Biotechnology and Genetic Engineering Reviews - Vol. 27, 159-184 (2010)

Quorum sensing: implications on Rhamnolipid biosurfactant production

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Abstract

Quorum sensing (QS) has received significant attention in the past few decades. QS describes population density dependent cell to cell communication in bacteria using diffusible signal molecules. These signal molecules produced by bacterial cells, regulate various physiological processes important for social behavior and pathogenesis. One such process regulated by quorum sensing molecules is the production of a biosurfactant, rhamnolipid. Rhamnolipids are important microbially derived surface active agents produced by *Pseudomonas* spp. under the control of two interrelated quorum sensing systems; namely *las* and *rhl*. Rhamnolipids possess antibacterial, antifungal and antiviral properties. They are important in motility, cell to cell interactions, cellular differentiation and formation of water channels that

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Abbreviations: QS: quorum sensing; AI: autoinducer; HSL: homoserine lactone; AHL: acyl homoserine lactone; PQS: pseudomonas quinolone signal; sRNA: small ribonucleic acid; RL: rhamnolipid; HAA: 3-(3-hydroxyalkanoyloxy) alkanoic acid; CTAB: cetyl trimethyl ammonium bromide; TLC: thin layer chromatography; GC: gas chromatography; MS: mass spectroscopy; FTIR: fourier transform infrared spectroscopy; ATR: attenuated total reflectance; NMR: nuclear magnetic resonance; SDS: sodium dodecyl sulfate; CMC: critical micelle concentration; LPS: lipopolysaccharide; TMV: tobacco mosaic virus; PAI: pseudomonas autoinducer; FAD: flavin adenine dinucleotide; NAD: nicotinamide adenine dinucleotide; TDP: thiamine di-phosphate; mN/m: milli-Newton per meter

are characteristics of *Pseudomonas* biofilms. Rhamnolipids have biotechnological applications in the uptake of hydrophobic substrates, bioremediation of contaminated soils and polluted waters. Rhamnolipid biosurfactants are biodegradable as compared to chemical surfactants and hence are more preferred in environmental applications. In this review, we examine the biochemical and genetic mechanism of rhamnolipid production by *P. aeruginosa* and propose the application of QS signal molecules in enhancing the rhamnolipid production.

Introduction

Quorum sensing (QS) is the mechanism by which bacteria engage in cell-to-cell communication using diffusible molecules based on a critical cell density (Williams *et al*., 2007). When the cell density increases these molecules referred to variously as autoinducers (Fuqua *et al*., 1997, Kleerebezem *et al*., 1997, Williams and Camara, 2009), pheromones or quoromones are produced that dictate the behavior of bacterial populations. QS signaling molecules, control diverse physiological processes; some of which are inter-related and under the control of multifaceted QS systems. For instance, in *P. aeruginosa,* exo-polysaccharide production (Davies *et al*., 1998), antibiotic resistance (Bjarnsholt *et al*., 2005) and biofilm formation (Davies *et al*., 1998, Hentzer *et al*., 2001) are all under the control of QS molecules. In addition to the aforementioned examples, certain *Pseudomonas* sp. also produces a surface active agent, viz. rhamnolipid, the production of which is regulated by QS molecules (Pearson *et al*., 1997).

Rhamnolipids have been extensively studied due to their antibacterial, antifungal and antiviral properties (Haferburg *et al*., 1987, Stanghellini and Miller, 1997, Syldatk *et al*., 1985). They are important in bacterial cell motility, cell to cell interactions, cellular differentiation and formation of water channels that are characteristics of *Pseudomonas* biofilms. Rhamnolipids also enable *Pseudomonas* spp. to access poorly soluble hydrophobic carbon sources and thereby facilitate their uptake (Maier and Soberon-Chavez, 2000, Nealson *et al*., 1970). These properties have encouraged the use of rhamnolipid compounds in environmental bioremediation of contaminated soils and polluted waters. In the medical scenario, they are important as antimicrobials, healing of wounds and in organ transplants (Tatjana and Goran, 2007). Apart from the above applications, rhamnolipids are also used in cosmetics, pesticide removal, pharmaceutical, oil sludge recovery, enhanced oil recovery, household cleaning, agriculture and food industry. Morever, rhamnolipids are biodegradable and less toxic than many synthetic surfactants, and hence their use is highly favored (Hommel, 1990, Volkering *et al*., 1995).

In this review, we will focus on quorum sensing in detail and describe its role in rhamnolipid production, with particular reference to *Pseudomonas aeruginosa.* The role of quorum signaling in rhamnolipid biosynthesis, bacterial physiology and ecology is described. We have also discussed the application of quorum signaling molecules in enhancing the production of rhamnolipids.

Quorum Sensing

QUORUM SENSING MOLECULES

Quorum sensing has received a great deal of attention, primarily due to the diverse roles it plays in regulating bacterial physiology (Miller and Bassler, 2001, Waters and Bassler, 2005). QS implies that bacteria sense each other by detecting a threshold accumulation of the secreted signals. The signal molecules are well documented in both Gram positive and Gram negative bacterial species. However, there seems to be a significant difference in the signal molecules amongst these bacterial groups. In the Gram-positive bacteria, QS is associated with a number of linear and post-translationally modified peptide based signal molecules, such as the peptide lactones and peptide thiolactones which are found in *Bacillus subtilis*, *Enterococcus* spp., and *Staphylococcus aureus*. The chemical structures of the Gram-positive QS peptides vary greatly in the number of residues and the type of modifications. The biosynthesis pathways are however more complex in Gram-positive bacteria than the AHL molecules in Gram-negative bacteria, because of the post translational modifications of the peptides and their inability to diffuse across the membranes. Interestingly, to date the largest studied most complex peptide signal molecules produced by a few Gram-positive bacterial species are the lantibiotics. These molecules possess antimicrobial activity, as shown by nisin produced by *Lactobactococcus lactis* (Lubelski *et al*., 2008). Another emerging class of compounds in *Staphylococcus aureus*; *Enterococcus faecalis*; *Listeria monocytogenes* and other *Staphylococci* are the type I autoinducing peptide. These QS molecules play an important role in the Gram-positive bacterial physiology (Miller and Bassler, 2001, Waters and Bassler, 2005).

