Rhamnolipid biosurfactants: evolutionary implications, applications and future prospects from untapped marine resource

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Abstract
Rhamnolipid-biosurfactants are known to be produced by the genus Pseudomonas, however recent literature reported that rhamnolipids (RLs) are distributed among diverse microbial genera. To integrate the evolutionary implications of rhamnosyl transferase among various groups of microorganisms, a comprehensive comparative motif analysis was performed amongst bacterial producers. Findings on new RL-producing microorganism is helpful from a biotechnological perspective and to replace infective P. aeruginosa strains which ultimately ensure industrially safe production of RLs. Halotolerant biosurfactants are required for efficient bioremediation of marine oil spills. An insight on the exploitation of marine microbes as the potential source of RL biosurfactants is highlighted in the present review. An economic production process, solid-state fermentation using agro-industrial and industrial waste would increase the scope of biosurfactants commercialization. Potential and prospective applications of RL-biosurfactants including hydrocarbon bioremediation, heavy metal removal, antibiofilm activity/biofilm disruption, and greener synthesis of nanoparticles are highlighted in this review.

Introduction
Surface active agents produced by certain microorganisms are known as biosurfactants and bioemulsifiers. These are amphipathic structures containing both the hydrophobic as well as the hydrophilic domains. Chemical surfactants are artificially synthesized through various chemical processes and can be obtained according to the desired structure of the molecules. The chemicals used in the synthesis of these synthetic surfactants are highly toxic to the environment. Therefore, emphasis is placed on the synthesis of biosurfactants and bioemulsifiers as greener alternatives. Non-toxicity and biodegradability, along with biocompatibility and specificity, renders these compounds indispensable alternatives of chemical surfactants. Biosurfactants have the ability to reduce surface tension, and act as emulsifiers (Banat, 1995) whereas bioemulsifiers do not have the property of reducing surface/interfacial tension. Various substances which are inexpensive and easily available including agricultural wastes, glycerol, sugar, oil and hydrocarbons can be used as substrate for production of biosurfactants. The chemical nature of biosurfactants is mostly glycolipids and lipopeptides. Structural derivatives of biosurfactants include glycolipopolysaccharides, fatty acids, neutral lipids and phospholipids.

Keywords
Biosurfactants, biosurfactant-applications, evolutionary implications, microbial-production, rhamnolipid

Sophorolipids, fatty acids, neutral lipids and phospholipids (Chen et al., 2006; Desai & Banat, 1997). These biosurfactants either attach themselves to the surface of the cells or are secreted into the culture medium (Chen et al., 2007). Biosurfactants are smart biomolecules with wider applications in environmental cleaning, food industry, antimicrobial, antitumor, oil-recovery and nanotechnology (Kiran et al., 2010c; Khopade et al., 2012; Kryachko et al. 2013; Ławniczak et al., 2013; Sachdev & Cameotra, 2013).

The genera Pseudomonas was focused as the main producer of rhamnolipid (RL) biosurfactants, however recent literature reported that diverse microbes are capable of producing RL and/or structurally similar biosurfactants. Based on the number of rhamnose moieties, mono- and di-rhamnolipids are differentiated. RL-biosurfactants are structurally glycolipid derivatives, in which one or two molecules of rhamnose are linked to one or two molecules of β-hydroxydecanoic acid. RL-biosurfactants can be considered as smart biomolecules as they have wider functional implications in hydrocarbon degradation, quorum sensing, biofilm formation and disruption, biological activity and now as nanoparticle stabilizing agents.

Various aspects of microbial biosurfactants were recently reviewed. Notably, Marchant & Banat (2012) reviewed glycolipid biosurfactants and their commercial exploitation for Microbially Enhanced Oil Recovery (MEOR) and other industrial applications. Chrzanowski et al. (2012) reviewed RL-mediated uptake of hydrophobic substrates by bacterial
cells and the natural role of RL-biosurfactants. The application of biosurfactants in agriculture and bioremediation was comprehensively reviewed by Sachdev & Cameotra (2013) and Lawniczak et al. (2013). Henkel et al. (2012) reviewed possible development of cost-effective and sustainable RL production using renewable substrates such as agro-industrial byproducts or wastes. Understanding the gene regulation networks and systems biology approaches are potentially important for sustainable production methods. Müller et al. (2012) reviewed RL from Pseudomonas and Burkholderia focusing on systems biology approaches and heterologous production. The complex gene regulation network involved in RL biosynthesis and regulatory factors, including quorum sensing systems, were comprehensively reviewed by Reis et al. (2011). Satpute et al. (2010) reviewed biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms. This review would be unique on evolutionary implications and phylogenetic diversity of rhamnoacyl transferases which ultimately envisages the distribution of RLs among various microbial genera. This implication can bring out new insight on exploring the huge microbial diversity and extreme environments for novel RLs. An insight on the exploitation of marine microbes as potential source of RL biosurfactants is highlighted in this review. Biofilm disruption and greener synthesis of nanoparticles are emerging applications of RL-biosurfactants. Therefore, this article is focused on the diversity of microbes producing RLs, biosynthesis, phylogenetic diversity and evolutionary implications of rhamnoacyl transferases, applications of RL-biosurfactants, potential marine microorganisms as a novel source of biosurfactants, and future considerations were comprehensively reviewed and considered along with research data.

Microbial diversity of RL producers

Pseudomonas species have been reported as a unique source of RLs. However, several isolates from microorganisms of variable distance in evolution also seems to be RL producers. First, several bacterial species, apart from P. aeruginosa, are potentially capable of synthesizing RLs (Gunther et al., 2005, 2006; Onbasli & Aslim, 2009). Some belong to a similar assortment category of bacterial genera Gamma proteobacteria and Acinetobacter calcoaceticus (order: Pseudomonadales) was reported as RL producers (Rooney et al., 2009). Other bacterial members have also been reported as RL producers from different taxonomic species and they belong to completely different orders, such as the phylum Gammaproteobacteria; e.g. Pseudoxanthomonas sp. (order: Xanthomonadales; family: Xanthomonadaceae; Nayak et al., 2009) and Enterobacter sp., and Pantoea sp. (order: Enterobacteriales; family: Enterobacteriaceae; Rooney et al., 2009; Vasileva-Tonkova & Gesheva, 2007).

