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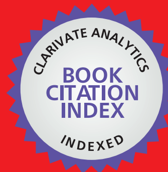

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Biosurfactants: Production and Applications

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1. Introduction

Great emphasis has recently been given to the environmental impacts caused by chemical surfactants due to their toxicity and difficulty in being degraded in the environment [1]. Increasing environmental concerns, the advance in biotechnology and the emergence of more stringent laws have led to biosurfactants being a potential alternative to the chemical surfactants available on the market [2, 3]. Although biosurfactants have promising use in bioremediation processes, their industrial scale production is currently difficult due to high raw-material costs, high processing costs and low manufacturing output [3]. As a result, the current research challenges are to increase the yield and to reduce the cost of raw materials [4].

The number of publications and patents involving biosurfactants has recently increased considerably [5]. Although many biosurfactants and their manufacturing processes have been patented, only some of them have been commercialized. EC-601 (EcoChem Organics Company), a dispersive agent of water-insoluble hydrocarbons containing rhamnolipids, and PD5 (Pendragon Holdings Ltd), an additive for fuels based on a mixture of rhamnolipid biosurfactants and enzymes, are examples of biosurfactant-based products commercially available [6]. Several studies have aimed to optimize the biosurfactant production process by changing the variables that influence the type and amount of biosurfactant produced by a microorganism. Important variables are carbon and nitrogen sources [7], potential nutrient limitations and other physical and chemical parameters such as oxygen [6], temperature and pH [4]. Recent studies have also focused on *in situ* production from renewable substrates, resulting in the so-called new generation of biosurfactants production [3], as well as metabolic engineering strategies and strain improvements to enhance the metabolic fluxes towards the product [4].

Among the biological surfactants, rhamnolipids reportedly have a good chance of being adopted by the industry as a new class of renewable resource-based surfactants [5]. Strain-engineering may be a promising strategy to improve manufacturing output, and the produc-

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tion by recombinant and non-pathogenic strains has been shown as possible and favorable [8]. Rhamnolipids have been the focus of many studies and are the better characterized biosurfactants in terms of production, metabolic pathways and gene regulation. Several bacterial species have been reported to produce the glycolipidic biosurfactants rhamnolipids [9]. In *Pseudomonas aeruginosa*, these biosurfactants are the products of the convergence of two metabolic pathways; the biosynthesis of dTDP-L-rhamnose – formation of lipopolysaccharide (LPS) – and the diversion of the β-hydroxydecanoyl-ACP intermediate from the FASII cycle by RhlA. The enzymes rhamnosyltransferases RhlB and RhlC catalyse the transfer of dTDP-L-rhamnose to either HAA, or to a previously generated mono-rhamnolipid, respectively [10]. High carbon to nitrogen ratio, exhaustion of nitrogen source, stress conditions and high cell densities are among the conditions that favor higher levels of production [11]. Rhamnolipids production in *P. aeruginosa* is tightly controlled by mulitple layers of gene regulation that respond to a wide variety of environmental and physiologic signals, and are capable of combining different signals to generate unique and specific responses [12].

Biosurfactants can potentially replace virtually any synthetic surfactant and, moreover, introduce some unique physico-chemical properties. Currently, the main application is for enhancement of oil recovery and hydrocarbon bioremediation due to their biodegradability and low critical micelle concentration (CMC) [13]. The use of biosurfactants has also been proposed for various industrial applications, such as in food additives [14], cosmetics, detergent formulations and in combinations with enzymes for wastewater treatment [13, 15].

In this chapter we intend to present an introduction of biosurfactants and their various applications with emphasis on bioremediation. Due to the relevance of rhamnolipids compared to other biosurfactants, their metabolic pathways and genetic regulation in *P. aeruginosa* will be revised. We additionally will discuss the critical factors and parameters for improved production of rhamnolipids.

2. Application of biosurfactants

Biosurfactants are potentially replacements for synthetic surfactants in several industrial processes, such as lubrication, wetting, softening, fixing dyes, making emulsions, stabilizing dispersions, foaming, preventing foaming, as well as in food, biomedical and pharmaceutical industry, and bioremediation of organic- or inorganic-contaminated sites. Glycolipids and lipopeptides are the most important biosurfactants (BS) for commercial purpose (Table 1). Shete et al. (2006) [16] mapped the patents on biosurfactants and bioemulsifiers (255 patents issued worldwide) showing high number of patents in the petroleum industry (33%), cosmetics (15%), antimicrobial agent and medicine (12%) and bioremediation (11%). Sophorolipids (24%), surfactin (13%) and rhamnolipids (12%) represent a large portion of the patents, however, this may be underestimated since many patents do not specify the producer organism restricting to the specific use of the BS only.

