, 2013). Rogers et al. (2014) reported a risk of dengue in some areas of Europe.

The use of chemical insecticides to control insect vectors is contrary to the recent global focus on developing products with low environmental impacts. Although population control is currently achieved using temephos for larvae and pyrethroids for adult mosquitoes (Chavasse and Yap, 1997), resistance to these compounds has been observed.

Potential negative effects of using chemical insecticides have led to research and development of products that are less harmful to the environment (Mittal, 2003). Biological pesticides produced by bacteria are effective against mosquitoes at low doses and do not affect other biological control agents (Walton and Mulla, 1992). This study evaluated the larvicidal, insecticidal, and repellent activities of rhamnolipids to verify their potential use as substitutes for insecticides that are currently used for *A. aegypti* control.

MATERIALS AND METHODS

RHAMNOLIPIDS PRODUCTION

The *P. aeruginosa* strain LBI 2A1 was used to produce rhamnolipids. Culture media and growth conditions were previously described by Müller et al. (2010). Fermentation using sunflower oil as a carbon source was performed in a bioreactor. Rhamnolipids isolated from sunflower oil were used in the experiments.

RHAMNOLIPIDS EXTRACTION

Fermented broth was centrifuged at 4000 rpm for 30 min, and equal volumes of cell-free supernatant and n-hexane were thoroughly mixed in a volumetric flask. The mixture was then allowed to settle until the organic and aqueous phases separated. The organic phase was removed and 85% H_3PO_4 1:100 (v/v) was added to the aqueous phase to precipitate rhamnolipids. Biosurfactants were extracted with ethyl acetate 1:1.25 (v/v). The mixture was shaken for 10 min, allowed to settle, and the upper phase was removed. This extraction process was repeated using the lower phase extract. Extracted rhamnolipids were concentrated using a rotary evaporator, and the viscous yellowish product was dissolved in methanol and concentrated again by evaporation of residual solvent at 45°C.

STATISTICAL ANALYSIS

Data were compared by one-way analysis of variance followed by quadratic regression test when significant differences were found at $P = 0.05$ (Sokal and Rohlf, 1995). The software used was Sisvar 5.3.

MOSQUITO AND LARVAL MAINTENANCE

The Center of Zoonosis Control (Santa Bárbara D'Oeste, São Paulo, Brazil) provided the parental *A. aegypti* specimens. The mosquitoes were maintained in plastic cages in a room with controlled temperature at 27 ± 2°C and a photoperiod of 12 h. Adults were fed 10% glucose w/v.

For egg and larval acquisitions, blood feeding was performed using a laboratory mouse sedated with ketamine (approved by the Ethics Committee on Animal Use, Protocol n°: 3698, Decision from CEUA N° 022/2011). After the blood feeding, 600 ml flasks with 300 ml of mineral water were placed in the cages. A 12 × 4 cm wood palette was used for oviposition. Flasks were filled with water until eggs were submerged. Larval feeding was performed with fish food (Löwenberg Neto and Navarro-Silva, 2004).

LARVICIDAL EVALUATION

A total of 40 ml of either rhamnolipids or control solution with 10 *A. aegypti* larvae in the third instar of development were added to 60 ml flasks. Small holes in the flask caps allowed for gas exchange. Control (mineral water) and solutions containing 50, 100, 200, 300, 400, 600, 800, 900, and 1000 mg/L rhamnolipids were evaluated. Experiments were performed in duplicate. Larvicidal activity was quantified based on time of permanence at the surface and the bottom and the numbers of attempts to stay on the surface, these factors were quantified in real time observations at intervals of 3 or 6 h. The larvae were considered dead when they did not show any sign of activity and movement for more than 3 h.

REPELLENCY EVALUATION

The following rhamnolipids concentrations, which demonstrated optimal performance in larvicidal evaluation, were used to evaluate repellency: 400, 800, and 1000 mg/L. Mice were sprayed with 2,400 μL of the solutions and labeled according to the type of treatment. Each cage contained a mouse for each treatment and a total of 25 mosquitoes, with an observation time of 40 min. The number of mosquitoes that landed on each mouse was counted as host attraction. Tests were performed four times, and the mosquitoes' activity was quantified in real time by the researchers.

INSECTICIDAL EVALUATION

The concentrations evaluated for insecticidal activity were the same as in the repellency test. Experiments were conducted in triplicate, with each cage containing 20 mosquitoes and was sprayed whit 3,200 μL of rhamnolipids solution. Dead mosquitoes from each treatment were chosen randomly, for analysis in optical microscopy.

RESULTS

LARVICIDAL ACTIVITY

Larvicidal activity was observed at all rhamnolipids concentrations evaluated, with a minimum mortality rate of 50%. Rhamnolipids concentrations of 800, 900, and 1000 mg/L killed all larvae at 18 h ($P = 0.00$; $R^2 = 0.92$), while 400 and 600 mg/L solutions required exposure for 48 h to reach 100% mortality ($P = 0.00$; $R^2 = 0.86$; **Figure 1**).

To evaluate if larvicidal activity was related to a reduction in surface tension and respiratory activity of the larvae, the residence time on the surface and the number of unsuccessful attempts to remain at the interface of the water surface were quantified (**Figure 2A**).

In solutions containing 50 and 100 mg/L rhamnolipids, larvae tended to stay longer on the surface compared to the control treatment. However, the larvae required more than one attempt to remain on the surface in the correct position to perform gas exchange (**Figure 2B**).

At concentrations greater than 400 mg/L, length of time on the surface was shorter than observed in controls. The number of attempts to settle on the surface increased proportionally to rhamnolipids concentration.

To verify the loss of larval activity over time, the time of larval permanence at the bottom of the flask was quantified (**Figure 3**).

Larvae in the control group had the lowest length of time at the bottom of the flask, with an average of 30.6 s. At concentrations of 800–1000 mg/L, the hydrostatic balance was broken between the

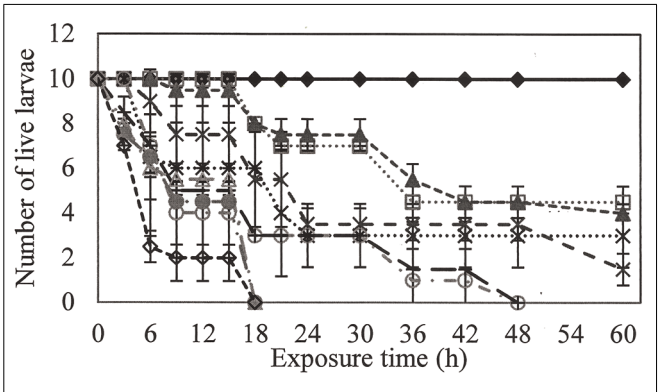

FIGURE 1 | Number of live larvae over time when exposed to different concentrations of rhamnolipids. Ten larvae of *Aedes aegypti* were exposed to: Control (-), 50(-□-), 100(-▲-), 200(-×-), 300 (-✱-), 400(-○-), 600(-•-), 800(-▽-), 900(-●-) e 1000(-◆-) mg/L of rhamnolipids.

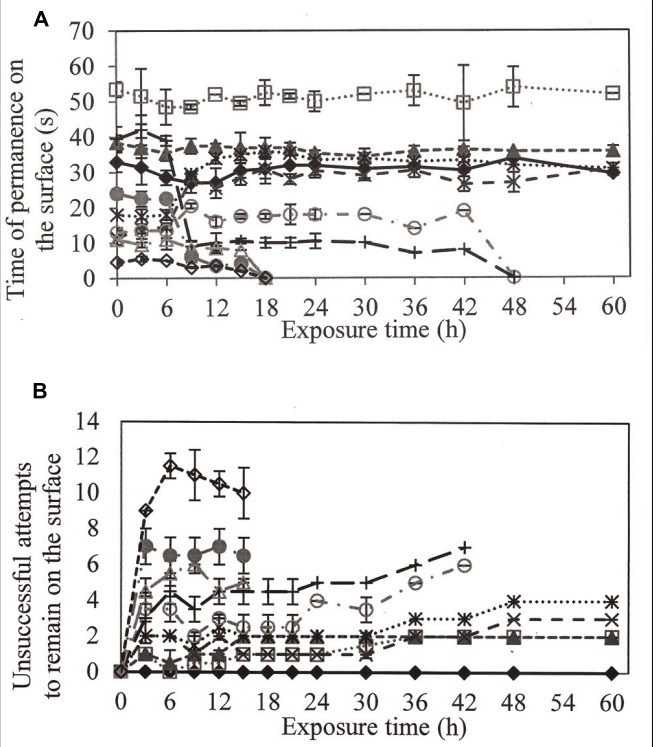

FIGURE 2 | (A) Time of permanence on the surface of the larvae to perform the gas exchange and **(B)** Number of unsuccessful attempts of the larvae in remain on the surface, when in contact with different solutions of rhamnolipids : Control (-), 50(-⊡-), 100(-▲-), 200(-✕-), 300 (-✱-), 400(-○-), 600(-+-), 800(-▵-), 900(-●-) e 1000(-◆-) mg/L of rhamnolipids.

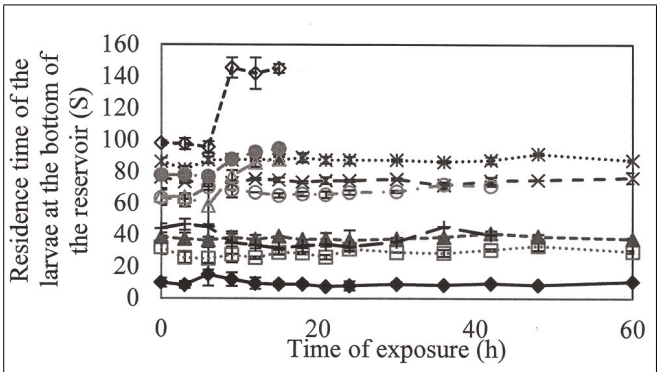

FIGURE 3 | Time that the *A. aegypti* larvae remained in the bottom of the reservoir along the time when exposed to different concentrations of rhamnolipids: Control (-), 50(-⊡-), 100(-▲-), 200(-✕-), 300 (-✱-), 400(-○-), 600(-+-), 800(-▵-), 900(-●-) e 1000(-◆-) mg/L of rhamnolipids.

water and larvae, as evidenced by an increase in the length of time at the bottom, culminating in death after 18 h.

REPELLENCY

The repellent property of rhamnolipids produced by *P. aeruginosa* was demonstrated given that mosquitoes had a higher incidence of landing on mice in the control group. The host attraction attempts

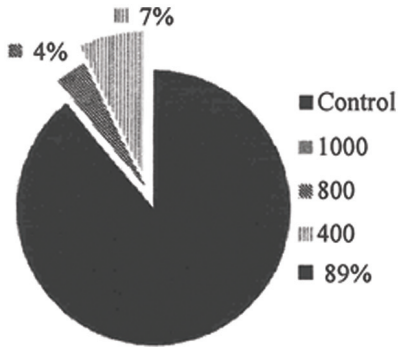

FIGURE 4 | Percentage of mosquitoes that landed on the mice and practiced hematophagy, subjected to different solutions of rhamnolipids: Control, 400, 800 e 1000 mg/L of rhamnolipids ($P = 0.00$; $R^2 = 0.97$; all concentrations evaluated were significantly different at $P = 0.05$).

were reduced with increased concentrations of the biosurfactant (**Figure 4**).