In Gram-negative bacteria, the regulation of quorum sensing is under the control of the autoinducer (AI) molecules. These AI molecules belong to the biochemical class of acyl homoserine lactones, which are lipophilic in nature. Homoserine lactones are derived from S-adenosyl-methionine, which is one of the substrates for AHL synthesis and consists of a hydrophilic homoserine lactone head group and a hydrophobic acyl side chain that varies based on species. The side chain ranges from 4 to 18 carbons, with the most significant divergence in length and chemical composition occurring at the third carbon. These alterations in structure act to provide specificity to QS signals and facilitate communication between bacteria. The quorum sensing molecule, designated as autoinducer 1 (AI-1), includes *Lux* based quorum sensing systems present in Gram-negative bacteria such as *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa* and *Vibrio fischeri* (Engebrecht *et al*., 1983, Fuqua *et al*., 1994). Quorum sensing was first characterized in the marine bacteria *Vibrio harveyi* and *Vibrio fischeri* (Nealson *et al*., 1970, Nealson and Hastings, 1979). In *V. harveyi*, there are two types of density-dependent signaling systems that regulate bioluminescence activity consisting of autoinducer 1 and 2. The AI-1 (N-3-oxohexanoyl-L-homoserine lactone) molecule found in *V. fischeri* governs the induction of luminescence operon (Gilson *et al*., 1995). *V. harveyi* and *V. cholerae* have been reported to use the AI-1 quorum sensing circuit for intra-species communication. The essential characteristics of AI-1 systems are the biosynthesis of acylated homoserine lactones (AHLs) by an AHL synthase, encoded by *luxI* in *V. fischeri* or *luxI* homologs in other bacteria; and an AHL response regula-

tor, encoded by *luxR* (or luxR homologs). The N-octanoyl-L-homoserine lactone (AI-1) molecule in *V. fischeri* interacts with and activates the luminescence in *E. coli* via LuxR (Gilson *et al*., 1995). The other QS signal molecules designated as autoinducer 2 (AI-2) is observed in both Gram-negative and Gram-positive bacterial species and is suggested to mediate communication among and between species (Bassler *et al*., 1997, Schauder *et al*., 2001). AI-2 signal production occurs in bacteria that possess a *luxS* homologue. The AI-2 molecule in *Vibrio harveyi* is currently believed to be furanosyl borate diesters (Chen *et al*., 2002). Several other bacterial species can interact with the *Vibrio harveyi* AI-2 signaling pathway and the AI-2 modifying LuxS protein sequence is extremely conserved throughout the bacterial kingdom. The third type of autoinducer (AI-3) molecules are involved in cross talk and inter-kingdom signaling with the eukaryotic hormones (epinephrine/ norepinephrine). The AI-3 molecules are observed in *E. coli* O157:H7 and the host epinephrine cell signaling. This signaling activates transcription of virulence genes in enterohemorrhagic *E. coli* O157:H7 as well as intestinal cell actin rearrangement. The structure of AI-3 molecules is however yet not elucidated.

Apart from these autoinducer molecules, other non-AHL compounds such as indole, PQS, small RNA and secondary messengers are also involved in quorum sensing induction. Indole is produced and is reported to act as an extracellular signal in the induction of quorum in *E. coli* (Wang *et al*., 2005). Another molecule heptyl-hydroxy-quinolone, designated the *Pseudomonas* quinolone signal (PQS) found exclusively in *Pseudomonas* spp. is a part of the quorum sensing hierarchy. PQS acts as a link between *las* and *rhl* systems (McKnight *et al*., 2000). PQS is similar to AHLs with respect to size and its lipophilic nature. Most of the genes involved in the synthesis and regulation of PQS have been described in detail earlier, however the mechanism of activity is unknown (Cao *et al*., 2001, Deziel *et al*., 2004, Diggle *et al*., 2003, Gallagher *et al*., 2002). These molecules diffuse freely through the bacterial membrane and are internally sensed. Other molecules, such as the small RNAs also play a role in quorum sensing. It is becoming increasingly apparent that like other bacterial processes, integration of information by QS systems is regulated by noncoding small RNAs (sRNAs). These sRNAs are global regulators that act directly or indirectly to control gene expression by post-transcriptional mechanisms. sRNAs are important regulators involved in bacterial and eukaryotic developmental processes (Masse *et al*., 2003, Wienholds and Plasterk, 2005). Bejerano-Sagie and Xavier (2007) have recently reviewed the crucial role of small noncoding RNAs in the regulation of bacterial QS. Regulation by sRNAs rather than by proteins is presumed to be beneficial when a rapid response is required, because of the short time required to synthesize or degrade sRNAs compared with synthesizing and degrading proteins.

QS systems govern a diverse set of microbial processes, including antibiotic biosynthesis, swarming, swimming and twitching motility, plasmid conjugal transfer, biofilm formation (Davies *et al*., 1998), pathogenesis, production of biosurfactant, enzymes and other secondary metabolites (for reviews see Camara *et al*., 2002, Fuqua and Greenberg, 2002, Lazdunski *et al*., 2004, Miller and Bassler, 2004, Pappas *et al*., 2004, Whitehead *et al*., 2001). Recently, the study of QS systems has been extended to include implications in synthetic biology for population control (You *et al*., 2004), band detection (Basu *et al*., 2005) and predator-prey systems (Balagadde *et al*., 2008). In this review, we focus especially on the aspects of QS involved in the production of rhamnolipid.

QUORUM SENSING IN MICROBIAL COMMUNITIES

QS activities have been documented in biofilms for some time (Davies *et al*., 1998, McLean *et al*., 1997), although the magnitude of their role in biofilms depends on the nutritional environment (Shrout *et al*., 2006). As stated earlier, *P. aeruginosa* has several QS systems, including the AHL-mediated *las* and *rhl* systems, the PQS system (Mashburn and Whiteley, 2005); as well as the AI-2 QS system (Duan *et al*., 2003). Very recently, small regulatory RNA molecules have also been shown to influence QS regulation (Tu *et al*., 2008). These signals and many of the functions that they encode are quite important for biofilm development and bacterial interactions within microbial communities (Givskov *et al*., 1996, Parsek and Greenberg, 2000). Several years ago, Singh *et al*. (2000) investigated the AHL expression levels in planktonic and biofilm grown *P. aeruginosa*. They found that the ratio of 3-oxo-dodecanoyl homoserine lactone (3-oxo-C12 HSL, produced by the *lasI* gene product) to N-butanoyl homoserine lactone (C4 HSL, produced by the *rhlI* gene product) in planktonic populations was approximately 3:1. In laboratory-grown and clinically-obtained biofilms (sputum samples of cystic fibrosis patients), the ratio was reversed with C4-HSL being the predominant AHL (Singh *et al*., 2000). Among other things, C4-HSL regulates rhamnolipid biosynthesis (Ochsner and Reiser, 1995). Here, we focus on biofilm and microbial community features that are influenced by rhamnolipids.