Of the RL-producing microorganism, apart from Gammaproteobacteria, the foremost distinguished example is the RL-producing Burkholderia spp., a betaproteobacteria (Haussler et al., 1998). Myxococcus sp. (class Deltaproteobacteria) has been reported for the synthesis of an uncommon rhamnose containing glycolipid (rhamnoside) referred to as myxotyrosides. The unique feature of myxotyrosides was chemically elucidated as a tyrosine-derived core structure glycosylated with rhamnose acylated with uncommon fatty acids like (Z)-15-methyl-2-hexadecanoic and (Z)-2-hexadecanoic acid. The authors claimed that the sole molecule is associated with myxotyrosides RLs and equally acylated (Ohlendorf et al., 2008).

Actinobacteria is the other phylum with potential to synthesize RLs. RL-producing Antarctic Nocardioidei sp. A-8 was reported by Vasileva-Tonkova & Gesheva (2005). An isolate from hydrocarbon contaminated industrial waste water was identified as Renibacterium salmoninarum 27BN and was optimized for RL production (Christova et al., 2004). However, R. salmoninarum in general was known as potential fish pathogen. Yang et al. (2013) characterized new glycolipid derivatives from a phytopathogenic fungus Ustilago maydis. Invariably, non-pathogenic RL-producing species are essential for industrial scale production process, which could be a mandatory requirement for product safety and containment requirements of a bioprocess industry. These novel RL producing isolates have been found distributed inside and outside the category Gamma proteobacteria. This highlights the necessity for a wide spectrum of screening programs for RL producers that embrace completely different orders of Gamma proteobacteria or may be of alternative categories. Nevertheless, some authors anticipate that novel RL-producing isolates are found largely inside the category Gamma proteobacteria (Rooney et al., 2009). Isolating new RL-producing microorganism is helpful from a biotechnological purpose and to replace infective P. aeruginosa strains which ultimately ensure product safety and containment.

Biosynthesis of RLs

RL is a glycolipid that contains one or two molecules of rhamnose that are linked to one or two molecules of β-hydroxydecanoic acid in a side chain, and have been well studied (Abalos et al., 2001). RLs are chemically organic carbon compounds composed of lipid and sugar moieties (Figure 1A). The predominant forms of RLs include mono-RL or di-RL, with rhamnose sugars linked with 3-hydroxydecanoic acid moieties. The monoo- and di-RLs are obviously referred as RLs (R1) and RL (R2). The length of the carbon chains on the β-hydroxyacyl portion of the RL can vary significantly.

The literature reports that 10-carbon molecule chains are the predominant structural forms of RLs synthesized by P. aeruginosa (Deziel et al., 2000). The rhamnose molecules are commonly linked to a hydroxyl group of a hydroxydecanoic acid esterified by another hydroxy fatty acid. RLs are potential surface reducing agents; they can reduce surface tension of water to 25–30 mN/m (Guerra-Santos et al., 1984). Production of rhamnose containing glycolipids was first described in P. aeruginosa by Jarvis & Johnson (1949). l-Rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and l-Rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate, referred to as RL 1 and 2, respectively, were the principle glycolipids produced by P. aeruginosa (Desai & Banat, 1997). Based on carbon number and moieties, the RLs were elucidated as a mixture of RL1 (RhaC10C10), RL2 (RhaC10 C10), RL3 (Rha2C10) and RL4 (RhaC10). Lang & Wagner, 1987; Nitschke et al., 2005; Rahman et al., 2002; Syldatk &
Wagner, 1987). The strain P. fluorescens was grown on virgin olive oil media and produces RL instead of viscosin (Healy et al., 1996).

Most of the RLs are anionic and the biosynthesis occurs during late exponential and stationary phase of growth under limiting concentrations of iron and nitrogen (Guerra-Santos et al., 1984). Two biosynthetic genes, namely, rhlA and rhlB, encode rhamnosyl transferase I, which are organized in the operons. The functional domains rhl genes comprising an autoinducer synthesis protein (rhlI), the rhamnosyltransferase I subunit A (rhlA), transcriptional regulatory protein (algQ), alginate regulatory protein (algR2), autoinducer synthesis protein (lasI), ATP-dependent RNA helicase (rhlB), RNA polymerase sigma factor (rpoS), rhamnosyltransferase chain B, pseudolysin precursor, neutral metalloproteinase and hypothetical proteins. The active enzyme complex is localized in the cytoplasmic membrane, whereas the rhlAB protein is in the periplasm and rhlB crosses the membrane (Ochsner et al., 1994a). Two regulatory genes, namely, rhlR and rhlL, belong to the lux regulatory family and are located in the downstream of rhlA and rhlB (Ochsner et al., 1994b). The rhlR is a putative transcriptional activator and the rhlI directs the synthesis of the quorum sensing inducer N-butyryl homoserine lactone. RL biosynthesis is also regulated by a LasR–LasI regulatory system which also directs the synthesis of the quorum-sensing inducer N-homoserine lactone (Latifi et al., 1995, 1996; Pearson et al., 1997). The biosynthesis of RLs in Pseudomonas sp. is by three sequential reactions (Chávez et al., 2005; Abdel-Mawgoud et al., 2014). RhlA is involved in the synthesis of the fatty acid dimer moiety of RLs (Déziel et al., 2003) and loosely bound to the inner membrane (Rahim et al., 2001). Abdel-Mawgoud et al. (2010) studied Pseudomonas aeruginosa from L-rhamnose and R-3-hydroxyalkanoate. It was established that RL plays a significant role in the quorum sensing process of Pseudomonas including motility, cell–cell interactions, cellular differentiation and formation of water channels which were necessary for biofilm formation (Dusane et al., 2010b; Okkotsu et al., 2013). The application of quorum sensing molecules on the synthesis of RL biosurfactant by P. aeruginosa was demonstrated (Dusane et al., 2013).