Another factor evaluated was the time spent feeding by mosquitoes at different rhamnolipids concentrations (**Table 1**).

According to the results, the microbial metabolite produced by *P. aeruginosa* appears to reduce the residence time spent by mosquitoes in addition to reducing the total number of mosquitoes.

Insecticidal effects

The insecticidal activity of rhamnolipids against mosquitoes showed a 100% elimination of adults at the highest concentration evaluated compared to only 8% in the control group (**Table 2**).

Rhamnolipids are thought to disrupt the cuticle of the mosquito, as shown in **Figure 5**.

DISCUSSION

The rhamnolipids solutions evaluated in this study have surface tension values ranging between 31.4 and 38.7 mNm^{-1}. As a result, the larvae experience difficulties at the water/air interface, which reduces their respiratory efficiency and increases the number of attempts to stay at the surface. Lack of maintaining the correct

Table 1 | Average time of hematophagy on mice treated with different rhamnolipids concentrations: control, 400, 800, and 1000 mg/L ($P = 0.00$; $R^2 = 0.99$).

Rhamnolipids concentration ($mg.L^{-1}$)	Average length of stay on the mice (*s*)	SD
Control	23.08 (a)*	1.65
400	5.15 (b)	3.28
800	1.18 (c)	1.36
1000	0.00 (d)	0.00

**Means followed by the same letter in a columm are not significantly different at $P = 0.05$.*

Table 2 | Number of live *Aedes aegypti* adults after application of different rhamnolipids concentrations: control, 400, 800, and 100 mg/L.

Rhamnolipids concentration ($mg.L^{-1}$)	Initial number of mosquitoes	Average number of live mosquitoes	Deviation	% of elimination
Control	20	18.33	1.53	8
400	20	2.67	0.58	87
800	20	1.33	0.58	93
1000	20	0.00	0.00	100

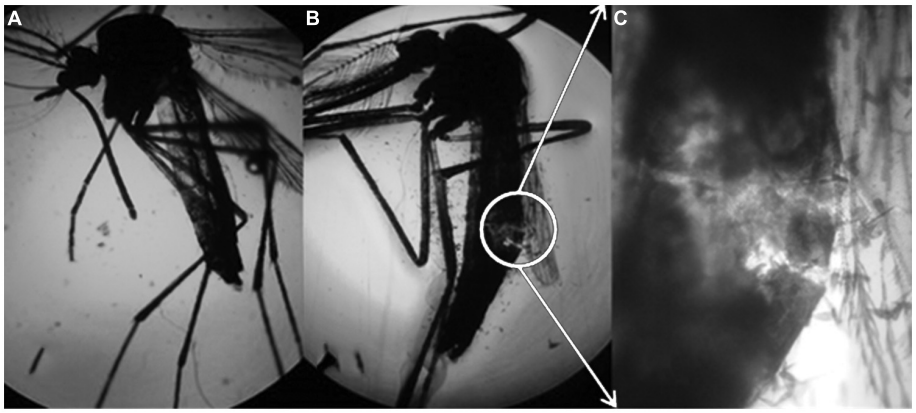

FIGURE 5 | (A) *Aedes aegypti* subjected to the control treatment (enhanced 50x), **(B)** *A. aegypti* subjected to the solution containing 1000 mg/L of rhamnolipids (enhanced 50x) and **(C)** expansion of figure (B) enhanced 100x.

siphon position during gas exchange was the cause of *A. aegypti* larval deaths. This is in agreement with that reported by Christophers (1960).

The air pockets in the trachea and tracheal trunks of *A. aegypti* larvae have a specific gravity close to that of water, which facilitates their ascent to the surface (Christophers, 1960).

The apex of the respiratory siphon is strongly hydrophobic, and secretions produced by the peri-spiracular glands are responsible for forming a non-wetting region when the structure is open and in contact with the surface (Christophers, 1960). Rhamnolipids likely interfere in this hydrophobic region, changing the wettability of the respiratory siphon and allowing water to flow in from the spiracular opening. Consequently, the hydrostatic balance is altered, which leads to difficulty returning to the surface. Furthermore, there is a greater energetic cost during transit due to the need for active swimming.

According to Davis et al. (2013), compounds produced by microorganisms are related to the feeding behavior of insects and may be powerful repellents. Verhulst et al. (2009) reported that odors produced by *P. aeruginosa* are not attractive to the mosquito species *Anopheles gambiae*.

The present study raises the hypothesis that the characteristic odor of rhamnolipids is recognized by *A. aegypti* adults as being unfavorable as a food source, thus leading to repellent behavior.

Rhamnolipids decreased the incidence of adult mosquitoes landing on mice and reduced the number of biting attempts.

Rhamnolipids led to breakup of the mosquito cuticle, leading to death. According to Kim et al. (2011), di-rhamnolipids act by thinning the cuticle in aphids. Dehydration of adjacent cell membranes also occurs, which separates cellular components and results in death.

Kamal et al. (2012) reported insecticidal activity of rhamnolipids against *Rhyzopertha dominica* and hypothesized that this biosurfactant acts on cuticular waxes and inter segmental membranes, which may also occur in *A. aegypti*.

Based on the results obtained in this study, rhamnolipids can replace currently used insecticides (e.g., temephos and pyrethroids). In addition to *A. aegypti* resistance against these insecticides, these chemicals are harmful to the environment and can lead to human health problems.

On the contrary rhamnolipids have been reported to be environmentally friendly and have low toxicity, which makes them suitable substitutes for chemical insecticides.

The rhamnolipids produced by *P. aeruginosa* LBI 2A1 have insecticidal activity against larvae and adults of *A. aegypti* and can be used as a repellent against mosquitoes. Therefore this microbial metabolite may be a new weapon to combat dengue and other arboviruses transmitted by *A. aegypti*.

ACKNOWLEDGMENT

We thank Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), for fellowships and financial support (Process 2011/15345-8).

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Conflict of Interest Statement: 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.

Received: 27 November 2014; accepted: 23 January 2015; published online: 16 February 2015.

Citation: Silva VL, Lovaglio RB, Von Zuben CJ and Contiero J (2015) Rhamnolipids: solution against Aedes aegypti? Front. Microbiol. ***6****:88. doi: 10.3389/fmicb.2015.00088*

This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology.

frontiers in Microbiology

OPINION
published: 07 April 2015
doi: 10.3389/fmicb.2015.00245

Bioemulsifiers are not biosurfactants and require different screening approaches

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Keywords: biosurfactants, bioemulsifiers, rhamnolipids, lipoproteins, lipopeptides, sophorolipids

The terms biosurfactant and bioemulsifier have often been used interchangeably to describe surface active biomolecules. However, it is important to note that there are marked differences between them especially based on their physico-chemical properties and physiological roles. Although bioemulsifiers and biosurfactants are both amphiphilic in nature and are produced by a wide range of microorganisms, each exhibit characteristic roles in nature. These microbial surfactants have recently received increased scientific attention due to their unique characteristics relative to chemically derived surfactants. Their unique features include; non-toxicity, biodegradability, biocompatibility, efficiency at low concentrations and their synthesis from natural substrates under mild environmental conditions.

OPEN ACCESS

Edited by:
Weiwen Zhang, Tianjin University, China

Reviewed by:
Kamaljeet K. Sekhon Randhawa, Aalborg University Copenhagen, Denmark
Wael Ismail, Arabian Gulf University, Bahrain

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Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *05 December 2014*
Accepted: *13 March 2015*
Published: *07 April 2015*

Citation:
Uzoigwe C, Burgess JG, Ennis CJ and Rahman PKSM (2015) Bioemulsifiers are not biosurfactants and require different screening approaches. Front. Microbiol. 6:245. doi: 10.3389/fmicb.2015.00245

The Physiological Roles of Biosurfactants and Bioemulsifers are Based on their Physicochemical Properties

The chemical composition of biosurfactants and bioemulsifiers is different and this may contribute to their specific roles in nature and biotechnological applications. Biosurfactants are generally low molecular weight microbial products composed of sugars, amino acids, fatty acids and functional groups such as carboxylic acids. The glycolipids (rhamnolipids, sophorolipids, trehalose lipids) consist of different sugars linked to β-hydroxy fatty acids while lipopeptides (surfactin, iturin, fengycin) consist of cycloheptapeptides with amino acids linked to fatty acids of different chain lengths. These molecules are amphiphilic in nature and this property allows them to dissolve in both polar and non-polar solvents (Perfumo et al., 2009; Satpute et al., 2010; Smyth et al., 2010a,b). Biosurfactants are known for their excellent surface activity which involves lowering the surface and interfacial tension between different phases (liquid-air, liquid-liquid, and liquid-solid); possessing a low critical micelle concentration (CMC) and formation of stable emulsions. The ability to lower surface and interfacial tension is by adsorption of biosurfactant onto the different phases causing more interaction and mixing of dissimilar phases. The CMC is the minimum concentration of biosurfactant required to yield the minimum surface tension in water and form micelles. They can act as wetting, foaming and solubilizing agents in different industrial processes. In oil polluted environments (solid or liquid), biosurfactants can enhance the effective dispersion and bioavailability of hydrophobic pollutants for microbial access and degradation by the process of micelle solubilization. They have the ability to mobilize hydrophobic molecules bound on solid substrata increasing the flow rate. Biosurfactants produced in a growth associated manner (trehalose lipids) confer increased cell surface hydrophobicity on the producing organism. Cell surface hydrophobicity is essential for easy access and

subsequent uptake of hydrophobic substrates by microbial cells (Perfumo et al., 2009; Satpute et al., 2010). These biomolecules are therefore suitable agents for different bioremediation technologies.

Bioemulsifiers are higher in molecular weight than biosurfactants as they are complex mixtures of heteropolysaccharides, lipopolysaccharides, lipoproteins and proteins (Perfumo et al., 2009; Smyth et al., 2010a; Sekhon-Randhawa, 2014). They are also known as high molecular weight biopolymers or exopolysaccharides. Similar to biosurfactants, these molecules can efficiently emulsify two immiscible liquids such as hydrocarbons or other hydrophobic substrates even at low concentrations but in contrast are less effective at surface tension reduction. Therefore, they can be said to possess only emulsifying activity and not surface activity. They are also involved in solubilization of poorly-soluble substrates, thus increasing their access and availability for biodegradation. However, in an oil polluted environment, these molecules play a specific role of binding tightly to dispersed hydrocarbons and oils preventing them from merging together. This process is known as stabilization of emulsion and has been attributed to the high number of reactive groups exposed in their structures. Bioemulsifiers are able to stabilize emulsions by increasing their kinetic stability and this property has increased their usefulness in the cosmetics, food, pharmaceutical and petroleum industries. Reports have shown that the efficient emulsifying activity of bioemulsifiers is a function of their chemical composition (Calvo et al., 2009; Monteiro et al., 2010). According to Willumsen and Karlson (1997), surface active biomolecules are categorized into surfactants and emulsifiers, while surfactants play the role of surface tension reduction, emulsifiers are involved in formation and stabilization of emulsions. However, some biomolecules possess both surfactant and emulsifying properties which contributes to their unique functions and broad industrial uses.