Rhamnolipids function as biosurfactant molecules (Davey *et al*., 2003). In this fashion they have been predicted to facilitate uptake of poorly soluble, hydrophobic compounds. Work from the laboratories of PA Holden (2002) and RM Miller (1994, 1995) have shown the emulsifying nature of rhamnolipids that enables *Pseudomonas* sp to facilitate hydrocarbon utilization. Interestingly, one study showed that the *P. aeruginosa* outer membrane was removed in the presence of rhamnolipids, such that the hydrophobic membrane interior could bind directly to lipids (Al-Tahhan *et al*., 2000).

During the process of biofilm development and maturation, surface-attached cells will aggregate into microcolonies that are surrounded by regions of few cells referred to as water channels (Davey and O'Toole, 2000, Sauer *et al*., 2002). During the aggregation process, surface-colonized *P. aeruginosa* move across the substratum by a combination of twitching motility, which involves type IV pili (O'Toole and Kolter, 1998); and swarming, which involves cell elongation, hyper-flagellation and differentiation (Kohler *et al*., 2000). Rhamnolipids play a role in the swarming process, acting both as surface wetting agents and as chemotaxis stimuli. In swarming but not swimming, rhamnolipids function as chemoattractants whereas the chemically related, hydroxy alkanoic acids function as chemorepellants (Tremblay *et al*., 2007). Swarming can be blocked by branched chain fatty acids, which presumably compete with rhamnolipids (Inoue *et al*., 2008).

Rhamnolipids are also important for the formation of water channels in mature biofilms as shown by Davey *et al*. (2003). During this study, *rhl* mutants, unable to synthesize rhamnolipids, formed biofilms lacking the characteristic architecture (micro-colonies and water channels). Co-culture of the *rhl* mutants with wild type *Pseudomonas* could partially rescue the biofilm structural phenotype. Overproduction of rhamnolipids caused an inhibition of biofilm formation, blocked cellular aggregation, and also blocked secondary colonization onto preformed biofilms by other

planktonic bacteria (Davey *et al*., 2003). Rhamnolipids have also been associated with cell dispersal from biofilms (Boles *et al*., 2005, Pamp and Tolker, 2007).

One notable feature of biofilms is the protection that is offered to their component cells from antimicrobial agents and external forces including predation and the immune system (Costerton *et al*., 1987). Rhamnolipids do play a role in the chemical ecology of biofilms. Rhamnolipid production within *P. aeruginosa* biofilms has been shown to cause the rapid killing of polymorphonuclear leukocytes during experimental lung infections of mice (Jensen *et al*., 2007). From a microbial competition perspective, rhamnolipids, produced by *P. aeruginosa* have been shown to be able to disrupt preformed biofilms of *Bordetella bronchiseptica* (Irie *et al*., 2005). Production of these biosurfactants is not always beneficial to *P. aeruginosa*. Kohler *et al*. (2007) showed that the action of the antibiotic, azithromycin, was enhanced in the presence of rhamnolipids, presumably as these compounds facilitated the transport of the antibiotic across the bacterial membrane. Although they do have varied roles within biofilms (Pamp and Tolker, 2007), rhamnolipids are an important component of *Pseudomonas* biofilm development, structure, and functions.

Biosurfactant

Biosurfactants are surface active agents that have been receiving increasing attention on account of their unique properties such as their mild production conditions, lower toxicity, and higher biodegradability, compared to their synthetic chemical counterparts (Rosenberg and Ron, 1999). Biosurfactants are produced by bacteria or yeasts from variety of sources such as sugars, glycerol, oils etc. Biosurfactants are classified as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, and polymeric or particulate compounds (Desai and Banat, 1997). The hydrophobic portion of the molecule may be long-chain fatty acids, hydroxyl fatty acids or α-alkyl-β-hydroxyl fatty acids. The hydrophilic moiety can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol. One such biosurfactants that have been extensively studied is rhamnolipid (Lang and Wullbrandt, 1999).

RHAMNOLIPID BIOSURFACTANT

Rhamnolipid production is a characteristic of *P. aeruginosa* and was first described by Jarvis and Johnson (1949), however recently other *pseudomonads*. *P. putida* and *P. chlororaphis*, as well as *Burkholderia pseudomallei* have been reported to produce a variety of rhamnolipids (Gunther *et al*., 2005, Haussler *et al*., 1998, 2003, Tuleva *et al*., 2002). The production of rhamnolipids is species specific. Some species produce a mono-rhamnolipid, others produce a di-rhamnolipid and yet others produce a mixture of rhamnolipids, all of which vary in lipid chain lengths. The rhamnolipids are composed of a polar head group and one or more non-polar tail. *P. aeruginosa* produces four types of rhamnolipids, including a mixture of homologous species of RL1 ($RhC_{10}C_{10}$), RL2 (RhC_{10}), RL3 ($Rh_2C_{10}C_{10}$) and RL4 (Rh_2C_{10}) (Rahman *et al*., 2002). The length of the carbon chains found on the β-hydroxy portion of the rhamnolipid can vary significantly; however, in case of *P. aeruginosa* ten carbon molecule chains are the predominant form (Deziel *et al*., 2000). Rhamnolipid as

well as their precursor, 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs), display tensioactive properties (Deziel *et al*., 2003), which further facilitate their medical, environmental and industrial applications. Rhamnolipid induces a remarkably larger reduction in the surface tension of water from 72mN/m to values below 30mN/m and it also reduces the interfacial tension of water/oil systems from 43mN/m to values below 1mN/m. Rhamnolipids also show an excellent emulsifying activity with a variety of hydrocarbons and vegetable oils (Abalos *et al*., 2001). Rhamnolipids solubilize hydrophobic molecules, such as long-chain hydrocarbons, and allow their use as a carbon source and in addition facilitate the interactions between cells by promoting aggregation (Herman *et al*., 1997).