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Biosurfactant class		Microorganism	Application
Glycolipids	Rhamnolipids	*P. aeruginosa and P. putida*	Bioremediation
		P. chlororaphis	Biocontrol agent
		Bacillus subtilis	Antifungal agent
		Renibacterium salmoninarum	Bioremediation
	Sophorolipids	*Candida bombicola and C. apicola*	Emulsifier, MEOR, alkane dissimilation
	Trehalose lipids	*Rhodococcus spp.*	Bioremediation
		Tsukamurella sp. and Arthrobacter sp.	Antimicrobial agent
	Mannosylerythritol lipids	*Candida antartica*	Neuroreceptor antagonist, antimicrobial agent
		Kurtzmanomyces sp	Biomedical application
Lipopeptides	Surfactin	*Bacillus subtilis*	Antimicrobial agent, biomedical application
	Lichenysin	*B. licheniformis*	Hemolytic and chelating agent

Table 1. Major types of biosurfactants.

Improvement of detection methods together with increased concerns with environmental issues are pushing researchers and policymakers towards more environmentally friendly solutions for waste management and replacements for non-biodegradable substances. Organic aqueous wastes (e.g., pesticides), organic liquids, oils (e.g., petroleum-based) and organic sludges or solids (e.g., paint-derived) are common environmental organic chemical hazards and are source of soil and aquatic contaminations that are normally difficult to be removed. Another commonly found environmental hazard are the heavy metals, such as lead, mercury, chromium, iron, cadmium and copper, which are also linked to activities of our modern society. The remediation of contaminated sites is usually performed via soil washing or in situ flushing, in case of soil contamination, and bioremediation or use of dispersants, in case of aquatic areas. Soil washing/flushing is heavily dependent on the solubility of the contaminants, which can be very challenging when dealing with poorly soluble hazards. Hydrophobic contaminants usually require use of detergents or dispersants, both in soil or aquatic environment, and the process is often followed by their biodegradation. Heavy metal, however, cannot be biodegraded and are converted to less toxic forms instead. Hence, the commonly found combination of inorganic and organic contamination demands a complex remediation process. High hydrophobicity and solid-water distribution ratios of some pollutants result in their interaction with non-aqueous phases and soil organic matter. Those interactions reduce dramatically the availability for microbial degradation, since bacteria preferentially degrade chemicals that are dissolved in water [17].

Bioremediation is a process that aims the detoxification and degradation of toxic pollutants through microbial assimilation or enzymatic transformation to less toxic compounds [18]. The success of this process relies on the availability of microbes, accessibility of contaminants and conduciveness of environment. A typical bioremediation process consists of application of

nutrients (containing nitrogen and phosphorous), under controlled pH and water content, together with an emulsifier and surface-active agents. Biostimulation is the bioremediation based on the stimulation of naturally indigenous microbes by addition of nutrients directly to the impacted site, whereas bioaugmentation is based on addition of specific microbes and nutrients to the impacted site. Bioaugmentation has been subject of several reports including use of genetically engineered microorganisms (reviewed in Gentry et al., 2004 [19]). Biostimulation success relies on microorganism targeting the pollutant as a primarily food source, which is supported by available electron donors/acceptors and nutrients (reviewed in Smets & Pritchard, 2003 [20]).

The bioavailability of a chemical in general is governed by physical-chemical processes such as sorption and desorption, diffusion and dissolution. Microorganisms improve bioavailability of potential biodegradable nutrients by production of biosurfactants [21], and the success of microbes in colonize a nutrient-restricted environment is often related to their capacity of producing polymers with surfactant activity.