The combination of polysaccharide, fatty acid and protein components in bioemulsifiers confers upon them better emulsifying potential and ability to stabilize emulsions. It is also important to note that some efficient bioemulsifiers consists of only polysaccharides and proteins. Emulsan is a lipopolysaccaharide bioemulsifier with a molecular weight of 1000 kDa produced by *Acinetobacter calcoaceticus* RAG-1. It is one of the most widely studied emulsifiers from bacteria. In pure form, emulsan shows emulsifying activity at low concentrations (0.01–0.001%). It increases the bioavailability of poorly soluble substrates in aqueous environments for microbial access and degradation by coating the hydrophobic substrate to form a minicapsule. The producing bacterium can also have direct access to hydrophobic substrates but the emulsifying activity is exhibited by secreted emulsan. However, this emulsifier can efficiently emulsify mixtures of aliphatic and aromatic hydrocarbons in balanced proportions but cannot emulsify their pure forms. The emulsifying activities of emulsan are therefore attributed to its fatty acid components which act as multiple sites for binding different hydrophobic phases (Choi et al., 1996; Ron and Rosenberg, 2001).

A bioemulsifier also known as emulsan produced by *Acinetobacter calcoaceticus* BD4 has been reported. BD4 Emulsan consists of a polysaccharide-protein complex without a lipid moiety thereby varying from RAG-1 Emulsan. BD4 emulsan exhibited its optimum emulsification of different hydrocarbons when the two components (polysaccharides and proteins) were mixed together but had no emulsifying activity when these components existed in separate forms (Kaplan and Roseberg, 1985). Kaplan et al. (1987) also explained the mechanism of emulsifying activity by the protein-polysaccharide mixture. The protein being the hydrophobic part binds the hydrocarbons in a reversible form while the polysaccharide attaches to the protein to produce a stable oil-in-water emulsion. Lukondeh et al. (2003) also described bioemulsifiers as amphiphilic, with the polysaccharide polymer as the hydrophilic part covalently attached to the protein hydrophobic part.

Furthermore, alasan is an anionic alanine-containing bioemulsifier produced by *A. radioresistens* KA53. This bioemulsifier is a complex of alanine, polysaccharides and proteins with a molecular mass of 1 MDa. It exists as cell-bound and secreted proteins and can efficiently emulsify a variety of hydrocarbons including; long chains, alkanes, aromatics, poly aromatic hydrocarbons (PAHs), paraffins and crude oils. Alasan can facilitate solubilization of PAH by aggregating them into oligomer molecules and this mechanism increases their solubility by 20-fold, thereby speeding up biodegradation. The protein components in the alasan molecule have been associated with its ability to emulsify various hydrocarbons unlike emulsan. This is a 45 kDa protein with highly hydrophobic regions folded over in loops contributing to emulsification and solubilization activity of alasan (Navon-Venezia et al., 1995). The contribution of protein components to the emulsifying potential of bioemulsifiers have also been reported in the literature. Sar and Rosenberg (1983) have also demonstrated that the protein content of bioemulsifier plays an important role in the emulsification activity.

Mannoproteins are glycoproteins extracted from the cell walls of many yeasts. These molecules are classified as structural and enzymatic mannoproteins depending on their chemical compositions and specific functions in living systems. Structural mannoproteins are the most abundant and are composed of a small protein portion linked to a greater carbohydrate portion (mannopyranosyl) while enzymatic mannoproteins have more protein moieties in their structures. These molecules are not only effective emulsifiers but have been associated with stimulation of host immunity by activating immune cells and proteins as well as triggering the production of antibodies (Casanova et al., 1992; Oliveira et al., 2009). A mannoprotein bioemulsifier from *Kluyveromyces marxianus* has been reported to form a 3-month old stable emulsion in corn oil (Lukondeh et al., 2003). Alcantara et al. (2014) reported a mannoprotein bioemulsifier from *Saccharomyces cerevisiae* 2031 consisting of 77% carbohydrate and 23% protein. A bioemulsifier with 53% protein, 42% polysaccharide and only 2% lipid has been reported from *Acinetobacter* sp. by Jagtap et al. (2010). These mannoprotein emulsifiers were capable of forming stable emulsions with different hydrocarbons, organic solvents and waste oils, suggesting their applications as cleaning agents.

Chemical components such as uronic acid have also been associated with the emulsifying capability of bioemulsifiers. *Halomonas eurihalina* produces an exopolysaacharide (EPS) that

TABLE 1 | Physicochemical properties of biosurfactants and bioemulsifiers.

Biosurfactant	Class	Microbial origin	Physicochemical properties	Physiological roles	References
Low molecular weight Glycolipid	Rhamnolipids	*Pseudomonas aeruginosa DS10-129*	One or two rhamnose sugars linked to 3-hydroxydecanoic acid. ST- 28 mN/m EI24- 53–73%	Bioremediation technology	Rahman et al., 2002
	Sophorolipids	*Candida bombicola Candida tropicalis*	Disaccharide sophoroses (2-O-β-D-glucopyranosyl-D-glucopyranose) linked to fatty acids. ST- 32.1–34.2 mN/m	-Detergent additive for enhanced performance and stain removal -Hard surface cleaning, Antibacterial activity.	Develter and Lauryssen, 2010; Joshi-Navare et al., 2013
	Trehaloselipid	*Rhodococcus wratislaviensis BN38 Norcardia farcinica BN26*	Non-reducing disaccharide with two glucose units linked in an α,α-1,1-glycosidic linkage ST-24.4 mN/m EI- 23-70%	Bioremediation of polluted sites, Antitumor activity	Tuleva et al., 2008; Christova et al., 2014
Lipopeptides	Surfactin	*Bacillus subtilis K1 Bacillus siamensis*	Heptacyclic depipeptides consisting of two acidic amino acids, four hydrophobic amino acids and C_{13-17} β-hydroxyfatty acids ST- 22–27.9 mN/m	-Enhanced oil recovery -Antibacterial -Antiviral -Antimycoplasma -Antitumoral -Anticoagulant -Enzyme inhibition	Ongena and Jacques, 2007; Varadavenkatesan and Ramachandra, 2013; Pathak and Keharia, 2014
	Iturin	*Bacillus subtilis K1 Bacillus amylofaciens*	Cycloheptapeptide with seven amino acids and C13-16 β-amino fatty acids ST-30–37 mN/m EI- 32–66%	-Antifungal -Biopesticides	Arrebola et al., 2010; Pathak and Keharia, 2014
	Fengycin	*Bacillus subtilis*	Cyclic deca-depsipeptides with C14-21 β-hydroxyfatty acid and 10 amino acids	-Strong fungitoxic agent against filamentous fungi, Immunomodulating activities	Arrebola et al., 2010; Pathak and Keharia, 2014
High molecular weight-Bioemulsifiers	RAG-1 Emulsan	*Acinetobacter* sp. *ATCC 31012 (RAG-1)*	Lipopolysaccharides. Lipid moiety (unsaturated fatty acid of C10-18) Polysaccharide moiety (D-galactosamine, D-galactosaminuronic acid, di-amino-6-deoxy-D-glucose)	-Increase surface area and bioavailiability of poorly-soluble substrates -Binding to toxic heavy metals	Choi et al., 1996; Ron and Rosenberg, 2001
	BD4 Emulsan	*Acinetobacter calcoaceticus BD4 13*	Protein-polysaccharide complex. Polysaccharide moiety (repeating heptasaccharides of L-rhamnose, D-glucouronic acid, D-mannose)	Stabilizes oil-in-water emulsions	Kaplan and Roseberg, 1985; Kaplan et al., 1987
	Alasan	*Acientobacter radioresistens KA53*	Alanine-containing polysaccharide and proteins	Emulsification and solubilization activity	Navon-Venezia et al., 1995; Walzer et al., 2006
	Mannoproteins	*Saccharomyces cerevisiae Kluyveromyces marxianus*	Polysaacharide and proteins	Formation of stable emulsion with hydrophobic substrates Stimulation of immune system	Casanova et al., 1992; Lukondeh et al., 2003
	Uronic acid bioemulsifiers	*Halomonas eurihalina Klebsiella* sp.	Polysaccharides-proteins-uronic acids	Emulsification and detoxification of hydrocarbons	Martínez-Checa et al., 2002; Jain et al., 2013

can efficiently emulsify hydrocarbons. This EPS is rich in uronic acids containing less carbohydrate and protein components. The uronic acid has been associated with the ability of EPS to emulsify and detoxify hydrocarbons (Martínez-Checa et al., 2002). Jain et al. (2013) also reported on a bioemulsifier with a molecular weight of 2716 kDa, consisting of mainly total sugars, uronic

acids and proteins produced by *Klebsiella* sp. On a general note bioemulsifiers have been associated with a number of potential applications including: remediation of oil polluted water and soil; enhanced oil recovery and clean-up of oil contaminated vessels and machineries; heavy metal removal (Monteiro et al., 2010; Zheng et al., 2012; Panjiar et al., 2014); formation of stable emulsions in food and cosmetics industries (Campos et al., 2014); and therapeutic activities (antibacterial, antifungal, pesticidal and herbicidal agents) (Ahmed and Hassan, 2013). The physico-chemical properties of bioemulsifiers and biosurfactants are presented in **Table 1**.

At this point, it is important to note that the ability to reduce surface and interfacial tension stands as the distinctive contrast between biosurfactant and bioemulsifiers. These molecules can both form stable emulsions but it is still unclear why bioemulsifiers do not show significant changes in surface/interfacial tension between different phases (liquid-air, liquid-liquid, liquid-solid). This outstanding contrast between biosurfactants and bioemulsifiers is especially important for accurate screening and identification procedures from microbial culture broths.

Screening for detection of biological surfactants in culture media has often been based on the measurement of surface and interfacial tension. Other methods include drop collapse, oil displacement, haemolysis tests and use of the emulsification index E_{24} (Cooper and Goldenberg, 1987), emulsification activity (Rahman et al., 2002, 2010; Satpute et al., 2008), and bacterial adhesion to hydrocarbon assay (BATH) also known as cell surface hydrophobicity (Rosenberg et al., 1980). According to Satpute et al. (2008), these methods are insufficient for the identification and differentiation of bioemulsifiers from biosurfactants. This is due to the fact that bioemulsifiers are best known for emulsification of liquids without significant changes in surface/interfacial tension of their growth medium or between different phases. In addition, experimental reports have shown that surface tension measurements and emulsification index/activity screening methods do not correlate. These methods have often resulted in elimination of bioemulsifiers since they do not exhibit significant changes in surface/interfacial tension and may give negative results during screening tests (Ellaiah et al., 2002). Emulsification index E_{24} and emulsification activity are screening tests for measuring the emulsification capacity of any surface active molecule with different hydrocarbons (Jagtap et al., 2010). BATH is an indirect screening method to detect cell-bound emulsifying agents produced by microorganisms. Cell-bound surface active agents are important in hydrocarbon assimilation during biodegradation and bioremediation of polluted environment. When microorganisms show low surface hydrophobicity it is an indication that emulsifying agents have been released extracellularly into the production media.