Rhamnolipids are extensively used in the production of fine chemicals, characterization of surfaces and surface coatings, as additives for environmental remediation and as biological control agents (Stanghellini and Miller, 1997). Rhamnolipids have been regarded as virulence factors (Kownatzki *et al*., 1987) and antimicrobials (Abalos *et al*., 2001), and are implicated in the development of biofilms (Davey *et al*., 2003) and along with HAAs, have been documented to be crucial for *P. aeruginosa* swarming motility (Deziel *et al*., 2003, Kohler *et al*., 2000).

METHODS FOR RHAMNOLIPID DETECTION AND QUANTIFICATION

In the last few decades, extensive research has been conducted in the area of biosurfactants. This has lead to the development of various techniques to detect, quantify and enhance the production of biosurfactants. Various techniques, especially with respect to rhamnolipid biosurfactant, are mentioned here. Each of these methods has been described in a recent review by Heyd *et al*. (2008) and other references stated below.

i. Surface tension reduction (Guerra-Santos *et al*., 1984, Haussler *et al*., 1998).
ii. Hemolytic activity (Siegmund and Wagner, 1991).
iii. Colorimetric method using Cetyl Trimethyl Ammonium Bromide (CTAB) plate assay (Siegmund and Wagner, 1991).
iv. Methylene blue complexation method (Pinzon and Ju, 2009).
v. Rhamnolipid estimation using Anthrone reagent (Helbert and Brown, 1957).
vi. Rhamnolipid estimation using Orcinol method (Chandrasekaran and Bemiller, 1980, Koch *et al*., 1991).
vii. Thin layer chromatography (TLC) (de Koster *et al*., 1994, Rendell *et al*., 1990).
viii. Gas Chromatography (GC) (Arino *et al*., 1996, Van Dyke *et al*., 1993).
ix. High performance liquid chromatography (HPLC) (Deziel *et al*., 1999, Lepine *et al*., 2002, Mata-Sandoval *et al*., 1999).
x. Mass spectrometry (MS) (Deziel *et al*., 1999).
xi. Fourier transform infrared spectroscopy (Borgund *et al*., 2007, Gartshore *et al*., 2000) and Attenuated Total Reflectance (ATR) FTIR (Leitermann *et al*., 2008).
xii. NMR spectroscopy (Choe *et al*., 1992, Monteiro *et al*., 2007).

Combinations of these methods are generally used for the detection and estimation of rhamnolipid production.

Applications of rhamnolipid biosurfactant

Compared to chemical surfactants, biological biosurfactant possess numerous attributes that make them invaluable in both environmental and industrial settings (Hommel, 1990, Rahman and Gakpe, 2008, Volkering *et al*., 1995). Noordman and Janssen (2002) claimed the degradation of hexadecane by rhamnolipid with rates higher as compared to other biosurfactants. Urum *et al*. (2006), on the other hand compared the effectiveness of biosurfactant rhamnolipid, saponin and sodium dodecyl sulfate (SDS). They found that rhamnolipid and saponin aided crude oil degradation almost equally, whilst SDS was found to be ineffective. Rhamnolipid is therefore the best biosurfactant since it is produced naturally via microbial activity, while SDS is a synthetic surfactant.

Rhamnolipids in particular are biotechnologically important due to their antibacterial, antifungal and antiviral activities (Haferburg *et al*., 1987, Stanghellini and Miller, 1997, Syldatk *et al*., 1985). Rhamnolipid biosurfactants increases membrane permeability of the bacterial cells thereby causing cell death. The biosurfactant probably forms molecular aggregates in surface bacterial membranes, leading to the formation of trans-membrane pores (King *et al*., 1991). Studies conducted by Sotirova *et al*. (2008) showed the rhamnolipid biosurfactant complex termed PS mediates permeabilizing effects on Gram-positive and Gram-negative bacterial strains, namely *B. subtilis* and *P. aeruginosa*. They reported that at lower concentrations of rhamnolipid biosurfactant close to CMC, the growth of bacterial cells is not influenced, however concentrations greater than CMC exhibit toxic conditions for *B. subtilis* cells but not for *P. aeruginosa* as evident from the levels of extracellular proteins. The biosurfactant enhanced levels of extracellular protein in *B. subtilis* cells compared with those of *P. aeruginosa*, which confirmed the higher susceptibility of Gram-positive cells to the effect of the studied biosurfactant. It is evident that the outer membrane of Gram-negative bacteria have lipopolysaccharide (LPS), porin channels, and murein lipoprotein, all of which are absent in Gram-positive bacteria. Also, the outer membrane functions as an efficient permeability barrier that is able to exclude biosurfactant molecules. The permeability barrier property is largely caused by the presence of the LPS layer. Increased cell permeability induced by rhamnolipid biosurfactant was most likely caused by the release of LPS from the outer membrane (Al-Tahhan *et al*., 2000, Sotirova *et al*., 2007). Rhamnolipids have been used as emulsifying agents for the transport of drugs to the site of action. Rhamnolipids in combination with the antibiotic, azithromycin facilitated destruction of the bacterial cells by increasing the bacterial membrane permeability (Kohler *et al*., 2007). *P. aeruginosa* rhamnolipid mixture was found to inhibit a majority of pathogenic bacteria such as *A. faecalis*, *E. coli*, *Micrococcus luteus*, *Mycobacterium phlei*, *Serratia marcescens* and *S. epidermidis*. The marine bacterium, *B. pumilus* cell adhesion and biofilm disruption was also achieved using rhamnolipids (unpublished data).

Rhamnolipids also show antifungal activity against *Aspergillus niger*, *Aureobasidium pullulans*, *Chaetonium globosum* and *Penicillum crysogenum* (Abalos *et al*., 2001, Rahman and Gakpe, 2008). The zoosporicidal activity of mono and dirhamnolipids against phytopathogens is reported by Stanghellini and Miller (1997). Stanghellini and coworkers (1998) patented their work on rhamnolipid biosurfactants produced by *Pseudomonas sp.* able to rapidly kill zoospores by rupturing the plasma membrane of three representative plant pathogenic microorganisms; namely *Pythium aphaniderna-*

tum, *Plasmopara lactucae-radicis*, and *Phytophthora capsici*. Rhamnolipid-producing strains also provide control of *Pseudoperonospora cubensis*, the causal agent of downy mildew of cucurbits, by intercalating into the plasma membrane and thereby destroying the cell structure. Rhamnolipids inhibit the growth of algal species including *Heterosigma akashivo* and *Protocentrum dentatum* (Wang *et al*., 2005). Rhamnolipids also display antiviral properties against pathogens like TMV and potato X virus (Haferburg *et al*., 1987). They can be used as adjuvants for vaccines. McClure and Schiller (1992) reported the enhancement of phospholipase C activity after addition of rhamnolipids. Tatjana and Goran (2007) patented their work on rhamnolipids as effective in wound healing, treating burn shock, atherosclerosis, organ transplants, depression, schizophrenia and cosmetics (Tatjana and Goran, 2007). Rhamnolipids are however also responsible for increasing the virulence factors secreted by *P. aeruginosa* that affect the structure of human airway epithelium in the early stages of infection (Zulianello *et al*., 2006).