The best-studied biosurfactant are the glycolipids, which contain mono- or disaccharides linked to long-chain aliphatic acids or hydroxyaliphatic acids. Rhamnolipids are better known glycolipid class, which are normally produced as a mixture of congeners that varies in composition according to the bacterium strain and medium components, which provides specific properties to rhamnolipids derived from different isolates and production processes [7]. This class of biosurfactant has been implied in several potential applications such as in bioremediation, food industry, cosmetics and as an antimicrobial agent. Several reports have been shown rhamnolipids to be efficient in chelating and remove/wash heavy metals, perhaps due to the interaction between the polar glycosidic group with the metal ions. Whereas their interaction with organic compounds increases their bioavailability or aids their mobilization and removing in a washing treatment. Rhamnolipids have been shown to be effective in reducing oil concentration in contaminated sandy soil [22] and their addition at relatively low concentration (80 mg/L) to diesel/water system substantially increased biomass growth and diesel degradation [23]. Interestingly, rhamnolipids combined with a pool of enzyme produced by Penicillium simplicissimum enhanced the biodegradation of effluent with high fat content from poultry processing plant, suggesting a synergistic interaction between biosurfactant and enzymes in waste treatment [15].

2.1. Surfactants in removal of hydrophobic pollutants

Highly hydrophobic contaminants can bind very tightly to soil, therefore inaccessible to biodegradation. Surfactants have the potential to promote desorption of the contaminants from soil. Usually, 1-2% (w/w) of surfactant is used for washing contaminant soil, whereas in aqueous solution the concentration of surfactant can be as low as 10 times less than in soil. Rhamnolipids were effective in removing polycyclic aromatic hydrocarbons (PAHs) and pentachlorophenol from soil with 60-80% removal efficiency, which varied with contact time and biosurfactant concentration [24, 25]. Addition of rhamnolipid biosurfactants to phenanthrene-degrading bacteria (Pseudomonas strain R and isolate P5-2) increased phenanthrene mineralization in Fallsington sandy loam with high phenanthrene-sorption capacity, and

addition of this biosurfactant at concentrations above the CMC resulted in enhanced phenanthrene release from soil [26]. Interesting, phenanthrene pseudosolubilization was increased in the presence of less hydrophobic PAHs [27]. The explanation for this cooperative effect could be that less hydrophobic compounds were accommodated at the interfacial region of a hydrophobic core, consequently, the interfacial tension between the core and water was reduced, and the reduced interfacial tension may support a larger core volume for the same interfacial energy [17]. Moreover, rhamnolipids were shown to enhance partitioning rate of PAHs such as fluorene, phenanthrene and pyrene [24].

Mixture of different surfactants often presents better properties than the individual surfactants, due to synergistic effect. An improved strategy to surfactant-enhanced remediation (SER) is to apply mixture of surfactants at reduced concentration of individual surfactants, which reduces the cost while maintaining the efficiency of remediation [17]. Solubilization of hydrophobic contaminants is improved by using mixtures of anionic and nonionic surfactants, which was shown to exhibit synergistic interaction, suggesting that appropriate combinations of surfactants have the potential to enhance the efficiency of soil washing and flushing and to facilitate the bioavailability of pollutants [28, 29].

Trehalose lipids produced by *Rhodococcus erythropolis* were shown to have good solubilization capacity for hydrophobic compounds such as phenanthrene, and great potential for applications in bioremediation of sites contaminated with PAHs [30]. PAHs sorption/desorption ratio was reduced with combined treatment with chemical surfactant (Tween 80) and biosurfactants under thermophilic conditions, and substantial amount of PAHs were desorbed from soil into the aqueous phase when surfactant concentration was above CMC [31]. Combining Tween 80, Triton X100 and biosurfactants from P. aeruginosa strains resulted in effective enhanced solubility of phenanthrene at 50 ^{0}C compared with addition of the individual surfactants [32]. Interesting, degradation of phenanthrene was completely inhibited for all the surfactants tested at concentration higher that their CMC, suggesting that the combination of surfactant and biosurfactant has potential in bioremediation however it requires a research in a case-by-case basis. The phenanthrene-degrading strain B-UM lacks the ability to produce surfactants for dissolution of phenanthrene, and the direct adhesion of cells to phenanthrene surface might be the major pathway for B-UM to take up this PAH [33]. As expected, addition of surfactants inhibited the phenanthrene degradation by B-UM, and similar effect were found with addition of Triton X100 to *Arthrobacte*r sp. growing on n-hexadecane [34], suggesting that surfactant can inhibit biodegradation of hydrocarbons by de-adhesion of cells from the liquid/solid-water surfaces preventing cells to contacting the surface of phenanthrene, therefore, the degradation is impaired. Kumar et al. (2006) [35] isolated and characterized a *P. putida* strain named IR1. It was shown that this strain is capable of utilizing up to four-ring PAHs but not hexadecane and octadecane as a sole carbon and energy source, and the authors identified the presence of both tension-active and emulsifying activities, suggesting that IR1 produces biosurfactants on both water miscible and immiscible substrates. Rhamnolipids together with anthracene-degrading bacteria had a dramatic increase in the solubility of anthracene by the bacterium strains and, interesting, it was observed the metabolism of biosurfactant by one of the strains which appears to be an important event on this process [36]. Addition of Tween 60, nonionic surfactant, to a