Using the emulsification index E_{24} test, Viramontes-Ramos et al. (2010) identified six isolates that could efficiently emulsify different hydrocarbons (more than 50% against diesel, decane, kerosene and motor oil) without showing a significant reduction in surface tension of their culture broths. The emulsifying potential of surface active compounds from different yeasts showing no reduction in surface tension, has been evaluated using the emulsification index test (Amaral et al., 2006; Monteiro et al., 2010). Toledo et al. (2008) reported the growth and production of exopolymers by three bacteria, *Bacillus subtilis* 28, *Alcaligenes faecalis* 212, and *Enterobacter* sp. 214 on glucose media amended with different hydrocarbons. These biopolymers exhibited high emulsifying activity but without reduction in surface tension of their culture media. *Candida tropicalis* grown on n-hexadecane produced an extracellular emulsifier with surface tension of 49.5 mN/m but was capable of emulsifying various hydrocarbons including aromatic hydrocarbons (Singh and Desai, 1989). Souza et al. (2012) identified a potent bioemulsifier produced by *Yarrowia lipolytica* which exhibited high emulsification values with hydrocarbons, with no reduction in surface tension.

Concluding Remarks

A number of literature reports have regarded all microbial surfactants as biosurfactants even when bioemulsifiers have been identified. These two types of surfactants are closely related especially in their ability to form stable emulsions. However, screening methods for identification of microbial surfactants based on surface tension reduction are bound to eliminate producers of bioemulsifiers and retain producers of biosurfactants. Therefore, the opinion of authors in this review is that bioemulsifiers are not biosurfactants (they both emulsify, but only biosurfactants have the surfactant effect of reducing surface tension) and current research toward identification should be based on a broad spectrum of tests and not only on surface tension measurements, which are often used as primary tests. In addition, there is minimal research on the discovery and characterization of new emulsifiers from microorganisms due to the lack of a clear picture of the distinguishing features between biosurfactants and bioemulsifiers. Biosurfactants and bioemulsifiers are both unique microbial products showing advantageous features and may become future substitutes for chemically produced ones. We have noted that bioemulsifiers have often been erroneously eliminated or mis-identified in the past but, since these molecules have great potential for green technology, carefully designed screening methods will be an essential step toward the discovery of novel microbial emulsifiers.

Acknowledgments

PR wishes to thank Commonwealth Scholarship Commission (UK) for PhD studentship award (CU, award ref: NGCS-2012-335).

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Conflict of Interest Statement: 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.

frontiers in MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 16 December 2014
doi: 10.3389/fmicb.2014.00711

Oil degradation and biosurfactant production by the deep sea bacterium *Dietzia maris* As-13-3

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Recent investigations of extreme environments have revealed numerous bioactive natural products. However, biosurfactant-producing strains from deep sea extreme environment are largely unknown. Here, we show that *Dietzia maris* As-13-3 isolated from deep sea hydrothermal field could produce di-rhamnolipid as biosurfactant. The critical micelle concentration (CMC) of the purified di-rhamnolipid was determined to be 120 mgL^{-1}, and it lowered the surface tension of water from 74 ± 0.2 to 38 ± 0.2 mN m^{-1}. Further, the alkane metabolic pathway-related genes and di-rhamnolipid biosynthesis-related genes were also analyzed by the sequencing genome of *D. maris* As-13-3 and quantitative real-time PCR (Q-PCR), respectively. Q-PCR analysis showed that all these genes were induced by *n*-Tetradecane, *n*-Hexadecane, and pristane. To the best of our knowledge, this is first report about the complete pathway of the di-rhamnolipid synthesis process in the genus *Dietzia*. Thus, our study provided the insights into *Dietzia* in respects of oil degradation and biosurfactant production, and will help to evaluate the potential of *Dietzia* in marine oil removal.

Keywords: *Dietzia*, biosurfactant, di-rhamnolipid, hydrocarbon degradation, genome sequence, biosynthesis pathway

INTRODUCTION

Biosurfactants (BS) are surface activity compounds possessing both hydrophilic and hydrophobic moieties (Satpute et al., 2010). Due to the diversity of microorganisms and their metabolites, microorganisms produced many kinds of biosurfactants with different structure and physico-chemical properties. Based on structure diversity, biosurfactants can be classified into glycolipid, phospholipids, fatty acids, neutral lipids, lipopeptides, etc. (Bharali and Konwar, 2011). Biosurfactants may have several advantages over their chemically synthesized counterparts: high reliability and excellence even at extreme temperatures, pH, and salinities, lower toxicity, low critical micelle concentration (CMC) value, and biodegradablity. Those advantages make biosurfactants the most ideal substitute for the chemically synthesized surfactants (Plante et al., 2008).

Up to now, hundreds of biosurfactants have been identified, among which rhamnolipids (RLs) have been extensively studied. RLs are glycosides that are composed of a glycon and a aglycon part linked to each other via O-glycosidic linkage. The glycon part is composed of one or two rhamnose moieties linked to each other through α-1,2-glycosidic linkage (Edwards and Hayashi, 1965). Several kinds of different homologs of rhamnolipid have been reported (Abdel-Mawgoud et al., 2010), and mainly produced by bacteria of *Pseudomonas* and *Burkholderia* reported by far. Later, several other bacteria were also able to produce RLs as well, including *Renibacterium salmoninarum* (Christova et al., 2004), *Cellulomonas cellulans* (Arino et al., 1998), *Nocardioides* sp. (Vasileva-Tonkova and Gesheva, 2005), and *Tetragenococcus koreensis* (Lee et al., 2005).

RLs have a wide range of application including enhanced biodegradation of diesel and oil (Lang and Wullbrandt, 1999; Maier and Soberon-Chavez, 2000; Wang et al., 2008), and bioremediation of organic and heavy metal polluted sites (Mulligan, 2005). Besides, RLs are broadly used in the cosmetic industry for products such as moisturizers, toothpaste (Desai and Banat, 1997), and also be used in medical industry for their antimicrobial and antiviral properties (Ito et al., 1971; Lang and Wullbrandt, 1999; Haba et al., 2003). Those features make RLs a promising product.

Members of the genus *Dietzia* have been confirmed as alkane degraders (Rainey et al., 1995; Yumoto et al., 2002; von der Weid et al., 2007; Wang et al., 2011). In addition, *Dietzia* strains have the ability to degrade polycyclic aromatic compounds, including naphthalene (von der Weid et al., 2007), phenanthrene (Al-Awadhi et al., 2007), benzoate (Maeda et al., 1998), fluoranthene (Kumar et al., 2011). Recently, it was reported that two isolates of *Dietzia* can use alkane as the sole carbon to produce biosurfactants. Wax ester-like compounds were produced by *D. maris* WR-3 as biosurfactants (Nakano et al., 2011). Different kinds of biosurfactant were produced by *Dietzia* sp.

DQ12-45-1b when using different alkanes as sole carbon source (Wang et al., 2013). However, the chemical characterization and properties of the biosurfactants have not been investigated in details.

In this paper, we reported an biosurfactant-producing strain *D. maris* As-13-3 isolated from deep sea hydrothermal field. When using *n*-hexadecane as the sole carbon source, it could produce di-rhamnolipid as biosurfactant. To further explore the mechanism of di-rhamnolipid biosynthesis, the genome of *D. maris* As-13-3 was sequenced and analyzed. The di-rhamnolipid biosynthesis-related genes were identified. These results bring new insights into the genetic and physiology of the genus *Dietzia*.

MATERIALS AND METHODS

STRAIN AND CULTIVATION

Strain As-13-3 was obtained from Marine Culture Collection of China (MCCC) with accession number as MCCC 1A00160, which was originally isolated from deep sea hydrothermal field environment of Southwest Indian Ocean (Chen and Shao, 2009). For the production of biosurfactant, it was cultivated in mineral salts medium (MSM; pH 7.4), which is composed of $(NH_4)_2SO_4$, 10 g L^{-1}; KCl, 1.1 g L^{-1}; NaCl, 30 g L^{-1}; $FeSO_4$, 2.8×10^{-4} g L^{-1}; KH_2PO_4, 3.4 g L^{-1}; K_2HPO_4, 4.4 g L^{-1}; $MgSO_4$, 7.0 g L^{-1}; yeast extract, 0.5 g L^{-1}; trace elements solution, 0.5 ml L^{-1}; and 2% (v/v) *n*-hexadecane as the sole carbon source. The trace elements solution contained (per liter): $ZnSO_4$, 0.29 g; $CaCl_2$, 0.24 g; $CuSO_4$, 0.25 g; and $MnSO_4$, 0.17 g, it was filtered with a 0.22-μm pore membrane. All chemicals were analytical grade unless specified, *n*-hexadecane was 98% pure and purchased from Fluka (Buchs, Switzerland). Bacteria were cultured in a 1L Chemostat (180 rev min^{-1}) at 28°C for 7–10 days.

CHEMICALS

All solvents and reagents used in this study are AR grade. The purity of all hydrocarbons used in this study is over 99% and checked by instrumental analysis.

NUCLEIC ACID EXTRACTION

Bacterial genomic DNA for strain As-13-3 was extracted using Axygen® AxyPrep™ Bacterial Genomic DNA Miniprep Kit (Axygen American).

A 2 mL sample was collected from each culture in the exponential phases using the RNA Bacteria Protect Reagent (Qiagen, Valencia, CA, USA). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol and subsequently treated with DNase I (Invitrogen, Carlsbad, CA, USA). The RNA yield was estimated using a Nanodrop UV Spectrometer (Thermo Scientific, Wilmington, DE, USA).

PHYLOGENETIC ANALYSIS

Strain As-13-3 was identified on the basis of its phylogenetic and physiological characteristics. The 16S rRNA gene was amplified from the genomic DNA. The 16S rRNA sequences were aligned and a phylogenetic tree was constructed using the Neighbor-Joining method.

SURFACE ACTIVITY TEST

Surface tension of the culture and its supernatant was measured using a DU Nouy ring tensiometer (model JZ-200A; Chengde Precision Testing Machine Co. Ltd., Hebei, China) according to McInerney et al. (1990). Pure water added with *n*-hexadecane at a final concentration of 2% (v/v) was used as a control. The surface tension value was the average of three repeats of the same culture.

EFFECT OF CARBON SOURCES ON BIOSURFACTANT PRODUCTION

In order to investigate effect of carbon sources on the production of biosurfactant, strain As-13-3 was inoculated into MSM supplemented with different carbon source (2% v/v or m/v) including Glycerol, Glucose, Sodium citrate, Sodium acetate, Sodium pyruvate, *n*-Dodecane, *n*-Tetradecane, *n*-Hexadecane, Pristane, and Olive oil, and this culture was shaked (180 rev min^{-1}) at 28°C for 7 days, then the OD_{600} and surface tension of culture broth were tested, respectively.