In environmental bioremediation, rhamnolipids play a significant role in the treatment of soils contaminated with industrial waste, crude oils, polyaromatic hydrocarbons, refinery products, pesticides and heavy metals (Rahman *et al*., 2003). The ability of a rhamnolipid mixture produced by *P. aeruginosa* UG2 to solubilize the pesticides atrazine, trifluralin, and coumaphos was compared with a chemical surfactant Triton X-100. It was observed that the values of maximum micellar solubilization capacities for trifluralin and coumaphos in Triton X-100 were double those for the rhamnolipid mixture, whereas atrazine maximum micellar solubilization capacity value for the rhamnolipid biosurfactant was in the same range as that for the synthetic surfactant (Mata-Sandoval *et al*., 2000).

Muller-Hurtig *et al*. (1993) and Finnerty (1994) reviewed the possible use of biosurfactants in soil remediation. Rhamnolipids can alter the physicochemical properties of oil, thereby facilitating the removal of oil from contaminated soils. Rhamnolipids have also shown to enhance the cell surface hydrophobicity of *P. aeruginosa* cells by inducing removal of lipopolysaccharides, thereby increasing the uptake of hydrophobic compounds by the cells (Al-Tahhan *et al*., 2000). Heavy metals are included on the EPA's list of priority pollutants (Mulligan *et al*., 2001). Heavy metals pose a persistent problem at many contaminated sites and are being added to soil, water, and air in increasing amounts from a variety of sources including industrial, agricultural and domestic effluents. Heavy metals can be removed from contaminated areas through direct binding of the rhamnolipids to the metals forming a stable complex, which is subsequently removed from the soil (Ochoa-Loza *et al*., 2001).

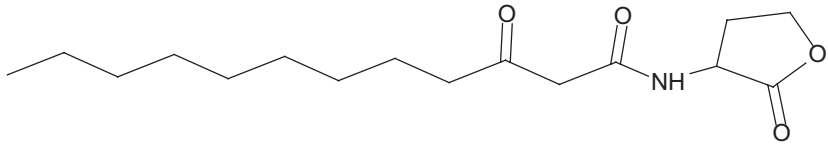
In food industries, rhamnolipids serve as a good source of rhamnose sugar that acts as a precursor for high quality flavor components (Linhardt *et al*., 1989). Van Haesendonck and Vanzeveren (2004) reported the application of rhamnolipid for volume enhancement and for texture modification in bakery and pastry products.

Rhamnolipids and quorum sensing

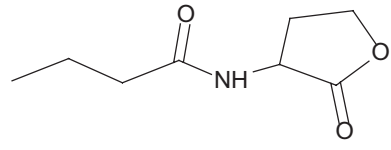
QUORUM SENSING MOLECULES IN RHAMNOLIPID PRODUCTION

P. aeruginosa possesses two interrelated QS systems, namely the *las* and *rhl,* (Gambello and Iglewski, 1991, Passador *et al*., 1993, Toder *et al*., 1991) that regulate different processes including rhamnolipid expression, enzyme production, pyocyanin

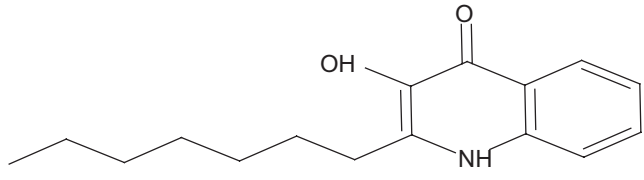
pigment production and maintenance of biofilm architecture (Davies *et al*., 1998, de Kievit and Iglewsky, 2000, Rumbaugh *et al*., 2000, Smith and Iglewski, 2003). Production of rhamnolipid is governed by three QS molecules: the twelve carbon *Pseudomonas* autoinducer 1 (PAI-1) [N-(3-oxododecanoyl) homoserine lactone also known as 3-oxo-C_{12}-HSL] (Pearson *et al*., 1994), *Pseudomonas* autoinducer 2 (PAI-2) [N-butyryl homoserine lactone known also as C_4-HSL] (Pearson *et al*., 1995), and PQS, [2-heptyl-3-hydroxy-4-quinolone] (Pesci *et al*., 1999) (Fig. 1).



PAI-1 (3-oxo-C12- HSL)



PAI-2 (C4-HSL)



PQS

Figure 1. Quorum sensing molecules in *Pseudomonas aeruginosa* PAO1. The three quorum sensing molecules denoted as (a) *Pseudomonas* autoinducer PAI-1 [N-(3-oxododecanoyl) homoserine lactone] also known as 3-oxo-C_{12}-HSL; (b) *Pseudomonas* autoinducer PAI-2 [N-butyryl homoserine lactone] known as C_4-HSL and (c) *Pseudomonas* Quinolone Signal (PQS), [2-heptyl-3-hydroxy-4-quinolone] coordinates the cellular activities.