Rhodococcus rhodochrous strain in liquid media was also shown to improve biodegradation of fluorene by being an additional carbon source to the bacterial cells [37]. Moreover, the combination of biostimulation and bioaugmentation was shown to result in significant removal of phenanthrene under Antarctic environmental conditions. The authors combined a complex organic source of nutrients (fish meal), a surfactant (Brij 700) and a psychrotolerant PAH-degrading bacterial consortium and it was shown that the combined treatment is more efficient than the biostimulation or bioaugmentation isolated [38].

2.2. Surfactants in petroleum industry

Indigenous or injected biosurfactant-producing microorganisms are exploited in oil recovery in oil-producing wells. Microbial enhanced oil recovery (MEOR) is often implemented by direct injection of nutrients with microbes that are able of producing desired products for mobilization of oil, by injection of a consortium or specific microorganisms or by injection of the purified microbial products (e.g., biosurfactants). These processes are followed by reservoir repressurization, interfacial reduction of tension/oil viscosity and selective plugging of the most permeable zones to move the additional oil to the producing wells. Oil recovery was showed to be increased by 30-200 % with injection of biosurfactants, bacteria (e.g., *P. aeruginosa*, *X. campestri*, B. *licheniformis*) and nutrients [18]. However, application of MEOR requires a thoroughly research on a case-by-case basis taking in account the physical-chemical conditions and soil and rock formation characteristics. The characteristics of the oil that has been already recovered from the well will also impact the MEOR application. MEOR is a powerful technique to recover oil, especially from reservoirs with low permeability or crude oil with high viscosity, but the uncertainties on the results and costs are a major barrier to its widespread.

Oilfield emulsions are formed at various stages of petroleum exploration, production and oil recovery and processing, and represent one of the major problems for the petroleum industry [39], which requires a de-emulsification process in order to recover oil from theses emulsions. Traditionally, de-emulsification is obtained by centrifugation, heat treatment, electrical treatment and/or chemicals [39]. Biosurfactants have the potential to replace the use of chemical de-emulsifiers in situ, saving on transport of the oil emulsion and providing a more environmentally-friendly solution. Among the bacteria species, Acinetobacter and Pseudomonas species are the main de-emulsifiers in the mixed cultures [40]. The microorganisms exploit the dual hydrophobic/hydrophilic nature of biosurfactants or hydrophobic cell surfaces to disrupt the emulsions. Glycolipids (e.g., rhamnolipids), glycoproteins, phospholipids and polysaccharides are among the microbial tools to displace the emulsifiers from the oil-water interface [4]. The major advantage of using microorganisms or their products over chemical products is the disposal of the de-emulsifier in the aqueous phase and its removal from the oil phase, since emulsion formation is required in further processing steps. Microbes and biosurfactants are in general readily biodegradable, which allows a cheap and easy removal of the de-emulsifier after this process.

Surfactants have potential application for oil recovery from petroleum tank bottom sludges and facilitating heavy crude transport through pipelines. It was shown that rhamnolipids can

be used to remove soaked oil from the used oil sorbents [30]. More than 95% of oil removal was achieved using commercial rhamnolipids (JBR215, Jeneil Biosurfactant Company, USA), and the main factor affecting oil removal were the sorbent pore size and washing time.

2.3. Washing and bioremediation of crude oil-contaminated environments

Petroleum hydrocarbons are an essential raw material in our current society, but they also constitute a major environmental pollutant that is very difficult to be bioremediated. Crude oils have very low water solubility, high adsorption onto soil matrix and present limited rate of mass transfer for biodegradation [2]. Oil-contaminated soil is especially difficult for bioremediation since oil excess forms droplets or films on soil particles, which is a powerful barrier against microbial degradation [41]. Bioavailability of contaminants in soil to the metabolizing organisms is influenced by factors such as desorption, diffusion and dissolution. Biosurfactants are produced to decrease the tension at the hydrocarbon-water interface aiming to pseudosolubilize the hydrocarbons, thus increasing mobility, bioavailability and consequent biodegradation [42]. Several biosurfactant are produced by a diversity of microorganisms in order to survive in an oil-rich environment, and this adaptation process selected for surfactants with highly adaptable phycal-chemical properties. Biosurfactants are, therefore, very suitable for applications in the oil industry and this is reflected in the market, where the large majority of biosurfactants produced are in petroleum-related applications [21]. The applications are, in general, in oil recovery, oil spill management, MEOR and as oil dispersants and demulsifiers [18].