**Evolutionary implications of rhamnosyl transferases**

The wide diversity of rhamnosyl transferases encoded by different genes separately or genes in an operon makes it difficult to identify the exact physiological function of this enzyme and its production solely from its primary sequence. Evolutionary relatedness of these enzymes would probably act as clue in determining the purpose of this enzyme in the producer organism. The blueprint of rhamnosyl transferase has been explored in two major clades of life, Plantae and Bacteria according to progress of research in this area. Rhamnosyl transferases (RTs, Figure 1B) are enzymes, in common, responsible for the transfer of rhamnose (an oligosaccharide) residue to a peptide or to a glycopeptides in different evolutionary levels of organisms.

**Pseudomonas putida** KT2440 can produce 7.3 g/L of RL (Andreas et al., 2011) within an hour, but the function of the RL is still inconclusive and incomplete evidence of their functionality. Production of such large amount of compound in a bacterial cell factory seems to be a mere waste of energy if it has no function, probably genetic selection would probably avoid the expression of the gene which produces a compound of no function and have no advantage of survival. Same way, the evolution and existence of rhamnosyl transferases must have a unique requirement by the organism, the environment or in certain cases may be of a cryptic category.

In bacteria (e.g. *Pseudomonas aeruginosa*), rhamnosyl transferases is encoded by the rhlAB operon (Ochsner et al., 1994a, 1996) which catalyses the transfer of thymidine diphosphate-L-rhamnose to 3-(3-hydroxyalkanoyloxy) alkanonic acid moieties (Déziel et al., 2003; Zhu & Rock, 2008) and subsequently producing RL (RL), a biosurfactant. RhlB is the catalytic protein of the rhamnosyl transferase. RhlA is crucial for RL synthesis, but its function is indefinite. RhlA 32.5 kDa is an inner-membrane 32.5 kDa protein that harbors a putative signal sequence and has been found to be located in the periplasm (Ochsner et al., 1994b; Rahim et al., 2001). It is most likely involved in the production or transport of rhamnosyl transferase precursor substrates or in the stabilization of the RhlB protein (Ochsner et al., 1994a). Ochsner et al. (1994a) later described RhlB, based on the analysis of the amino acid sequences they deduced from rhlB as a protein with at least two putative membrane-spanning domains, which would allow anchoring in the inner membrane. They also partially purified RhlB from the membrane fraction and determined its size to be around 47 kDa (Ochsner et al. 1994a). Rahim et al. (2001) were the first to identify rhlC, which encodes for the second rhamnosyl transferase. They found that RhlC contains a transmembrane hydrophobic region, suggesting that it is also an inner membrane bound protein. RhlC specifically converts mono-RL into di-RL (Rahim et al., 2001). They suggested that both mono- and

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**Figure 1.** Illustrative structures. A. Chemical structures of mono and di-rhamnolipids. B. 3D structure of rhamnosyl transferase.
di-RLs are synthesized at the cytoplasmic side of the inner membrane before being transported to the extracellular milieu (Rahim et al., 2001). Finally, it should be mentioned, that the rhlA, rhlB, and rhlC genes have been recently identified in the RL-producing species *Burkholderia thailandensis* and *B. pseudomallei* (Dubeau et al., 2009). Interestingly, and in contrast with *P. aeruginosa*, where rhlC is separate from rhlAB, in these species, they are grouped together in a gene cluster. Furthermore, this RL synthesis gene cluster is duplicated on the chromosome of these bacteria, and both copies are functional and contribute to RL production (Dubeau et al., 2009).

Indeed, as the opportunistic pathogen *P. aeruginosa* was well-established as the primary RL-producing bacterium, many other bacterial species has been reported to produce RLs in recent years. Diversity of rhamnolipid secretion as the virulence/pathogenicity factor was demonstrated (Pantazaki & Choli-Papadopoulou, 2012), but this fact is beyond the scope of this review. Still, the RL production capability is indeed restricted to a limited number of species, and that reports are subjective. RL is a surface-active glycolipid initially synthesized as exoprodacts, the most intensively explored and extensively analyzed (Chávez et al., 2005; Maier & Soberón-Chávez, 2000; Ochsner et al., 1996). The physiological function of biosurfactants synthesized by *P. aeruginosa* is still remaining an enigma (Abdel-Mawgoud et al., 2010). In plants, glycosyl transferases (RT’s also involved) are involved in secondary metabolism and are a large group of enzymes classified as the glycosyltransferase family 1 (Lombard et al., 2014). RTs have been studied in many bacterial and plant species and a growing number of genes have been isolated and functionally characterized (Abdel-Mawgoud et al., 2010; Darvishi et al., 2011; McCue et al., 2007). Rhamnosyl transferases appear to be ubiquitous and distributed widely in both prokaryotes and eukaryotes.

To integrate the evolutionary implications of rhamnosyl transferase among various groups of microorganisms, a comprehensive comparative motif analysis was performed among bacterial producers. All 204 RT amino acid sequences retrieved from the GenBank was subjected to multiple sequence alignment using ClustalW to determine the presence and comparative localization of various motifs of varied length in the complete primary sequence of RT protein. We first carried out phylogenetic analyses to produce an unrooted tree using the neighbor-joining method (Saitou & Nei, 1987). The statistical reliability was conducted by bootstrapping 1000 replicates (Felsenstein, 1985). The evolutionary distances were computed using a number of differences methods (Tamura et al., 2011) and are in the units of the number of amino acid differences per sequence. The analysis involved 204 amino acid sequences. A phylogenetic tree of the sampled RT sequences was constructed based on the full length of amino acid sequences (Figure 2) as well as on the basis of various pfm domain sequences for all the 204 RTs from different organisms. Ancestral states were inferred using the Maximum Likelihood method (Nei & Kumar, 2000) under the JTT matrix-based model (Jones et al., 1992). The tree shows a set of possible amino acid (states) at each ancestral node based on their inferred likelihood at site 1 (data not shown). However, this output was used to interpret the clustering and the ancestral node of each cluster and the subclasses.