Genetic basis of rhamnolipid production

The production of rhamnolipids is under the control of two quorum sensing systems, namely the *las* and *rhl* QS systems. In *P. aeruginosa*, the *las* operon consists of two transcriptional activator proteins, the LasR and LasI, which directs the synthesis of N-3-oxododecanoyl homoserine lactone (PAI-1 or 3-oxo-C_{12}-HSL) autoinducer. Induction of the *lasB* gene that encodes the elastase enzyme and other virulence genes requires the expression of LasR and PAI-1 autoinducer. The production of rhamnolipid is regulated by the *rhl* system (Johnson and Boese-Marazzo, 1980). The synthesis of rhamnolipids takes place under the coordinated guidance of *rhlAB* genes that

encodes a group of enzymes termed the rhamnosyltransferases (Ochsner *et al*., 1995). Rhamnolipid is a complex synthesized by two enzymes namely, rhamnosyltransferase 1 and rhamnosyltransferase 2. The *rhl* system consists of transcriptional activator proteins RhlR and RhlI, which regulates the synthesis of a QS molecule, N-butyryl homoserine lactone (PAI-2 also called the C_4-HSL) (Ochsner *et al*., 1994b, Pearson *et al*., 1995). The transcriptional activator RhlR activates the transcription of *rhlAB* operon genes, coding for rhamnosyltransferase 1 (Ochsner *et al*., 1994b), and another gene, *rhlC* encoding for the rhamnosyltransferase 2 (Rahim *et al*., 2001). The genes involved in the production of rhamnolipid are mentioned in table 1. With increase in bacterial cell density, the induction of *las* quorum sensing system takes place resulting in an increase in the concentration of PAI-1 (3-oxo-C_{12}-HSL) autoinducer molecule. This quorum sensing molecule (PAI-1) then binds to the transcriptional activator site LasR and forms the LasR–PAI-1 complex. The LasR–PAI-1 complexes induces genes controlled by the *las* quorum-sensing system, including a negative regulator gene *rsaL*, *rhlR* (Ochsner *et al*., 1994b, Pearson *et al*., 1995) and *pqsH*, required for PQS production (Mashburn and Whiteley, 2005). The activity of these signals is dependent upon their ability to dissolve in and freely diffuse through the aqueous solution.

Table 1. Quorum sensing systems prevalent in *P. aeruginosa* suggesting the genes and functions assisting rhamnolipid production.

Quorum sensing system	Genes involved	Enzyme product
las system	*las*I	Autoinducer synthesis, LasI synthase
	*las*R	Transcriptional regulator, LasR synthase
	*las*A	LasA protease precursor
	*las*B	Elastase LasB
rhl system	*rhl*I	Autoinducer synthesis protein, RhlI synthase
	*rhl*R	Transcriptional regulator, RhlR synthase
	rhlAB	Rhamnosyltransferase 1
	*rhl*C	Rhamnosyltransferase 2
	*rhl*G	β-ketoacyl reductase
pqs system	*pqs*A	coenzymeA ligase
	*pqs*B	Homologous to β-keto acyl carrier protein synthase
	*pqs*C	Homologous to β-keto acyl carrier synthase (3-oxoacyl-[acyl-carrier protein])
	*pqs*D	3-oxoacyl-[acyl-carrier protein] synthase III
	*pqs*E	Quinolone signal response protein
	*pqs*H	Probable FAD-dependent monooxygenase
	*pqs*L	Probable FAD-dependent monooxygenase

P. aeruginosa produced rhamnolipid biosurfactant that enhances the solubility of PQS in aqueous solutions (Calfee *et al*., 2005). However unlike other QS, the hydrophobic

PQS is transported primarily through outer membrane vesicles (Mashburn and Whiteley, 2005), the formation of which are PQS-induced (Mashburn-Warren *et al*., 2008, Mashburn-Warren *et al*., 2009). PQS (3, 4-hydroxy-2-heptylquinoline), as mentioned earlier acts as a link between *las* and *rhl* quorum sensing systems (Muller-Hurtig *et al*., 1993). Using mutants deficient in the synthesis of PQS, the cells of *P. aeruginosa* make less rhamnolipid than the wild type strains (Diggle *et al*., 2003). PQS here either directly or indirectly induces the *rhlI* gene which directs the production of PAI-2 (C_4-HSL) quorum sensing molecule that binds to and activates RhlR (McKnight *et al*., 2000). The operon, *rhlAB* that encodes these enzymes responsible for rhamnolipid production is controlled at the transcriptional and translational levels by RhlR and C4-HSL (Ochsner and Reiser, 1995). The RhlR–PAI-2 complex induces genes controlled by the *rhl* quorum sensing system for the production of rhamnolipid. The *las* system controls the expression of transcriptional activator RhlR (Fig. 2). Along with this an important gene, *rhlG* is involved in the synthesis of β-hydroxyacid moiety of rhamnolipids (Campos-Garcia *et al*., 1998). A QS hierarchy therefore exists in *P. aeruginosa las* and *rhl* systems for the synthesis of rhamnolipid.

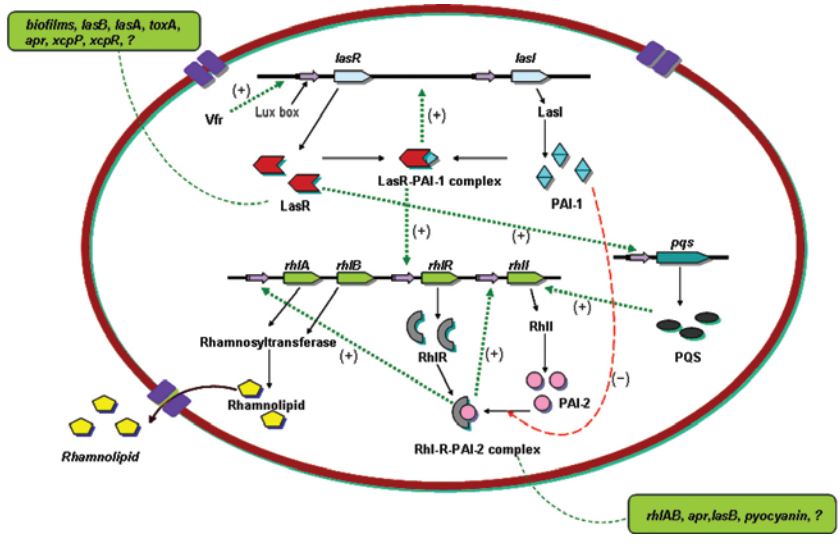


Figure 2. Schematic representation of the *las* and *rhl* genes and quorum sensing molecules in *Pseudomonas aeruginosa* for the production of rhamnolipid. The *las* system produces transcriptional activators, LasR and LasI (producing PAI-1). The *rhlA* and *rhlB* genes are arranged as an operon and are clustered with *rhlR* and *rhlI*. These genes, *rhlABRI* directs the synthesis of rhamnosyltransferase and transcriptional activators, RhlR and RhlI (producing PAI-2), which are responsible for the production of rhamnolipids. Vfr induces *lasR* and the concentration of PAI-1 increases where it binds to and activates LasR (Albus *et al*., 1997). The autoinducer PAI-1 (encoded by *lasI*) binds with LasR and forms a LasR-PAI-1 complex. This complex regulates the transcription of *rhlR*. *rhlR* produces RhlR protein, which binds to the PAI-2 autoinducer resulting in RhlR-PAI-2 complex that interacts with the *rhlA* promoter (*lux* box) to begin transcription of the rhamnolipid producing *rhlAB* gene. Here (+) indicates transcriptional activation and (–) indicates transcriptional repression of the concerned genes.