CELL SURFACE HYDROPHOBICITY

Cell surface hydrophobicity rates of strain As-13-3 were measured by bacterial adhesion to organic compounds according to Rosenberg (Rosenberg et al., 1980). Briefly, the cultures (5 ml) were centrifuged at 8000 rpm for 5 min. The cell pellets were washed twice with 3 ml AB buffer composed of $K_2HPO_4{\cdot}3H_2O$, 22.2 g; KH_2PO_4, 7.26 g; and $MgSO_4{\cdot}\ 7H_2O$, 0.2 g, per liter H_2O at pH 7.4, followed by resuspension in AB buffer to ensure the absorbance of the cell suspension at 600 nm of 0.5–0.6 (OD of the initial cell suspension). Hydrocarbons (0.2 ml), including *n*-Dodecane, *n*-Tetradecane, *n*-Hexadecane, Pristane, Toluene, and Paraffins, were added to 1.2 ml of the cell suspension, mixed thoroughly, transferred into a round-bottom test tube (i.e., 10 mm), and vortexed for 2 min. The mixture was then kept undisturbed at room temperature for 1 h, to achieve phase separation. The lower aqueous phase was then carefully removed and its OD values were measured at 600 nm (OD of the aqueous phase). Hydrophobicity was expressed as the percentage of adherence to the hydrocarbon, which was calculated as follows: 100 × (1-OD of the aqueous phase/OD of the initial cell suspension). The cells grown in LB medium were used as a negative control.

EMULSIFICATION INDEX (E_{24})

The emulsification index (E_{24}) of supernatant samples was determined according to Burgos-Díaz et al. (2011). Generally speaking, 2 mL of organic matter was added to 2 mL of culture supernatant, then vortexed at a high speed for 2 min. The mixture was then allowed to stand still for 24 h prior to measurement. Emulsification activity was defined as the height of the emulsion layer divided by the total height and expressed as percentage. Data represent the mean of three independent experiments.

BIOSURFACTANT EXTRACTION AND PURIFICATION

The biosurfactant extraction and purification process was done according to Qiao and Shao (2010). The culture broth was first centrifuged for 20 min at 12,000 rpm at 4°C, a hydrophobic layer floating on the surface was scraped out and washed with three volumes of hexane to remove alkanes. Then the crude material was extracted with chloroform/methanol (v/v 1:1) for three times. The solvent was then removed by rotary evaporation at

35°C under reduced pressure, and the crude extract was stored at −20°C until subjected to further purification.

The crude extract was further purified using a silica gel column, the column was washed with the following solvent systems with increasing polarity: chloroform; methanol/chloroform (95:5, v/v); methanol/chloroform (90:10, v/v); methanol/chloroform (80:20, v/v); 100% methanol. The eluates demonstrated to have the highest surface activity was further separated by a Sephadex LH-20 gel column. The velocity of flow was about 12 s per drop, and flow solvent was chloroform: methanol (1:1, v/v). After purification by using Sephadex LH-20 gel column, the eluates exhibited a high surface activity were collected and pooled together for further purification.

THIN LAYER CHROMATOGRAPHY (TLC) ANALYSIS

The eluate was further analyzed by TLC on silica gel F_{254} with the following solvent system: chloroform/methanol/water (80:15:2, v/v/v).

Four reagents were used to test the category of the surfactant: phenol/sulfuric acid reagent for glycolipids; 0.2% ninhydrin reagent for lipopeptides; cobalt chloride/acetone reagent for phospholipids; and bromocresol green for lipid-organic acids.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS

HPLC analysis of the biosurfactant was done according to Bharali and Konwar with slight modification (Bharali and Konwar, 2011). The components of partially purified biosurfactant were fractionated using a gradient elution HPLC (Waters 2487) with a UV and Evaporative Light-scattering Detector (ELSD). A Chromolith Fast Gradien RP-18e, with a dimension of 50–3 mm was used. An acetonitrile-water gradient containing 0.1% trifluoroacetic acid was used, starting with 5% B increase to 100% B within 0.8 min, 100% B for 1.1 min. The mobile phase was kept at a flow rate of 1.5 ml/min and the sample injection volume was 10 μl. All fractions eluted from the HPLC column were collected at different retention times. The fractions were then evaporated at room temperature to remove all of the solvent part to obtain a purified biosurfactant. The fraction having the height reduction in surface tension of water was selected and further characterized.

CHEMICAL CHARACTERIZATION OF THE PURIFIED BIOSURFACTANT: LCQ-MS AND NMR

Mass spectrometer (MS) characterization and detection of the biosurfactant was carried out according to Qiao and Shao (2010). The purified biosurfactant was characterized by using a LCQ quadrupole ion-trap MS (Finnigan MAT, San Jose, CA, USA) with electrospray ionization (ESI). Standard solutions and samples were infused into the mass spectrometer at a flow rate of 10 μL min^{-1}. In the ESI source, the nitrogen sheath and auxiliary gas flows were maintained at 50 and 5, respectively; these refer to arbitrary values set by the software. The heated capillary temperature was 275°C, and the spray voltage was set to 5000 V. The positive ion mode was used and scans were run over the 100–1000 m/z range.

The purified biosurfactant was dissolved in denatured chloroform ($DCCl_3$) and analyzed with nuclear magnetic resonance (NMR). The NMR spectra were recorded on Bruker 400 MHz NMR spectrometer at 25°C.

DETERMINATION OF CRITICAL MICELLE CONCENTRATION (CMC)

For the determination of CMC value, different aqueous concentrations of biosurfactants were prepared. Surface tensions were measured using a DU Nouy ring tensiometer as described before. And then the surface tension-concentration plots were drew, the CMC value was determined as the intersection of linear component of the curve drawn between the surface tension and the concentration of biosurfactant, and it was expressed in 5 mg l^{-1}. For calibrating the instrument, it was subjected to the determination of surface tension of the pure water at 25 ± 1°C (Makkar and Cameotra, 1997).

DEGRADATION OF HYDROCARBON AND GC-MS ANALYSIS

The strain As-13-3 was inoculated into mineral salts medium MSM supplemented with different carbon source (1% v/v), and this culture was shaking (180 rev min^{-1}) at 28°C for 10 days to test the hydrocarbon degradation rate. Non-inoculated flasks were served as controls. Residual hydrocarbons were extracted three times from the cultures by shaking vigorously with an equal volume of *n*-hexane. The *n*-hexane was concentrated by rotary evaporation at 35°C under reduced pressure, then the concentrated extracts were subjected to gas chromatography-mass spectrometry (GC-MS). In GC-MS analyses, the GC temperature program was 70–300°C at 4°C min^{-1} with a 15-min hold time. In GC-FID analysis, the program was 50–100°C at 6°C min^{-1}, then to 300°C at 4°C min^{-1} with a 15-min hold time. Helium was used as the carrier gas at a constant pressure of 180 kPa. Also standard curve of different hydrocarbon was drawn under the same condition, and the concentration of residue hydrocarbon was calculated according to the standard curve.

The percentage of hydrocarbon degradation was calculated as follows: 100 × (the hydrocarbon concentration of experimental group/the hydrocarbon concentration of the control group). The experimental data are presented in terms of arithmetic averages of at least three replicates, and the standard deviations are indicated by error bars.

SEQUENCING OF THE GENOME OF STRAIN AS-13-3

The genome sequencing of strain As-13-3 was performed with a Solexa paired-end sequencing technologies (Bentley et al., 2008). Genomic libraries containing 3-kb inserts were constructed. A total of 5,545,034 paired-end reads were generated with an Illumina Solexa Genome Analyzer IIx (Illumina, San Diego, CA) to reach a depth of 182.9-fold coverage, and 69.9% of the reads were assembled into 123 scaffolds by using the Burrows–Wheeler Alignment (BWA) tool (Li and Durbin, 2010), including 188 non-redundant contigs. Protein encoding genes were predicted by Glimmer 3.0 (Delcher et al., 2007). The analysis of the genome was performed as described previously (Feng et al., 2008; Li et al., 2011). The genome sequence was also submitted to the Integrated Microbial Genomes (IMG) server (http://img.jgi.doe.gov) of the Joint Genome Institute (JGI)

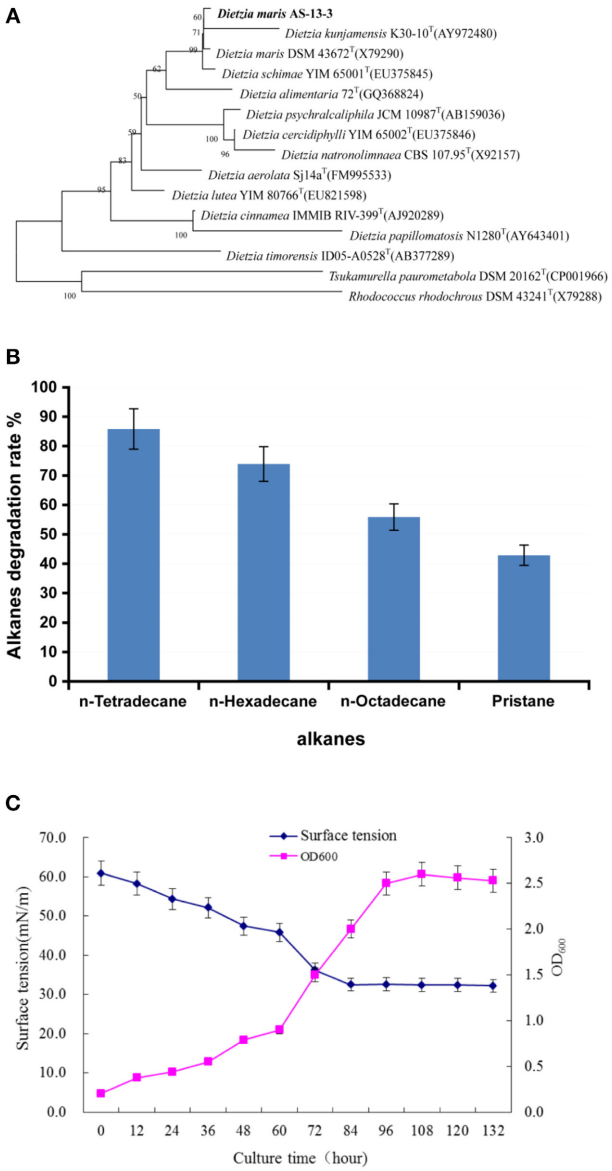

FIGURE 1 | The characterization of the strain As13-3. (A) Neighbor-Joining tree showing the phylogenetic positions of strain AS-13-3 and representatives of some other related taxa, based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0.005 nucleotide substitution rate (Kunc) units. **(B)** Degradation of individual *n*-alkanes by AS-13-3. **(C)** Relationship between cell growth and biosurfactants production during the growth of strain AS-13-3. Data are the means of three separate experiments. The error bars represent the S.D.

for deep analysis and genome comparison (Markowitz et al., 2010).

REAL-TIME PCR

Approximately 4 μg of RNA was reversely transcribed using 20 ng of random primers (Invitrogen, Carlsbad, CA, USA) and PrimeScript™ Reverse Transcriptase (Takara, Dalian, China). Control reactions were performed without reverse transcriptase to verify the absence of genomic DNA.