The phylogenetic tree was constructed based on the whole amino acid sequences of rhamnosyl transferases of various sources as shown in Figure 2 and Supplemental Figure 1. RTs from a diverse source of organisms produced a complicated tree, where they can be classified into ten groups or classes. The phylogenetic analysis revealed both deep evolutionary origin and the existence of more recent duplications for the rhamnosyl transferase proteins and formed ten main clusters from the root, where the plant genes and bacterial genes share a clade which indicates the deep evolutionary origin of RTs. Classes I–IX are exclusively of bacterial members and grouped according to the sequence identity and similarity. Class I constitutes Firmicutes (9) as subclass I and Proteobacteria (5 GP and 1 AP), Aquificae (1) and Bacteroides (1) as subclass II. Proteobacteria (Gamma and Alpha) and Archeae were clustered in Class II. Class III is a complex group where a diverse group of bacterial species have shared a common ancestor might be due to horizontal gene transfer, the extremophiles includes *Deinococcus*, clades together with Alpha, Beta, Gamma, delta Proteobacteria and *Bacteroides* were found to be in this subclass. Actinobacteria, Proteobacteria (Alpha proteobacteria, Beta Proteobacteria, Gamma Proteobacteria and Delta Proteobacteria), Firmicutes, *Deinococcus* clustered together and formed two subclasses as SC I and II. Subclass is actually the mixture of various different groups of bacterial clades like Alpha proteobacteria, Beta Proteobacteria, Gamma Proteobacteria and Delta Proteobacteria, *Deinococcus* and Firmicutes which describes the conservative nature of this rhamnosyl transferase and glycosyl transferase across various kinds of organisms and its deep evolutionary origin by depicting their presence in diverse bacterial populations.

Class IV is composed of three different groups of bacteria *Aquificae* (Gt 2 from Sulfurhydrogenibium yellowstonense SS-5 (ZP 04584268)), *Thermodesulfo vibrio* (Gt Thermodesulfo bacterium sp. OPB45 (YP 004627381)) and *Bacteroides* (Rt from Algoriphagus sp. PR1 (ZP 07719084) and *Cyclobacterium marina* DSM 745 (YP 004774294)). Class V majorly comprises Firmicutes (15) and at the minimum of Proteobacteria [Alpha (1), Beta (2) and Gamma (1), *Actinobacterium* (1) and *Flavobacterium* (1)]. Class VI is solely of Actinobacterial rhamnosyl transferases and glycosyl transferases (28). Beta Proteobacteria forms the Class VII consists of RTs from species of *Burkholderia* (8) and a *Flavobacterium* (ZP 08444900 Gt 2 Capnocytophaga sp. oral taxon 329 str. F0087) is the descendent of this cluster. Class VIII comprises three subclasses as SLI, II and III as Firmicutes (6), *Archeae* (4) and *Cyanobacteria* (3) and Acidobacteria (2), respectively. Subclass II formed by *Archeae* where *Archeae* YP 003735737 Rt *Halalkalicoccus jeotgali* B3 pairs with Bacteroides YP 445133 Rt *Salinibacter ruber* DSM 13855. Subclass III is shared by *Cyanobacteria* and Acidobacteria. Subclass IV is of alpha and gamma proteobacteria, where the descendent *Gamma* proteo ZP 05924756 putative *Vibrio* sp. RC341.

Class IX is the complex cluster of Proteobacteria (Alpha, Beta and Gamma), Firmicutes, Bacteroidetes, Archeae, *Thermodesulfovibrio* and *Deinococcus* as Class III and
the Apo Structure of Wsaf Chain B (2X0D) clustered in this class insisting the similarity of this structure derived rhamnosyl transferases with the group of organisms shared here in Class IX. The Class X (Subclass I and II) appeared to have evolved independently from the phylogenetic root and it is notable that in this class, Eukaryotes, i.e. Plantae and Prokaryotes such as Proteobacteria, Archeae and Bacteroides shared a clade together, indicating the possible horizontal gene transfer between bacteria and amniotic ancestor (Ross et al., 2001). Based on the phylogenetic analysis, it could be inferred that the RL biosynthetic pathways distributed almost all groups of microbes. This ubiquitous distribution can be attributed to the diverse natural role of RLs including uptake and biodegradation of poorly soluble substrates, immune modulators and virulence factors, antimicrobials, surface motility and biofilm formation (Abdel-Mawgoud et al., 2010).

Figure 2. A Phylogenetic tree of rhamnosyl transferases: the evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The analysis involved 204 amino acid sequences. Only bootstrap value higher than 50 are shown. Evolutionary analyses were conducted in MEGA5. The bacterial groups taken for study were proteobacteria (Alpha, Beta, Gamma, Delta), Firmicutes, Actinobacteria, Acidobacteria, Cyanobacteria, Archea Bacteroides and Deinococcus. Plant specific enzymes were included based on the existence.
Marine microbes as potential source of RL biosurfactants

Halotolerant extremophiles are required to produce biosurfactants with high tolerance for the bioremediation of oil spills. Rezanka et al. (2011) reported new RL-producing extremophilic strains such as the thermophilic bacteria, Thermus sp., T. aquaticus and Meiothermus ruber. RL-derivatives were characterized as mono-rhamnolipid and di-rhamnolipid homologues containing one or two 3-hydroxy-fatty acids, saturated, monounsaturated or diunsaturated, even- or odd-chain, up to unusual long chains with 24 carbon atoms (Rezanka et al., 2011). Exploring the marine environment would yield novel halotolerant biosurfactants, but reports from the marine environment are limited. Recent reports proposed marine microbes as potential sources of biosurfactants including RL-derivatives. Since the complete structure and chemical elucidation of glycolipid derivatives are not yet reported, this review is focused on glycolipid derivatives which may envisage RL biosurfactants from marine origin. For instance, the rhlB gene product (GenBank Accession Numbers: FJ372668 and FJ372667) was amplified from marine actinobacteria producing a biosurfactant, which was characterized with glycolipid moieties. It was found that the biosurfactant produced by marine actinobacteria was stable up to 5% NaCl (Kiran, 2009). A glycolipoprotein biosurfactant was isolated from Oceanobacillus sp. BRI sampled from Antarctica (Jadhav et al., 2013). This biosurfactant was reported to potentially emulsify different forms of petroleum hydrocarbons. Exploration of hydrothermal vents and extreme marine environments would bring out new biosurfactant derivatives active at extreme temperature, pressure, salinity, etc. Notably, the literature reported that biosurfactants with high-tolerance capacity have not been explored. A recent report on the isolation of cold-tolerant glycolipid biosurfactant supports the possibility that such high-tolerant biosurfactant could be obtained from extreme environments (Liu et al., 2013).