Purified rhamnolipid biosurfactants were applied in the removal of oil from contaminated sandy soil [22]. The authors optimized the biosurfactant and oil concentrations in the removal of oil applying statistical experimental design tool that generates a surface response. Sandy oil contained predominantly aromatic and paraffinic hydrocarbons (5-10% w/w) mixed with reduced concentration of rhamnolipid (6.3-7.9 g/L) resulted in removal of oil by up to 91 and 78%, respectively. Rhamnolipids, when added above CMC, enhanced the apparent aqueous solubility of hexadecane, the biodegradation of hexadecane, n-paraffins, octadecane, creosotes in soil and promoted biodegradation of petroleum sludges [43, 44]. Rhamnolipids produced by Nocardioides sp. A-8 allows this bacterium to grown on aromatic hydrocarbons or n-paraffin as sole carbon source by lowering the surface tension and emulsifying the aromatic hydrocarbons [45]. The authors found similar results for the strain Pantoea sp. A-13, which also produces rhamnolipid to grow on n-paraffin or kerosene. Both A-8 and A-13 strains were isolated together with other 15 aerobic microbial isolates from oil-contaminated sites in Antarctica and appear to be very promising source for application in accelerated environmental bioremediation at low temperatures.

Urum and Pekdemir (2004) [41] applied different biosurfactants (rhamnolipid, aescin, saponin, lecithin and tannin) in washing oil-contaminated soil and observed significant removal of crude oil with different concentrations of biosurfactant solution. Oil mobilization was the main cause for its removal, which was triggered by the reduction of surface and interfacial tensions, rather than oil solubilization or emulsification. This work was followed by a comparison of a biosurfactant, rhamnolipid, and a chemical surfactant, SDS, in removal of crude oil from soil

[46]. More than 80% of crude oil was removed with application of SDS or rhamnolipid, however, the biosurfactant was effective in a wider range of tested conditions. Urum et al (2006) [47] then compared the efficiency on oil removal from soil using rhamnolipid and saponin biosurfactants and SDS, and the results showed a preferential removal of oil by the surfactants. SDS was more effective for aliphatic than aromatic hydrocarbons, whereas biosurfactants removed more aromatics. These results provide insights on the formulation of surfactant combinations, suggesting that the strategy should consider the degree of aromaticity in the crude oil-contaminated soil. The combination of oil-degrading bacteria and biosurfactant or biosurfactant-producing bacteria has also been tested by research groups. *P. putida*, an oil-degrading bacterium, was co-cultured with a biosurfactant-producing bacterium and improved degradation was observed in both aqueous and soil matrix in comparison with to the individual bacterium cultures [35]. This treatment resulted in increased oil emulsification and also adhesion of hydrocarbon to the bacteria cell surface.

2.4. Other applications

Rhamnolipids have potential microbial activity. It has been shown that these biosurfactants are very efficient bacteriostatic agent against Listeria monocytogenes, an important food-related pathogen, and showed synergistic effect when combined with nisin, a broad-spectrum bacteriocin [48]. Both biosurfactants and surfactin were shown to be able to reduce bacterial adhesion to polystyrene surfaces more efficient than the chemical surfactant sodium dodecyl sulfate. Moreover, purified rhamnolipid inhibited virtually 100% of the growth of strongly adherent L. monocytogenes strain [49]. Moreover, rhamnolipids were shown to significantly reduce the rate of deposition and adhesion, in rinsed chamber with these biosurfactants, of several bacterial and yeast strains isolated from explanted voice prostheses [50]. Rhamnolipids are also known to remove heavy metals. It was shown that these biosurfactant was able to remove niquel and cadmium from soils with efficiencies of 80-100%, under controlled environment, and 20-80% removal was reported in field samples [51].