Biochemical pathway of rhamnolipid production

Initiation of quorum sensing is contingent upon the accumulation of sufficient signal molecules. Due to this stringency, quorum sensing is only initiated once the population

reaches a critical threshold (Fuqua *et al*., 1994, Pierson *et al*., 1994). In *P. aeruginosa,* rhamnolipid production initially depends on PAI-1 and PAI-2 diffusible molecules, which interact with the activators, LasR and RhlR at high bacterial cell densities. For more information refer to Fig. 3.

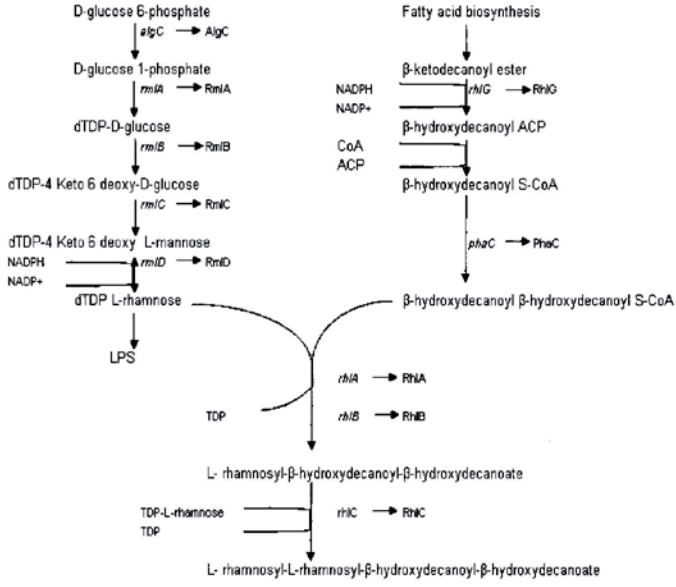


Figure 3. Biochemical pathway for rhamnolipid biosynthesis showing the genes and proteins involved in *Pseudomonas aeruginosa* PAO1 (Maier and Soberson-Chavez, 2000). The synthesis of rhamnolipid proceeds via the transfer of TDP-L-rhamnose. The 3-(3-hydroxyalkanoyloxy) alcanoic acid (HAA) is synthesized by RhlA enzyme and is then converted to mono-rhamnolipid by RhlB enzyme (Deziel *et al*., 2003, Ochsner *et al*., 1994b). The mono-rhamnolipid is converted to di-rhamnolipid by the RhlC enzyme (Rahim *et al*., 2001). CoA-β-hydroxyacids are the precursors of rhamnolipids. The *rhlAB* operon and *rhlC* genes are induced by homoserine lactone activated RhlR and are thus under the control of QS system (Ochsner *et al*., 1994b, Rahim *et al*., 2001). RhlR protein is known to activate *rhlG* transcription for rhamnolipid production. The biochemical synthesis of rhamnolipid is shown in fig. 3. Many QS molecules are known to regulate the synthesis of rhamnolipids. An autoinducer, N-butyryl homoserine lactone (PAI-2) present in *P. aeruginosa* restores rhamnolipid production in *P. aeruginosa rhlI* mutant (Pearson et al., 1995, Winson *et al*., 1995). PAI-2 and RhlR enhances the expression of *rhlI* in *E. coli* (Latifi *et al*., 1996). These finding suggests the importance of *rhlR* and *rhlI* quorum sensing components required for the auto-induction of rhamnolipid biosynthesis genes *rhlA* and *rhlB* (Pearson *et al*., 1997). The activator LasR-PAI1 complex induces the production of several virulence factors, such as the alkaline protease, exotoxin A and also regulates the expression of secretion proteins (Gambello *et al*., 1993, Morihara and Homma, 1985, Toder *et al*., 1991, 1994). The Rhl-PAI2 complex present in the biosynthesis of rhamnolipid induces expression of the LasA and LasB proteases as well as the secretion proteins (Xcp). In addition, the complex also controls expression of *rhlAB* and *rhlG* genes responsible for rhamnosyltransferase (Burger *et al*., 1963) and synthesis of hydroxyalkanoate for rhamnolipid production respectively (Campos-Garcia *et al*., 1998).

Enhancement of rhamnolipid production

P. aeruginosa is an opportunistic pathogen associated with cystic fibrosis and infections associated with severe burns. This bacterium is known for its resistance towards a variety of antibiotics and is one of the leading causes of nosocomial infections (Tummler *et al*., 1991). Numerous studies on *P. aeruginosa* and rhamnolipid

biosynthesis have improved the understanding of methods for the enhancement of rhamnolipid production.

NUTRITIONAL AND ENVIRONMENTAL CONDITIONS

In *P. aeruginosa,* rhamnolipid production occurs typically in late exponential or stationary phase (Guerra-Santos *et al*., 1986). The presence of nutrients, such as carbon and nitrogen, also play an important role in the production of rhamnolipids (Wu *et al*., 2008). *P. aeruginosa* growth and rhamnolipid production can occur using a range of different primary carbon sources. The highest level of rhamnolipid production in *P. aeruginosa* occurs when using vegetable based oils as carbon sources including soybean oil (Lang and Wullbrandt, 1999), corn oil (Linhardt *et al*., 1989), canola oil (Sim *et al*., 1997), and olive oil (Robert *et al*., 1989). Rhamnolipid production is dependent upon environmental and nutritional conditions. Environmental factors play a crucial role in influencing the productivity and efficacy of rhamnolipids. In general, as a biosurfactant, rhamnolipid activity is controlled by environmental conditions such as pH, salinity and temperature (Ilori *et al*., 2005, Inakollu *et al*., 2004, Jirasripongpun, 2002). Ilori *et al* (2005) pointed out that the chemical structure of biosurfactant gives benefit for hydrocarbon degradation and very unlikely to be disrupted due to extreme temperature and pH. Benka-Coker and Ekundayo (1996) highlighted the amount of oil might affect the biodegradation rate as well, due to poor aeration and lack of oxygen. The action of *Pseudomonas* in degrading oil is accelerated by the formation of rhamnolipid. The size and structure of hydrocarbon substrates may however slow down this oil degradation process.