Recently, marine sponge-associated actinobacteria was emerging as a potential source of biosurfactants, bioemulsifiers and bioactive secondary metabolites (Kiran et al., 2010a,b,c,d). Sponges (Porifera) are marine sedentary animals producing secondary metabolites (Faulkner, 2000). Sponges produce secondary metabolites (toxins) to ward-off predators and to protect themselves from pathogens (Pawlik et al., 2002). Although the bioactive potential of marine sponges was well-established, scaling-up/commercialization of these compounds was not successful due to failure to collect sufficient quantities of the candidate sponge. Sponges are sedentary animals that harbor microorganisms upto 40–50% of their biomass (Selvin et al., 2010). Symbiont hypothesis revealed that the associated microorganisms were producing secondary metabolites which were isolated from the marine sponges. Sponge-associated microorganisms can be a sustainable source of supply for marine bioprospecting. Among the bioactive molecules discovered from the sponge-associated microorganisms, biosurfactants are unique (Kiran et al., 2011).

Among the actinobacteria isolated from the marine sponge Dendrilla nigra (Figure 3) four strains such as Nocardiopsis lucentensis MSA04, Nocardiopsis sp. MSA13A, Brevibacterium casei MSA19 and Brachybacterium paraconglomeratum MSA21 produced glycolipid derivatives (Kiran et al., 2010a,b,c,d, 2011, 2014a). Based on the GC-MS analysis, the surface active compounds produced by the four marine actinobacteria was characterized as glycolipid (Table 1). The strain N. lucentensis MSA04 produced a glycolipid biosurfactant with a hydrophobic non-polar hydrocarbon chain identified as nonanoic acid methyl ester and hydrophilic moiety of sugar 3-acetyl-2,5-dimethyl furan. The strain Nocardiopsis sp. MSA13A produced a glycolipid biosurfactant with a hydrophobic non-polar hydrocarbon chain identified as hexacosanoic acid, propyl ester and hydrophobic sugar moiety, methyl-4-O-methyl-beta-D-xylopyranoside. The strain B. paraconglomeratum MSA21 produced glycolipid biosurfactant with a hydrophobic moiety of...
| Strain | Hydrophobic moiety                              | Hydrophilic moiety                          | Structure                          |
|--------|-----------------------------------------------|---------------------------------------------|------------------------------------|
| MSA4   | Nonanoic acid methyl ester                    | 3-Acetyl 2,5 dimethyl furan                 | ![Structure](image1)                |
| MSA13A | Hexacosanoic acid, propyl ester               | methyl-4-O-methyl-beta-D-xylopyranoside     | ![Structure](image2)                |
| MSA19  | (9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[(trimethylsilyl)oxy]methyl]ethyl ester | H-Cyclopenta[c]furan                      | ![Structure](image3)                |
| MSA21  | Dodecanoic acid, methyl ester                  | 3-Methyl-2-oxopropyl furan                  | ![Structure](image4)                |
dodecanoic acid, methyl ester and a hydrophilic sugar moiety of 3-methyl-2-oxopropyl furan (Kiran et al., 2014). The surface active compound produced by the strain *B. casei* MSA19 was grouped as RL, a most prominent biosurfactant mostly known to be produced by *Pseudomonas* sp. The peculiar crystalline structure of RLs was elucidated (Figure 4) from marine actinobacteria. Literature cited that the crystalline structure based studies was limited and Abdel-Mawgoud et al. (2008) reported the relevance of the crystalline structure based analysis on the illustration of the chemical nature of biosurfactants. The RL crystals from marine actinobacteria appeared to be fine rectangular in shape. These different crystalline shapes might be attributed to differences in the chemical composition of the RLs produced.

The reports available of RL biosurfactants from marine microbes are limited (Table 2). The potential of untapped vast microbial diversity would be a new beginning on the bioprospecting of novel RL derivatives. A marine sediment isolate *Inquilinus limosus* KB3 produced a biosurfactant capable of forming stable emulsions with various hydrocarbons and had the ability to enhance oil recovery, PAHs solubility, and antimicrobial activity (Saimmai et al., 2012). Biosurfactant produced by marine *Bacillus megaterium* was optimized for production using free Fe$^{2+}$ in the media (Rangarajan & Sen, 2013). *Pseudomonas nitroreducens* TSBMJ10, isolated from a mangrove ecosystem produced stable emulsions with aliphatic and aromatic hydrocarbon and it can be used for oil recovery and hydrocarbon degradation (Sousa & Bhosle, 2012). Marine isolates *Acinetobacter calcoaceticus*, and PG-12 *Alcanivorax dieselolei* showed high emulsification activity and biosurfactant production (Hassanshahian et al., 2012). A *Streptomyces* species B3, isolated from marine sediment samples produced glycolipid biosurfactants exhibiting antimicrobial activity (Khopade et al., 2012).

### Table 2. Rhamnolipid/glycolipid biosurfactant from marine origin.

| Name of microorganism | Application | References |
|-----------------------|-------------|------------|
| *Streptomyces* VITSSB2 | Microbial enhanced oil recovery process | Shubhrasekhar et al. (2013) |
| *Ochrobactrum* sp. *Brevibacillus* sp. | Marine oil spill bioremediation | Chen et al. (2013) |
| *Streptomyces* B3 | Antimicrobial | Khopade et al. (2012) |
| *Acinetobacter, Pseudomonas, Gordonia, Rhodococcus, Cobetia, Halomonas, Alcanivorax, Marinobacter and Microbacterium* | Uncharacterized biosurfactant with crude oil degradation potential | Hassanshahian et al. (2012) |
| *Bacillus megaterium, Corynebacterium kutscheri* and *Pseudomonas aeruginosa* | Bioremediation | Thavasi et al. (2011) |
| *Nocardia otitidiscaviarum* | Crude oil degradation | Vyas & Dave (2011) |
| *Serratia marcescens* | Biofilm disruption | Dusane et al. (2011b) |
| *Aspergillus* sp. MSF1 | Antimicrobial | Hema et al. (2010) |
| *Brevibacterium casei* MSA19 | Biosurfactant-mediated synthesis of nanoparticles | Kiran et al. (2010b) |
| *Nocardiopsis lucentensis* MSA04 | Bioremediation processes | Kiran et al. (2010a) |
| *Aspergillus ustus* MSF3 | Microbial enhanced oil recovery process | Kiran et al. (2009) |