MEL, a glycolipid, is a potent antimicrobial agent, especially against gram-positive bacteria. MEL has also been shown to induce growth arrest, apoptosis and differentiation of mouse malignant melanoma cell cultures [52, 53]. MEL-A produced by Pseudozyma antartica T-34 has been shown to have cell differentiation activities against human leukemia cells, mouse melanoma and PC12 cells [54]. Delivery of siRNA into the cytoplasm is still a challenge and a barrier for more gene silencing-based individualized drugs. MEL-A-containing cationic liposomes has been shown to directly deliver siRNA into the cytoplasm by the membrane fusion in addition to endocytotic pathway with better results compared with viral vectors in clinical applications [55].

Lipopeptides, such as surfactins, are particularly interesting because of their high surface activities and antibiotic potential. These biosurfactants have been reported as antibiotics, antiviral and antitumor agents, also as immunomodulators and inhibitors of specific toxins and enzymes. Surfactin, a cyclic lipopeptide, is known to be active in several biological activities, such as induction of ion channel formation, antiviral and antitumor, anti-inflammatory agent (reviewed by Rodrigues et al., 2006 [50]). Moreover, pre-coated vinyl urethral

catheters with surfactin were shown to reduce biofilm formation by several Salmonella species and other infectious bacteria [56]. Surfactin has been shown to be more efficient than chemical surfactants (sodium dodecyl sulphate or dodecylamine) in improving floatability of metal-laden sorbents under similar conditions [57]. Also, surfactin was shown to contribute to reduce colonization of pathogenic bacteria, such as *L. monocytogenes*, *Enterobacter sakazakii* and *Salmonella enteritidis*, when applied to solid surfaces prior to infection [58, 59].

3. Metabolism and gene regulation of rhamnolipid production

Biosurfactants are considered ideal for environmental application, due to their numerous advantages over their chemical counterparts, such as biodegradability and less toxicity [60]. Among the biosurfactants, rhamnolipids are the better studied and promising candidates for large scale production especially because of their notable tensoative and emulsifying properties [61-63].

3.1. Rhamnolipid production

P. aeruginosa produces two major types of rhamnolipid in liquid cultures: the monorhamnolipid, rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-C10-C10) and the dirhamnolipid, rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-Rha-C10-C10) [64]. Besides these, twenty-five rhamnolipid congeners have been described in *P. aeruginosa*, varying in chain length and/or extent of saturation, showing that the addition of a hydrocarbon chain to dTDP-L-rhamnose is not specific to the carbon chains [65].

In *Pseudomonas aeruginosa*, these biosurfactants are the products of the convergence of two metabolic pathways: the biosynthesis of dTDP-L-rhamnose and the diversion of the β-hydroxydecanoyl-ACP intermediate from the FASII cycle by RhlA to synthesize the fatty acid dimer moiety of rhamnolipids and free 3-[3-hydroxyalkanoyloxy) alkanoic acid (HAA). The rhamnosyltransferases RhlB and RhlC catalyse the transfer of dTDP-L-rhamnose to either HAA, or a previously generated mono-rhamnolipid, respectively [10].

Free HAAs also show surface-tension activities and have been directly related to the promotion of swarming motility [10]. Recent studies suggest that RhlA is responsible for diverting the β-hydroxydecanoyl-ACP intermediate from the FASII cycle by directly competing with FabA and FabI for this intermediate [66]. In addition, RhlA is the only protein required to convert two molecules of β-hydroxyacyl- ACP into an HAA. The diversion of the β-hydroxydecanoyl-ACP intermediate from the FASII cycle providing a substrate for the enzyme RhlAB to produce the hydrocarbon chain in the rhamnolipid molecule is an important step, both biotechnologically and clinically, but is still not fully understood.

3.2. Biosynthesis of dTDP-L-rhamnose

Rhamnose sugar is widely found in bacteria and plants, but not in humans. The activated L-rhamnose is derived from a glucose scaffold in four sequential steps, yielding deoxy-thymidine