SUPPLEMENTING EXOGENOUS QS MOLECULES

In mutants, unable to produce rhamnolipids, external addition of autoinducer molecules, N-acyl homoserine lactones regains the phenotype of rhamnolipid production. Kuniho *et al* (1998) found that autoinducer activity increased approximately ten-fold in fed batch system which strongly correlated with increased rhamnolipid production. Enhancement of rhamnolipid production occurred in the presence of either N-(3-oxohexanoyl)-L-homoserine lactone (OhDHL) or N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL). Overall, the presence of exogenous autoinducer increased rhamnolipid production five-fold, with maximal yields occurring during stationary phase of growth. Construction of rhamnolipid mutants has allowed for the identification of several genes that are essential for rhamnolipid biosynthesis. The first, identified in *P. aeruginosa,* comprises the *rhlAB* operon; here both the genes play an essential role in rhamnolipid production (Ochsner *et al*., 1994a). The *rhlAB* genes encode rhamnosyltransferase I complex involved in the formation of the RhlAB heterodimer (Ochsner *et al*., 1994b) and defects in either gene result in deficiencies in rhamnolipid production (Deziel *et al*., 2003). In addition to the two aforementioned genes, *rhlC* (encoding rhamnosyltransferase II, which adds the second rhamnosyl group to form RL2) is essential for the production of RL2, but is not essential for RL1 production (Rahim *et al*., 2001). A stable mutant strain would be a great advantage for rhamnolipid production by fermentation (Wang *et al*., 2007).

GENETIC ENGINEERING

Screening high rhamnolipid-producing microorganism from the natural environment is a good strategy; however engineering strains for rhamnolipid production is another alternative. Rhamnolipid production could also be effectively enhanced by cloning the wild-type *rhlI* gene into a suitable strain such as *E. coli*, or by the addition of *P. aeruginosa* cell-free spent supernatant containing the autoinducer molecules (Ochsner and Reiser, 1995). Rhamnolipid production has been shown to be transcriptionally regulated by quorum-sensing circuitry (Ochsner and Reiser, 1995, Heurlier *et al*., 2004). In a recent study, Cha *et al* (2008) were able to successfully increase rhamnolipid production by cloning both the *rhlAB* rhamnosyltransferase genes and the *rhlRI* quorum sensing system into *P. putida* to enhance rhamnolipid production.

In another study carried out by Wang and co-workers (2007) the novel transposome biotechnique was used. They integrated successfully the key genes of rhamnolipid biosynthesis into the chromosome of *P. aeruginosa* and *E. coli* cells, which were originally devoid of rhamnolipid production and the engineered strains, thus produced rhamnolipid. This technique would allow one to create a stable insertion mutation in a wide range of bacteria (Hoffman *et al*., 2000). Unlike plasmid-based engineered strain, transposon-based strains could exist stably under no drug-selection pressure, and the integration site of the targeted gene(s) would easily be confirmed by inverse-PCR, DNA sequencing, and alignment with a vast repository of genome information available from public database. The mechanism of gene regulation enables controlled production of rhamnolipid. Ochsner *et al* (1994b) reported that *rhl*R gene is essential in synthesizing rhamnolipids since the interruption at this locus contributed to the formation of rhamnolipid deficient mutants.

By using a suitable medium with the addition of QS molecules (AHL) at an early stage of bacterial growth along with genetically modified bacterial strains could be used for enhanced synthesis of rhamnolipid. A recent development in synthetic biology where synthetic molecules of quorum sensing are used for induction has shown numerous applications. Better understanding of the QS based synthetic networks is useful and has been applied in studies related to programming cell death in *E. coli* (Balagadde *et al*., 2005), constructing microbial consortia (Brenner *et al*., 2007), building of artificial intercellular communication and quorum-sensing behavior in prokaryotes (Bulter *et al*., 2004) and eukaryotes (Chen and Weiss, 2005). The topic is of significant interest and there is a need to explore it in great detail.

Conclusion

Rhamnolipids are effective biosurfactants with numerous applications. The production of rhamnolipids is under the control of quorum sensing. Over the past decade, significant strides have been made towards understanding the cell to cell communication, especially in the production of rhamnolipids biosurfactant. Evidence suggests that knowledge of cell to cell communication molecules and their role in biosurfactant production could be exploited to industrial scale production. There are numerous methods of enhancing rhamnolipids, however knowledge of the genes required for biosurfactant production can be critical for application in industry.

Currently, biosurfactants are unable to compete economically with chemically synthesized compounds in the market due to high production costs. Once the genes required for biosurfactant production have been identified, they can be placed under the regulation of strong promoters in nonpathogenic, heterologous hosts to enhance production. The production of rhamnolipids could be increased by cloning both the *rhlAB* rhamnosyltransferase genes and the *rhlRI* quorum sensing system into a suitable bacterium such as *E. coli* or *P. putida* and facilitate rhamnolipid production. Biosurfactants can also be genetically engineered for different industrial applications assuming there is a strong understanding of both the genetics and the structure-function relationships of each component of the molecule. Genetic engineering of surfactin has already been reported, with recent papers describing the creation of novel peptide structures from the genetic recombination of several peptide synthetases. Recent application of dynamic metabolic engineering strategies for controlled gene expression could lower the cost of fermentation processes by increasing the product formation. Therefore, by integrating a genetic circuit into applications of metabolic engineering the biochemical production can be optimized. Furthermore, novel strategies could be designed on the basis of information obtained from the studies of quorum sensing and biosurfactants produced suggesting enormous practical applications.

Acknowledgements

DHD would like to acknowledge Bhabha Atomic Research Centre (BARC) and University of Pune (UoP) collaborative research programme. PKSMR wishes to thank UK- Bioscience for Business KTN for the award of FROPTOP fund to explore the biocatalytic study of biosurfactant production from renewable resources. RJCM is funded by the Norman Hackerman Advanced Research Program of the Texas Higher Education Coordinating Board (003615-0037-2007).

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