### Screening of novel biosurfactants from marine environments

Bioprospecting of biosurfactants from marine microbes necessitate a convenient, rapid and reproducible method to screen potential producers with least/no possibility of a false positive. Biosurfactant production is always detected by measuring cell surface hydrophobicity (Pruthi & Cameotra, 1997), drop collapsing ability (Bodour & Maier, 1998), hemolytic activity (Yonebayashi et al., 2000), their surface activity (Desai & Banat, 1997) and lipase activity (Kiran et al., 2009; Figure 5). Hydrophobicity of the cell surface is an important factor in predicting bacterial cell adhesion to surfaces (Figure 6). The hydrophobic nature of the outermost surface of the microbial cells could be used to measure potential cell affinity to hydrophobic substrates. A direct correlation was established between cell hydrophobicity and biosurfactant production (Pruthi & Cameotra, 1997).
A rapid detection of rhamnolipid production was developed with the blue agar plate method using methylene blue and cetyl trimethyl ammonium bromide (Pinzon & Ju, 2009). In this method, rhamnolipids production can be observed as dark-blue halos around the colonies (Figure 5F). The diameter of the halos is dependent on the rhamnolipid concentration. Reports evidenced that due to their simplicity, they are widely used in the early days of related research (Guerra-Santos et al., 1984, 1986; Reiling et al., 1986). They are still used, especially for new rhamnolipid species (Haussler et al., 1998; Tuleva et al., 2002). Rhamnolipid can also be determined by colorimetric methods. This method is based on the reaction of rhamnose in the presence of a strong acid with anthrone which turned blue-colored at 625 nm (Helbert & Brown, 1957; Hodge & Hofreiter, 1962). The orcinol assay for rhamnolipid detection is one of the conventional methods still used in pre-screening of potential producers (Chandrasekaran & Bemiller, 1980).

A novel plate assay was developed by Burch et al. (2010) for the detection of biosurfactant production on agar plates. The atomized oil spray method was performed on LB or KB agar plates with pin head colonies grown overnight by inoculating fresh culture using sterile toothpicks. A uniform spread of tiny colonies could facilitate the assay more precisely. Atomized droplets of mineral oil misted on the plate using an airbrush dispenser. The positive colonies can be visualized as halos around the colony with an indirect source of light. This method can be used as high throughput formats for screening metagenomic libraries for biosurfactant producing clones.

Solid-state production as greener bioprocess for biosurfactant production

The greener production process of biosurfactants could be developed using industrial waste and spent crude oil as petroleum hydrocarbons. This review envisages the production of biosurfactants under solid-state fermentation from marine actinobacteria as an established example. Literature cited that the solid-state fermentation (SSF) has not been used for the production of biosurfactant from actinobacteria. The SSF was developed using agro-industrial and industrial waste to increase biosurfactant production by marine actinobacteria. SSF processes have been used extensively in Asian and African countries, however, their usage and popularization was well-recognized in Western and European countries (Krishna, 2005). The advantages of SSF include a low water requirement, raw materials and industrial waste as substrates and low energy requirement. Zhu et al. (2013) has developed a SSF using agro-industrial byproducts to optimize biosurfactant production by marine actinobacteria. The production of surfactin by Bacillus amyloliquefaciens XZ-173 was effectively optimized with critical control...
factors such as glycerol, water content, inoculum size and temperature.

The SSF was successfully developed to increase biosurfactant production substantially by marine actinobacteria (Kiran et al., 2010b). After optimization, the production was increased to 2.0- to 3.0-fold over the original isolate (wild strain). Detailed summary of appropriate substrate, factors influencing the production and the yield is presented in Table 3. Based on this data, the SSF would be the preferred process for the optimization and production of biosurfactants by marine actinobacteria. Agro-industrial wastes such as wheat bran, ground nut oil cake, rice bran, oil seed cake and industrial wastes such as furnished leather powder, diesel contaminated soil, petrol bunk soil, aavin™ (milk processing waste) pretreated sludge, aavin™ (milk processing waste) treated waste, tannery pre-treated sludge, treated molasses (distillery waste), pre-treated molasses (distillery waste) and tannery treated sludge were employed (Kiran, 2009). In this study, industrial and agro-industrial wastes such as oil seed cake, wheat bran, tannery treated sludge, tannery pretreated sludge, treated molasses and pretreated molasses were used in SSF. Among the six substrates used for the SSF development, each strain utilized different substrates for the biosurfactant production. The highly preferred substrate for the strains include: N. lucentensis MSA04 – wheat bran, Nocardiopsis sp. MSA13A – treated molasses, B. aureum MSA13 – pre-treated molasses, B. casei MSA19 – oil seed cake and B. paraconglomeratum MSA21 – tannery pre-treated sludge. The potential of industrial and agro-industrial wastes in the development of SSF for the production of biosurfactant from marine actinobacteria was demonstrated for the first time (Kiran, 2009). Industrial effluents have shown good promise

Table 3. Critical control factors influencing the biosurfactant production by marine actinobacteria in SSF.

| Strain   | Substrate                  | Factors influencing the production                                      | Yield after optimization |
|----------|----------------------------|--------------------------------------------------------------------------|--------------------------|
| MSA04    | Wheat bran                 | Kerosene (C), Beef extract (N), CuSO₄ and inoculum size                   | 2.5-fold                 |
| MSA13A   | Treated molasses           | Glucose (C), Yeast extract (N), FeCl₃ and inoculum size                   | 2.5-fold                 |
| MSA13    | Pre-treated molasses       | Glucose (C), Acrylamide (N), FeCl₃ and Inoculum size                      | 3-fold                   |
| MSA19    | Oil seed cake              | Glucose (C), Yeast extract (N), FeCl₃ and Inoculum size                   | 2-fold                   |
| MSA21    | Tannery pretreated sludge  | Glucose (C), Yeast extract (N), CuSO₄ and Inoculum size                   | 2-fold                   |

Figure 6. Cell aggregation potential of surface active compound produced by marine actinobacterial isolates. Control plate showing no cell aggregation whereas MSA04, MSA13A, MSA13, MSA19 and MSA21 showing biosurfactant induced cell aggregation.
as potential substrates for biosurfactant production (Desai & Banat 1997; Haba et al., 2000). Koch et al. (1988) constructed *P. aeruginosa* lacking the capacity to utilize lactose present in whey waste, by transferring the lac plasmid from *Escherichia coli* to *P. aeruginosa*, capable of RL biosurfactant production from whey. Babu et al. (1996) studied the specific growth rates of *P. aeruginosa* strain BS2 and specific formation rates of RL biosurfactants from distillery and whey wastes and synthetic medium. Zhang (2014) conducted a study on the development of fermentation techniques with seawater for the production of biosurfactant by salt resistant bacterial strain. Production of rhamnolipid was achieved in fermentation medium containing seawater with yeast extract and waste vegetable oil as carbon source. This study facilitates the possible development of scaled-up for rhamnolipid production process by marine isolates.