The primers for real-time PCR were designed using Primer Premier 5.0 (http://www.premierbiosoft.com/). The primer sequences are listed in Table S1 and were synthesized by Invitrogen (Shanghai, China). Quantitative real-time PCR was performed using IQ™ SYBR Green Supermix and the IQ™ 5 Multicolor Real-time PCR Detection System (Bio-Rad, California, USA). The reactions were performed in 96-well optical plates sealed with optical caps. The total reaction volume of 25 μl contained 12.5 μl of 2X SYBR® Green PCR Supermix (Bio-Rad, California, USA), the DNA template, primers at an optimized concentration and sterile water. The following program was utilized: 2 min at 50°C (uracil-N-glycosylase activation), 10 min at 95°C (activation of Taq polymerase) and 40 cycles of denaturation (10 s at 95°C), annealing and elongation (30 s at 56–61°C). Fluorescence data were acquired at the end of the elongation step. The specificity of the accumulated products was verified through melting curve analysis. In all of the experiments, appropriate negative controls were subjected to the same procedure to detect any possible contamination. The size and purity of the obtained amplicon and the absence of dimer formation were assayed through conventional agarose gel electrophoresis.

Table 2 | Effect of carbon sources on bacterial growth and biosurfactant production[a].

Carbon sources	OD_{600} of culture broths	Surface tension of culture broth (mN/m)
Glycerol	2.10	56.4
Glucose	1.10	55.4
Sodium citrate	1.27	61.1
Sodium acetate	2.10	59.1
Sodium pyruvate	1.35	59.1
n-Dodecane	2.04	40.1
n-Tetradecane	1.66	35.6
n-Hexadecane	1.22	33.2
Pristane	0.805	34.0
Olive oil	4.01	41.4
Control	0	62.0

[a] *Data are the mean of three separate experiments.*

Table 1 | The cell surface hydrophobicity of *D. maris* AS-13-3 grown on various hydrocarbon substrate[a].

Hydrocarbon	*n*-Dodecane	*n*-Tetradecane	*n*-Hexadecane	Pristane	Toluene	Paraffins
Hydrophobicity	40.7%	63.9%	61.2%	38.8%	73.3%	51.8%

[a] *Data are the mean of three separate experiments.*

STATISTICAL ANALYSIS

The 16S rRNA housekeeping gene was used as a reference gene to normalize gene expression in strain As-13-3. The relative fold change in mRNA quantity was calculated for the gene of interest in each sample using the $\Delta\Delta$Ct method. For each RNA preparation, at least three independent real-time PCR experiments were conducted. Data were analyzed by unpaired two-tailed Student's *t*-tests or One-Way ANOVA, followed by Tukey's multiple comparison test with GraphPad Prism software (San Diego, CA, USA). Data were expressed as mean ± SD derived from at least three independent experiments. Differences were considered significant at $P < 0.05$.

SEQUENCE ACCESSION NUMBERS

The sequences of the di-rhamnolipid biosynthesis-related genes of *D. maris* As-13-3 have been deposited in the NCBI database with the following accession numbers: KP202067 through KP202092.

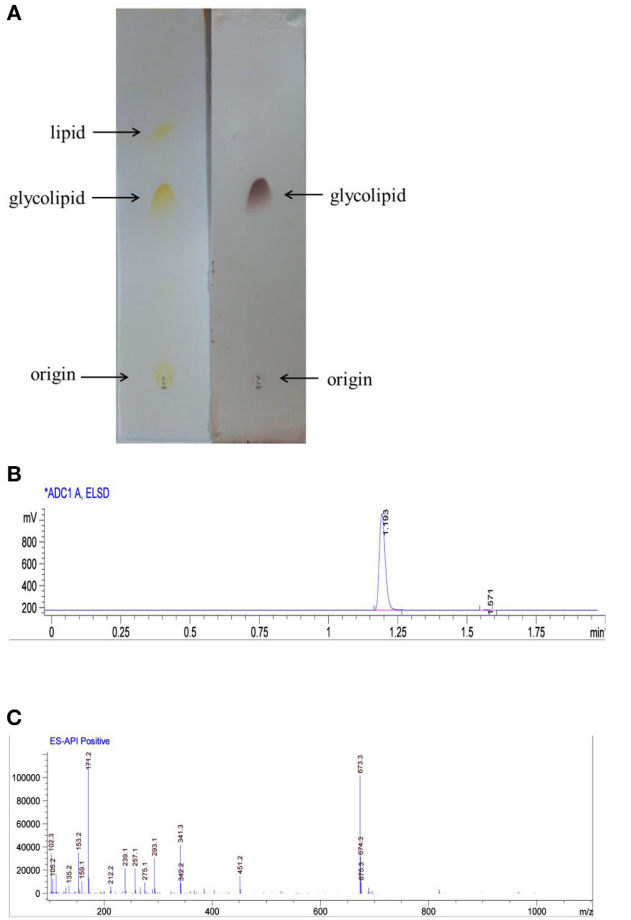

FIGURE 2 | Biosurfactant purification and structural characterization. **(A)** Thin layer chromatogrpahy analysis of glycolipid produced by AS-13-3 developed with chloroform–methanol–water(85:15:2,v/v/v). detected with iodine (plate A) and phenol–sulfuric acids (plate B). **(B)** HPLC analysis of biosurfacant produced by strain AS-13-3(ELSD detector). **(C)** Molecular mass spectra of the glycolipid produced by strain AS-13-3.

RESULTS AND DISCUSSION

THE CHARACTERIZATION OF THE STRAIN AS-13-3

Phylogenetic analyses showed that strain As-13-3 formed a stable clade with the type strains of all species in the genus *Dietzia*, and the highest 16S rRNA sequence similarity of 99.22% with

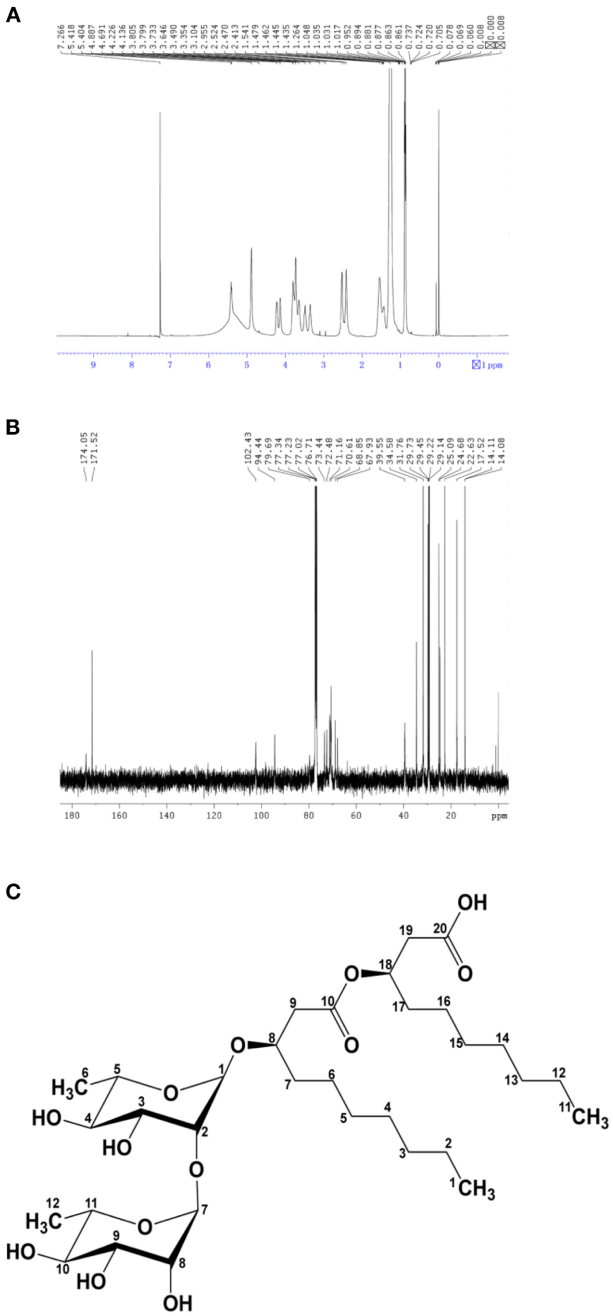

FIGURE 3 | Biosurfactant structural ananlyisis. (A) ^{1}H nuclear magnetic resonance spectrum of the lipopetide produced by strain AS-13-3. **(B)** ^{13}C nuclear magnetic resonance spectrum of the lipopetide produced by strain AS-13-3. The chemical groups represented by each peak are given in the **Table 4**. **(C)** Molecular structure of the di-rhamnolipid produced by strain AS-13-3.

Table 3 | The emulsification index (E_{24}) of the produced biosurfactant by AS-13-3 grown on various hydrocarbon substrate[a].

Hydrocarbon	Toluene	*n*-Hexane	Cyclohexane	*n*-Hexadecane	Pristane	Diesel
E_{24}	60.29%	55.88%	63.64%	55.88%	54.29%	63.64%

[a] *Data are the mean of three separate experiments.*

Table 4 | ^{1}H-NMR and ^{13}C-NMR spectrum of purified biosurfactant by strain AS-13-3.

Hydrogen atoms	^{1}H-NMR (ppm)	Carbon atoms (Sugar-portion)	^{13}C-NMR (ppm)
SUGAR-PORTION			
1′-H	4.89	1′-C,7′-C	102.21, 94.46
CH_3	1.21	2′-C,8′-C	73.46
2′,3′,4′,5′-H	3.63-4.23	3′-C,9′-C	–
2′,3′,4′,5′-H		4′-C,10′-C	–
		5′-C,11′-C	67.92
		6′-C,12′-C	17.55
FATTY ACID-PORTION			
CH_3	0.89	1-C,11-C	14.11
CH_2	1.20–1.45	2-C,12-C	34.81
CH_2CO_2	2.48	3-C,13-C	–
CO_2CH	5.35–5.42	4-C,14-C	–
O–CH	4.14	5-C,15-C	–
		6-C,16-C	–
		7-C,17-C	22.66
		8-C,18-C	73.46–67.92
		9-C,19-C	39.52
		10-C,20-C	174.14, 171.53

Dietzia maris DSM 43672T(X79290) (**Figure 1A**). Strain As-13-3 can utilize short-chain and middle-chain *n*-alkanes (from C_8 to C_{20}, data not shown), and vigorous degradation occurred from C_{14} to C_{18} and pristane (**Figure 1B**). Notably, strain As-13-3 can also utilize branched-alkanes pristane (2,6,10,14-tetramethylpentadecan), and the degradation rate was 42.87% in 10 days (**Figure 1B**). The cell surface hydrophobicity (CSH) of strain As-13-3 against different kinds of hydrocarbon substrate was also tested. The results showed that strain As-13-3 have the highest CSH toward toluene with hydrophobicity of 73.3%, and a relative high CSH toward *n*-hexadecane and *n*-tetradecane with the CSH of about 60%, respectively (**Table 1**). In contrast, strain As-13-3 exhibits a weak hydrophobicity toward dodecane and pristane with hydrophobicity of 40.7 and 38.8%, respectively (**Table 1**).

Alkanes and non-alkane carbon sources were used to monitor the biosurfactant production. The results demonstrated that strain As-13-3 produced biosurfactant only in the presence of alkanes such as *n*-tetradecane, *n*-hexadecane, and Pristane (**Table 2**). Otherwise, the surface tension of culture broth had no notable variation when using simple carbon sources such as glucose and glycerol (**Table 2**). Using *n*-hexadecane as the sole carbon source, the culture broth surface tension decreased during the cultivation period, reaching 33.1 mN m^{-1} after 5 days of cultivation, and then maintained at a constant level (**Figure 1C**).