**Applications of RL-biosurfactants**

**Hydrocarbon bioremediation**

Crude oil is a complex hydrocarbon composed predominantly of alkanes (saturated hydrocarbons), alkenes and alkyynes (both unsaturated), and aromatic hydrocarbons. Large amounts of hydrocarbon pollutants are spilled into the environment as a result of various anthropogenic activities. The majority of the accidental spills from oil exploration sites, oil tankers, pipelines (underwater and underground), spent marine lubricants and storage tanks are existing as a common occurrence. Hydrocarbon pollution is now emerging as a most prevalent ecological hazard since petroleum products are commonly used as energy sources and raw materials in a wide variety of industries. Although marine oil spills are a major concern, soil pollution by petroleum hydrocarbons is also a critical issue due to the consequences on agricultural production and environmental sustainability. Crude oil excreted from the oil and gas industries and lubricating oil commonly released from metal processing industry as rinsing baths are the major contaminants in oily wastewater leads to soil pollution (Allard & Neilson, 1997; Urum & Pekdemir, 2004; Zhang et al., 2009).

In a marine exploration, 55 bacterial strains were collected from the marine sponge *D. nigra*, of these only two strains are hydrocarbon degrader (Hema, 2014). Among these *Planococcus* sp. MMD26 had higher potential to degrade the hydrocarbons present in crude oil. In plate assay, *Planococcus* sp. MMD26 had a marked crude oil degrading activity as visualized by the clear zone that developed around the colony after 24 h of incubation at 37 °C. In the bacterial adherence to hydrocarbons (BATH) assay, the strain *Planococcus* sp. MMD26 had higher cell hydrophobicity (84%) to the hydrocarbons in crude oil. The MS data evidenced that saturated hydrocarbons of the crude oil mainly distribute between n-C8 and n-C23. N-alkanes between n-C10 and n-C16 ranges were distributed most abundantly in crude oil (Figure 7). Recently, simulated bioremediation of marine oil spills was developed with bacterial consortium amended with rhamnolipid biosurfactants (Chen et al., 2013). Rhamnolipids have been reported to increase the oil biodegradation efficiency by 5.6%. RL-biosurfactants have been reported to be effective on biodegradation of petroleum hydrocarbons (Mulligan, 2005). Chen et al. (2013) have proven RLs as potential modulators in the biodegradation process of petroleum hydrocarbons, particularly as biomarker in biodegradation of oil samples. The biosurfactants modulate the biodegradation process if the structure is similar to rhamnolipid (Hua et al., 2004). Based on the analogue similarity hypothesis, it was predicted that petroleum hydrocarbons might be biotransformed through co-metabolism with rhamnolipids.

There are few reports that suggest the mechanisms of action of rhamnolipids on the biodegradation of hydrocarbons. Crude oil hydrocarbons are insoluble in water and therefore microbes cannot utilize hydrocarbons as substrates. The first step of hydrocarbon utilization (degradation) by microorganisms is cell surface contact which can be achieved through micelle/emulsion. This will facilitate the uptake/intake of hydrocarbons across the cell membrane. Adhesion to oil is facilitated by cell surface hydrophobicity and pseudo-solubilization (emulsion) is the two types of hydrocarbon interaction during biodegradation (Kirschner et al., 1980). The hydrocarbon degrading bacteria assimilate hydrocarbons from emulsified oil which is eventually biotransformed into primary metabolites and/or other storage granules (e.g. polyhydroxy alkanoates). The uptake phenomenon was perhaps influenced by the composition and concentration of hydrocarbons available in the bioremediation site. Cameotra & Singh (2009) proposed an active pinocytosis-like mechanism for the bacterial systems for hydrocarbon uptake. In this report, rhamnolipid type of biosurfactant produced by *Pseudomonas* sp. showed the internalization of biosurfactant layered hydrocarbon droplet which was similar in appearance to active pinocytosis.

**Heavy metal removal**

RL-mediated bioremediation of heavy metals was emerging as a promising approach (Herman et al., 1995). Asçi et al. (2010) investigated the sorption/desorption of Cd and Zn using rhamnolipids biosurfactant. Both cationic and anionic biosurfactants are efficiently used for the removal of heavy metal from contaminated soil, but the mechanisms involved in both processes are differs. Cationic biosurfactants form complexes with anionic metals and this complex facilitates their easy and efficient removal according to Le Chatelier’s principle. Anionic biosurfactants removes the metal from surfaces by the methods of desorption. In this method, first the anionic biosurfactants complexes with metal ions and further this biosurfactant–metal combination would require to be recovered from soil matrix (Christofi & Ivshina, 2002). The anionic nature of rhamnolipids and their complexation ability can proficiently remove heavy metal ions from soil. Urum & Pekdemir (2004) describe the effective removal of mixed hydrocarbon and heavy metals simultaneously by rhamnolipids. The micelle and vesicular structure of rhamnolipids has been responsible for metal ion–rhamnolipids complex formation. Rhamnolipids forms a variety of micellar and vesicular structures that depend on pH of a solution. They form liposome like vesicles, lamella like structures or lipid aggregates and negatively charged micelles at low pH (<6.0), pH 6.0 and 6.6, above pH 6.8, respectively (Mulligan &
The surface activity of rhamnolipids is also sensitive to pH. The highest surface activity of rhamnolipids is at near neutral pH (7.0 and 7.5; Mulligan & Wang, 2006). In micelle formation, the hydrophobic end/non-polar tail of biosurfactant molecules are oriented toward the center and hydrophobic end/polar heads oriented toward the surrounding aqueous solution. These polar heads of micelles bind to metals and facilitate the metal ions into the solution. The metal ions in emulsion can be recovered by washing, pumping or flushing (Aşçi et al., 2007; Frazer, 2000). It was established that sponge-associated bacteria as potential indicator model for monitoring heavy metal pollution in the marine environment (Selvin et al., 2009). Literature reports positive effects of biosurfactant-mediated heavy metal removal, but negative effects of biosurfactants on soil microbial diversity and mobility of solubilized (micelle) heavy metals into groundwater warrants further research.