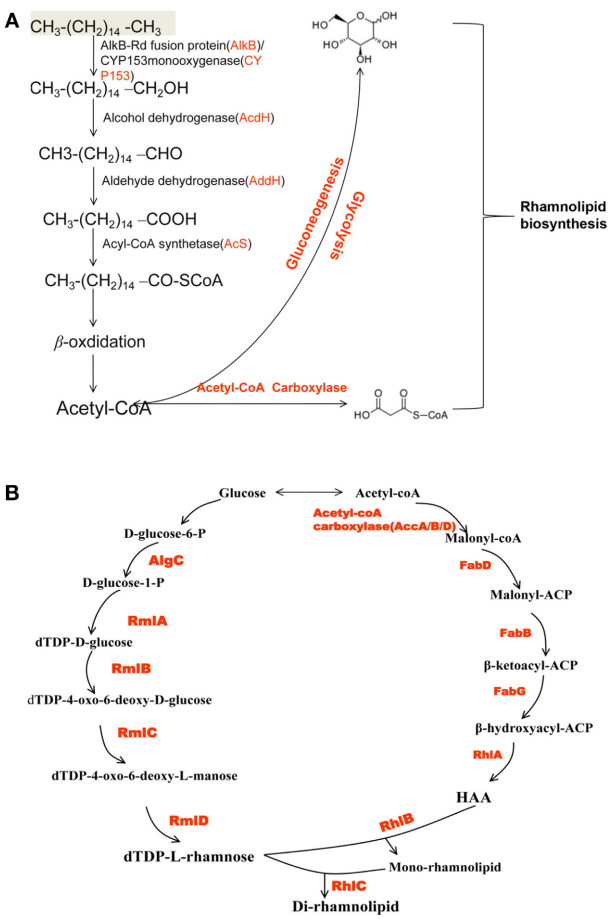

FIGURE 4 | Schematic overview of rhamnolipid biosynthesis pathways in strain AS-13-3. (A) The alkanes degradation pathways. AlkB: AlkB-Rub fusion alkane monooxygenase; CYP153: CYP153 alkane monooxygenase gene; acdH: alcohol dehydrogenase gene; addH: aldehyde dehydrogenase; acS: acyl-CoA synthetase. **(B)** The di-rhamnolipid biosynthesis pathways. AlgC: phosphomannomutase; RmlA: glucose-1-phosphate thymidylyltransferase; RmlB: dTDP-D-glucose 4,6-dehydratase; RmlC: dTDP-4-dehydrorhamnose 3,5-epimerase; RmlD: dTDP-4-dehydrorhamnose reductase; FabD: malonyl-CoA:ACP transacylase; FabH, FabB and FabF: b-ketoacyl-ACP synthetases; FabG: NADPH-dependent b-ketoacyl-ACP reductase; HAA: 3-(3-hydroxyalkanoyloxy) alkanoic acid; RhlA: 3-(3-hydroxyalkanoyloxy) alkanoate synthetase; RhlB: rhamnosyltransferase; RhlC: rhamnosyltransferase.

BIOSURFACTANT STRUCTURE AND CHARACTERISTICS

The partially purified biosurfactant that demonstrated to have the highest surface activity was further analyzed by thin layer chromatography and visualized with specific reagents. After phenol/sulfuric acid reagent staining a black–brown prominent spot was observed, the biosurfactant was identified as glycolipid

Table 5 | The alkanes degradation and rhamnolipids biosynthesis related genes identified in genome of *D. maris* AS-13-3.

Gene name	Functional description	Organism	Identity	GenBank ID
alkB	AlkB-Rd fusion protein	*Dietzia cinnamea* P4	100%	KP202090
tetR	TetR family transcriptional regulator	*Dietzia* sp. E1	95%	KP202073
fd	Ferredoxin reductase	*Dietzia cinnamea* P4	100%	KP202087
cyp153	Cytochrome P450 153A16	*Dietzia cinnamea* P4	99%	KP202088
fdR	2Fe-2S ferredoxin	*Dietzia cinnamea* P4	100%	KP202089
acdH	Alcohol dehydrogenase	*Dietzia cinnamea* P4	96%	KP202082
addH	Aldehyde dehydrogenase	*Dietzia cinnamea* P4	94%	KP202081
acS	Acyl-CoA synthetase	*Dietzia cinnamea* P4	91%	KP202084
algC	Phosphomannomutase	*Dietzia cinnamea* P4	85.65%	KP202070
rmlC	dTDP-4-dehydrorhamnose 3,5-epimerase	*Dietzia cinnamea* P4	97%	KP202076
rmlA	Glucose-1-phosphate thymidylyltransferase	*Dietzia cinnamea* P4	62%	KP202079
rmlB	dTDP-glucose 4,6-dehydratase	*Dietzia cinnamea* P4	95%	KP202080
rmlD	dTDP-4-dehydrorhamnose reductase	*Rhodococcus equi* ATCC 33707	92%	KP202078
accD1	Acetyl-CoA carboxylase	*Dietzia cinnamea* P4	97%	KP202072
accD2	Acetyl-CoA carboxylase	*Rhodococcus opacus* PD630	77%	KP202086
accA1	Acyl-CoA carboxylase alpha chain	*Dietzia cinnamea* P4	94%	KP202085
accB	Acyl-CoA carboxylase beta chain	*Dietzia cinnamea* P4	97%	KP202074
accA2	Acyl-CoA carboxylase alpha chain	*Dietzia cinnamea* P4	98%	KP202075
fabD	ACP-S-malonyltransferase	*Dietzia cinnamea* P4	95%	KP202083
fabB	Beta-ketoacyl-ACP synthase I	*Dietzia cinnamea* P4	89%	KP202067
fabG1	3-Ketoacyl-ACP reductase	*Dietzia cinnamea* P4	98%	KP202068
fabG2	3-Ketoacyl-ACP reductase	*Dietzia cinnamea* P4	98%	KP202069
fabG3	3-Ketoacyl-ACP reductase	*Dietzia cinnamea* P4	97%	KP202077
rhlA	3-(3-Hydroxyalkanoyloxy)alkanoic acids (HAAs) synthase	*Burkholderia cenocepacia*	96%	KP202092
rhlB	Alpha-3-L-rhamnosyl transferase	*Dietzia cinnamea* P4	92%	KP202071
rhlC	Alpha-3-L-rhamnosyl transferase	*Dietzia alimentaria*	90%	KP202091

(**Figure 2A**). The glycolipid product was future subjected to HPLC analysis and preparation, the retention time of the purified biosurfactant was 1.193 min (**Figure 2B**). LCQ-MS results revealed molecular ion peak with molecular masses of 673.3, and the glycolipid was assigned to the protonated molecular ion and to the adduct of sodium ions $[M+Na^+]$ (**Figure 2C**). Thus, the molecular weight of the glycolipid is 650.

The molecular structure of the glycolipid was further analyzed by ^{1}H-NMR (**Figure 3A**), ^{13}C-NMR (**Figure 3B**) and other 2D-NMR spectroscopies. The chemical shifts of the compound are summarized in **Table 4**. In ^{1}H-NMR, characteristic chemical shifts 1.21 ppm showed the presence of $–CH_3$ in sugar portion, and similarly 4.89 ppm for 1′-H, 3.63–4.23 ppm for 2′,3′,4′,5′-H. In fatty acid portion, 0.89 ppm in ^{1}H-NMR was supposed to be$–CH_3$ and peaks at 0.88–1.28 ppm revealed alkyl group in fatty acid portion. Besides, the chemical shifts at 2.48, 4.14, and 5.35 ppm represents hydrogen atom for$–CH_2–COO–$, –O–C–H, and $–COO–CH_2$, respectively. In the ^{13}C-NMR spectrum, the peak at 174.1 and 171.5 ppm were assigned to be carbonyl groups. All parameters such as ^{1}H-NMR and ^{13}C-NMR chemical shifts and other 2D-NMR spectroscopies indicated the molecular structure as that of L-rhamnosyl-L-rhamnosy-β-hydroxydecanoate (di-rhamnolipid) (**Figure 3C**).

The di-rhamnolipid showed satisfying emulsification ability with all the substrate tested including *n*-Hexane, *n*-Hexadecane, and Pristane, and the E_{24} value is about 50% (**Table 3**). The maximum emulsification index was obtained with cyclohexane and diesel, and the E_{24} value is about 60% (**Table 3**). The CMC value of the di-rhamnolipid is 120 mgL^{-1} (Figure S1). The surface tension of water was reduced by di-rhamnolipid from 74 ± 0.2 to 38 ± 0.2 mN m^{-1} (Figure S1). The CMC value and surface tension of di-rhamnolipid produced by strain As-13-3 is a little higher than *Pseudomonas aeruginosa* species produced RLs. In 2011, Bharali reported a *P. aeruginosa* strain OBP1 which could produce di-rhamnolipid, it reduced the surface tension of water to 31.1 mN m^{-1} with a CMC value of 45 mg L^{-1} (Bharali and Konwar, 2011). Also, it was reported that the RLs produced by *P. aeruginosa* species could reduce the surface tension of water from 72 to 29 mN m^{-1} with a CMC value in the range of 5–60 mg L^{-1} (Van Dyke et al., 1993). However, the di-rhamnolipid produced by strain As-13-3 exhibited better performance of surface actives compared with rhamnolipids produced by *Burkholderia thailandensis* which reduce the surface tension of water to 42 mN m^{-1} and displaying the CMC value of 225 mg L^{-1} (Dubeau et al., 2009).

BIOSYNTHESIS PATHWAY OF RHAMNOLIPID IN STRAIN As-13-3

Rhamnolipids (RLs) is the most intensively studied biosurfactant, and the biosynthesis have been clarified by many reports (Burger et al., 1963; Rehm et al., 2001; Deziel et al., 2003; Zhu and Rock, 2008). It was reported that the substrates for the biosynthesis of rhamnolipid were glucose for the biosynthesis of

rhamnose moiety and acetyl-CoA for the biosynthesis of lipid moiety (Koga, 1997; Madduri et al., 2001; Abdel-Mawgoud et al., 2011). The genes that played important roles in RLs biosynthesis process were also reported (Ochsner et al., 1994; Urs et al., 1994; Campos-Garcia et al., 1998; Messner, 1999; Soberón-Chávez, 2004). Generally speaking, RLs biosynthesis can be divided into three parts: biosynthesis of the fatty acid; sugar moieties and link the sugar and lipid (Gunther et al., 2005; Abdel-Mawgoud et al., 2011).

In this report, *n*-hexadecane was served as the sole carbon source in the medium, indicating that the raw material for the biosynthesis of rhamnolipid is derived from *n*-hexadecane. The genome of strain As-13-3 revealed a complete pathway for the biodegradation of alkanes by terminal oxidation (**Figure 4A**). The pathway genes including alkane hydroxylase genes: AlkB-Rub fusion protein gene (*alkB*) and CYP153 alkane monooxygenase gene (*cyp153*), alcohol dehydrogenase gene (*acdH)*, aldehyde dehydrogenase gene (*addH*), acyl-CoA synthetase gene (*acS*), and TCA cycle and gluconeogenesis related genes (data not shown) (**Table 5**). Quantitative real-time PCR (Q-PCR) analysis showed that *alkB* and *cyp153* expression were strongly induced by *n*-Tetradecane, *n*-Hexadecane, and pristane, respectively (**Figure 5A**). The *n*-Hexadecane increased *alkB* and *cyp153* by 49.6- and 29.2-fold, respectively. The *tetR*, fd, *fdR*, *acdH*, *addH*, and *acS* gene were induced moderately by *n*-Tetradecane, *n*-Hexadecane, and pristane, respectively (**Figure 5A**). Interestingly, the arrangement of alkane hydroxylase genes on the chromosome of strain As-13-3, with the presence of *alkB-rub* gene or *cyp153* gene, are very similar to that previously reported in the related *Dietzia* sp.DQ12-45-1b (**Figure 6**) (Nie et al., 2011, 2014).