**Biosurfactant-mediated disruption of biofilms**

The medical importance of biosurfactants was established due to their antibacterial, antifungal, antiviral and their anti-adhesive action against several pathogenic microorganisms (Meylheuc et al., 2001; Singh & Cameotra 2004). Recently, marine microbial products were evaluated as a potential source of anti-biofilm compounds (Dusane et al., 2010b, 2013). Sponge-associated marine bacteria are emerging as a potential source of novel biosurfactants (Gandhimathi et al., 2009). It has been hypothesized that the antimicrobial fouling process represents a chemical defense of host sponges mediated by the associated bacteria. Therefore, a biosurfactant produced by the sponge-associated marine actinobacterium was evaluated for the control of pathogenic biofilms. The glycolipid biosurfactant from marine *Brevibacterium casei MSA19* acts as a potential substance against biofilms produced by *Vibrio* spp., *E. coli* and *Pseudomonas* sp. In

![Figure 7. GC-MS peak showing the chemical components present in crude oil (A) before degradation and (B) after degradation (Hema, 2014).](image)
addition, this study explains the extraction, identification and characterization of the recovered biosurfactant from marine actinobacterium *B. casei* (Kiran et al., 2010). A glycolipid biosurfactant isolated from *Lysinibacillus fusiformis* S9 showed remarkable inhibition of biofilm formation by pathogenic bacteria such as *Escherichia coli* and *Streptococcus mutans*. At concentrations of 40 μg ml⁻¹, the biosurfactant was not bactericidal but effective on biofilm formation (Pradhan et al., 2014). Dusane et al. (2012) achieved effective disruption of *Yarrowia lipolytica* biofilm using RL biosurfactant. The biosurfactant produced by sponge-associated marine actinobacterium potentially inhibited the biofilm formation capacity of *Vibrio alginolyticus* (Figure 8). Dusane et al. (2010a, b) demonstrated that the biofilm formation by marine bacteria on the cooling system of atomic power station can be prevented by RL biosurfactants. Since the biofilm formation is a primary stage of destructive fouling in such cooling and marine structures, this study would enlighten innovative approaches in biofouling control as well (Dusane et al. 2011a).

**Biosurfactant-assisted greener synthesis of nanoparticles**

The surfactant mediated synthesis/stabilization of nanoparticles is emerging as a greener method is being considered to be a potential method for size-controlled synthesis of nanoparticles (Basnet et al., 2013; Fariasa et al., 2014; Kiran et al., 2011; Reddy et al., 2009). Biosurfactants can be used for high-performance nanomaterial production, since they easily form a variety of liquid crystals in aqueous solutions. A rhamnolipid biosurfactant was evaluated for its effect on the synthesis/stabilization of nanozirconia particles (Biswas & Raichur, 2008). Although the surfactant-mediated processes are highly effective, the processes are not cost-effective, since most of the available efforts are based on synthetic/commercial surfactants. The RL-templated stabilization process of nanoparticle synthesis was effective on the protection of size, monodispersity and salt effect (Saikia et al., 2013). Biosurfactant-templated synthesis and stabilization of nanoparticles are developing into a greener method (Figure 9; Kiran et al., 2010d). In this study, silver nanoparticles were stabilized by glycolipid biosurfactant produced by *B. casei* MSA19 under SSF using agro-industrial and industrial wastes as substrate. The synthesized silver NPs were uniform and stable for 2 months (Kiran et al., 2010d). In a previous report, a rhamnolipid biosurfactant was used as stabilizing agent of silver nanoparticles (Xie et al., 2006). The marine glycolipid mediated synthesis of silver nanoparticles would
be effective and advantageous over chemical surfactants. Production of nano-biosurfactants was achieved and it will help to replace synthetic nano-emulsions (Saeedi et al., 2014). A recent discovery showed that the presence of a rhamnolipid biosurfactant increased the resistance of E. coli to nanosilver (Xie et al., 2014). This study may facilitate a new line of research on enhanced nanosilver synthesis by engineered E. coli cells.

Future considerations and conclusion

The literature reports that the cost-effective greener processes for the enhanced production of rhamnolipid biosurfactants are limited, since the production exclusively relied on Pseudomonas and recombinant strains. Thus, new sources of potential producers are inevitable for the development of diverse production processes using industrial waste and/or raw materials. The novel producers would ultimately increase the scope of rhamnolipid application in marine and other extreme environments. From our recent studies on sponge-associated marine actinobacteria, it is evident that the culturable marine actinobacteria are novel source of rhamnolipid biosurfactants. Exploration of biosurfactants from marine sources requires further research to demonstrate potential applications and novel structural analogues. The application of biosurfactants in agriculture, including development of novel insecticides for stored produce, will have potential implications. Preliminary research findings (unpublished data) evidenced that stored grain pest *Sitophilus oryzae* can be controlled using glycolipid biosurfactant (Figure 10). This would ultimately increase the scope of green and safe methods to protect stored produce from invading pests.

At present, only <1% of microbes can be isolated using conventional culturing methods and departing from the huge number of microorganisms and their biochemical pathways are inaccessible. Metagenomics is an exploratory tool of biological diversity and provides a magnifying lens to study the uncultivable microbial community and approaches can be either be sequence driven or function driven (Handelsman, 2009). The development of high throughput metagenomic approaches would facilitate a comprehensive assessment of the potential exploitable microbial biodiversity their biochemical pathways within various marine ecosystems (Kennedy et al., 2011). High throughput function-driven metagenomic approaches would bring out novel leads including biosurfactants from marine environment.

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