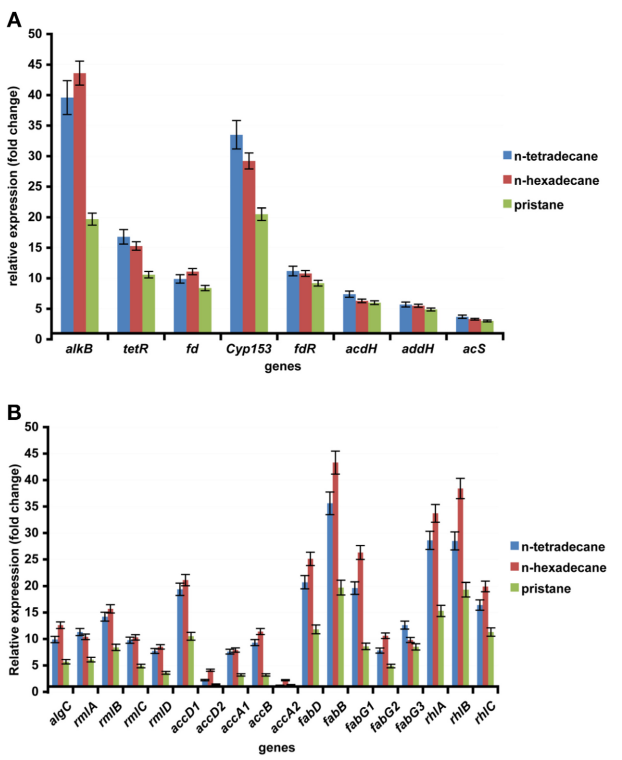

FIGURE 5 | The expression of alkanes degradation and biosynthesis of rhamnolipid related gene in the AS-13-3 strains grown on various carbon sources and in control cells grown on sodium acetate. The data are presented as the mean of three independent experiments. The error bars represent the S.D. **(A)** The expression of *alkB*, *cyp153, tetR, fd, fdR, acdH, addH*, and *acS* in AS-13-3 cells. **(B)** The expression of *algC, rmlA, rmlB, rmlC, rmlD, accA1, accA2, accB, accD1, accD2, fadD, fadB, fagG1, fadG2, fadG3, rhlA, rhlB*, and *rhlC* in AS-13-3 cells.

The lipid moiety of RL was previously reported to be generated through the classical pathway of fatty acid synthesis (Zhu and Rock, 2008). In genome of strain As-13-3, we also found fatty acid synthetases of type-II (FAS II) pathway, the key genes include *accA*, *accB*, *accD*, *fabB, fabD, and fabG* (**Table 5**). The acetyl-CoA for substrate via a series of enzymatic catalytic reactions (the FAS II pathway) changes into the β-hydroxy fatty acids (HAA), and the HAA was future used in the biosynthesis of RLs (**Figure 4B**). The expression profiles of three *fabG*, *fabB*, and *fabD* were also induced significantly by *n*-Tetradecane, *n*-Hexadecane, and pristane (**Figure 5B**); as well as the genes involved in fatty acid synthetases, like *accA1*, *accB,* and *accD1* (**Figure 5B**); however, *accA2* and *accD2* were not sensitive to the presence of alkanes (**Figure 5B**).

Almost all the genes involved in the biosynthesis of dTDP-L-rhamnose were also found based on genome analysis. These genes including *algC*, *rmlA*, *rmlB*, *rmlC*, and *rmlD*. Q-PCR analysis showed that these genes were induced significantly by *n*-Tetradecane, *n*-Hexadecane, and pristane, respectively (**Figure 5B**). Olvera et al. reported that *algC* mutant seriously impacted the production of RLs in *P. aeruginosa* (Olvera et al., 1999). Thus, in the production of rhamnolipid, *algC* plays an important role.

Glycosyltransferase constitutes a large family of enzymes that are involved in the biosynthesis of oligosaccharides, polysaccharides, glycoproteins, glycolipids and other glycoconjugates (Paulson and Colley, 1989; Breton et al., 2006). They catalyze the sugar residues from an activated donor substrate into saccharide and non-saccharide acceptors (Breton et al., 2006). In the biosynthesis of rhamnolipids, rhamnosyltransferase participates in the final steps, which link the dTDP-L-rhamnose and lipidic moiety of RLs to yield the final products (RLs), those genes including *rhlA*, *rhlB*, and *rhlC* (Rahim et al., 2001; Dubeau et al., 2009; Abdel-Mawgoud et al., 2011). In this report, *rhlA*, *rhlB*, and *rhlC* encoding rhamnosyl transferases that linked dTDP-L-rhamnose and β-hydroxy fatty acids (HAA) together were identified, respectively (**Figure 4B**). Q-PCR analysis showed that the *rhlA*, *rhlB*, and *rhlC* genes were induced significantly by all the tested alkanes, respectively (**Figure 5B**).

According to above experiment results, strain As-13-3 can use different hydrocarbons including branched alkane pristane as the raw material to produce biosurfactant (**Figure 4**). Q-PCR analysis showed that almost all above-mentioned genes were induced significantly by alkanes (**Figure 5**). In the case of strain *Dietzia sp*. DQ12-45-1b, it can produce kinds of biosurfactants corresponding to the presence of *n*-alkanes (Wang et al., 2013). When using *n*-hexadecane as the sole carbon source, two glycolipid compounds were detected, the glycolipids share the same saccharide moiety, but the fatty acid moiety were different, one

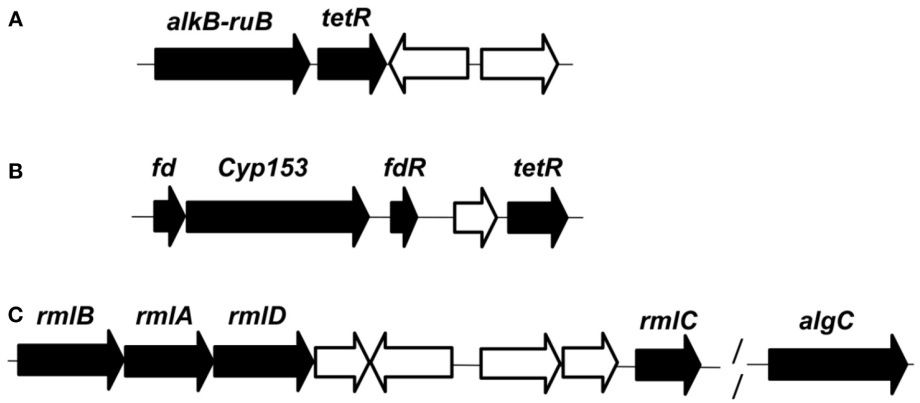

FIGURE 6 | Organization of the genes involved in the degradation of *n*-alkanes and synthesis of dTDP-L-rhamnose in genome of *D.maris* AS-13-3. (A): *alkB-rub* fusion gene. **(B)**: cyp-153 gene. **(C)**: rmlA/B/C/D and algC gene. Black, proteins involved in the degradation pathway or the synthesis pathway. White, proteins are not involved in the degradation pathway or the synthesis pathway. *alkB-rub*: FLAG-tagged AlkB-Rd fusion protein; *tetR*: TetR family transcriptional regulator; *fd*: 2Fe-2S ferredoxin protein; *cyp153*: cytochrome P450 153A16 monooxygenase; *fdR*: ferredoxin reductase;*rmlA*:glucose-1-phosphate thymidylyltransferase; *rmlB*:dTDP-glucose 4,6-dehydratase; *rmlC*:dTDP-4-dehydrorhamnose3,5-epimerase; *rmlD*:dTDP-4-dehydrorhamnosenose reductase; *algC*: phosphoglucomutase.

composed of palmitic acid, and the other composed of palmitic acid, myristic acid, octadecanoic acid, and two unsaturated fatty acids (Wang et al., 2013). In *n*-tetracosane culture, only one glycolipid was found, the fatty acid portion of the glycolipid was identified as a mixture of lauric acid, myristic acid, pentadecanoic acid, palmitic acid, octadecanoic acid, and *n*-nonadecanoic acid (Wang et al., 2013). However, in the case of strain As-13-3, when use *n*-hexadecane as the sole carbon source, only one glycolipid was found, and it was identified as Rha-Rha-C_{10}-C_{10}. We did not find these RL variants in strain As-13-3. Therefore, further investigations are required to identify the other biosurfactant in strain As-13-3, when use different alkanes as the sole carbon source.

BIOSURFACTANTS APPLICATION OUTLOOK IN STRAIN As-13-3

In the context of the interest in finding non-pathogenic RL-producing strains rather than pathogenic strains (e.g., *P. aeruginosa*) for commercial production purposes. Thus, strain As-13-3 for non-pathogenic RL-producing strain is more suitable for the industrially-safe production of RLs.

In commercial application of rhamnolipid, the main problem is high cost. There is a great need to develop an efficient rhamnolipid-producing strain and a low cost-effective processing technique. Considering the fact that strain As-13-3 has the advantage of growing fast (within 4 days), and capable of using waste oil as carbon source. Thus, it is promising in application in both bioremediation of oil contaminated environment and industrial production of RLs.

CONCLUSIONS

In this report, *D. maris* As-13-3 was confirmed as biosurfactant-producing strain with alkanes. When using *n*-hexadecane as the sole carbon source, the biosurfactant produced was identified as di-rhamnolipid. The molecular structure of the di-rhamnolipid was characterized as Rha-Rha-C_{10}-C_{10}, which exhibited satisfying performance of surface activity and emulsification activity. Several genes that played important roles in the process of rhamnolipid biosynthesis were identified including *alkB*, *cyp153*, *algC*, *rmlA*, *rmlB*, *rmlC*, *rmlD*, *rhlA*, *rhlB*, and *rhlC* etc. Further, a complete picture of the di-rhamnolipid synthesis process in strain As-13-3 is shown, which helps in future application in industrial production of RLs.

ACKNOWLEDGMENTS

This work was financially supported by COMRA program (No. DY125-15-R-01), the Project of the National Science Foundation of China (41106151, 41176154), the fund of National Infrastructure of Microbial Resources (No. NIRM2014-9), and the Scientific Research Foundation of Third Institute of Oceanography, SOA (2011036).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00711/abstract

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Conflict of Interest Statement: 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.

Received: 16 September 2014; accepted: 29 November 2014; published online: 16 December 2014.

*Citation: Wang W, Cai B and Shao Z (2014) Oil degradation and biosurfactant production by the deep sea bacterium Dietzia maris As-13-3. Front. Microbiol. **5**:711. doi: 10.3389/fmicb.2014.00711*

This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology.

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