di-phospho (dTDP)-L-rhamnose. The first enzyme in the dTDP-L-rhamnose pathway is glucose-1-phosphate thymidylyltransferase (RmlA, EC 2.7.7.24) which catalyzes the transfer of a thymidylmonophosphate nucleotide to glucose-1-phosphate. The catalytic activity of RmlA is allosterically regulated by the final product of the pathway, dTDP-L-rhamnose [67], which makes RmlA the regulatory sensor for the downstream pathway. RmlA is a homotetramer with the monomer consisting of three functional domains: one core domain that shares the sequence similarity with nucleotidyltransferases, and two other domains that contain the recognition and binding sites for the nucleotide and sugarphosphate [67]. The second enzyme, dTDP-D-glucose 4,6-dehydratase (RmlB, EC 4.2.1.46), catalyzes an oxidation of the C4 hydroxyl group of the D-glucose residue, followed by dehydration, leading to the formation of dTDP-4-keto-6-deoxy-D-glucose [68]. The third enzyme, dTDP-4-keto-6-deoxy-D-glucose 3, 5-epimerase (RmlC, EC 5.1.3.13), catalyzes a double epimerization reaction at the C3 and C5 positions of the 4-keto-6-deoxy-D-glucose ring [69]. Finally, dTDP-4-keto-6-deoxy-Lmannose reductase (RmlD, EC 1.1.1.133) reduces the C4 keto group of the 4-keto-6-deoxy-L-mannose moiety and leads to the formation of dTDP-L-rhamnose [69]. All four enzyme genes are organized as a single operon in P. aeruginosa, called *rmlBDAC*.

3.3. Regulation of rhamnolipids synthesis

Rhamnolipids production in *P. aeruginosa* is tightly controlled by multiple layers of gene regulation that respond to a wide variety of environmental and physiologic signals, and are capable of combining different signals to generate unique and specific responses [12]. High carbon to nitrogen ratio, exhaustion of nitrogen source, stress conditions and high cell densities are among the conditions that favor higher levels of production [11]. Apparently, the key regulatory targets for rhamnolipid production in *P. aeruginosa* are the regulation of the *rmlBDAC* and *rhlAB* operons. The *rhlAB* operon is transcriptionally and posttranscriptionally regulated by manifold factors, commonly associated to the quorum sensing system, some of which also participates of the regulation of *rmlBDAC* operon.

3.4. Role of quorum sensing systems

The quorum sensing (QS) system is a bacterial communication system characterized by the secretion and detection of signal molecules – autoinducers – within a bacterial population. When it reaches a population "quorum", in which the autoinducers threshold is achieved, the bacterial population coordinates its responses to environmental inputs. QS is a global regulatory system found in most bacterial species controlling several and diverse biological functions, such as virulence, biofilm formation, bioluminescence and bacterial conjugation [70]. The main components of a quorum sensing system are the QS signal synthase, the signal receptor (regulatory protein), and the signal molecule [71]. The complex autoinducer/regulatory protein modulates the activity of the QS-regulated genes. There are two known conventional QS systems in *P. aeruginosa*, las and rhl. The autoinducer synthases LasI and RhlI produce the homoserine lactones 3OC12-HSL and C4-HSL, respectively, which complex with their correspondent transcriptional regulators, LasR and RhlR, to modulate the transcription of 5-10 % of the entire P. aeruginosa genome [72]. A third distinct QS system is formed by the

transcriptional factor PqsR (also called MvfR) [73], responsible for activating the gene clusters pqsABCDE and phnAB both required for the production of Pseudomonas quinolone signal (PQS) and 4-hydroxy-2-alkylquinolones (HAQs), respectively, which is known to influence the production of QS-dependent factors, such as elastase, pyocyanin, PA-1L lectin, and rhamnolipids [74].

There has been postulated that sequences conserved in some promoters regions of RhlR and LasR-regulated genes are responsible by these regulation [75]. It can be verified that some QS-regulated genes belong specifically to *rhl* regulon, and some sequences RhlR-specific can be determined in promoters regions of some genes regulated by *rhl* system [76]. Although the *rhl* system has already been considered *las*-dependent [77], it has been shown that the expression of *rhl* system is maintained in a *lasR* mutant [72].

Rhamnolipid production in *P.aeruginosa* was shown to be directed linked to QS system by the transcriptional regulator RhlR. RhlR acts as an activator of *rhlAB* and *rmlBDAC* transcription when complexed to C4-HSL and as a repressor in the absence of the autoinducer [78, 79]. The *rhlR* gene is known to have four different transcription start sites [78]. In rich medium conditions, the expression of RhlR is dependent on LasR; however, under phosphate-limiting conditions, the expression of RhlR is regulated from multiple promoters through different transcriptional activators, including Vfr, RhlR, and the sigma factor σ54, and it is also known that low-phosphate condition upregulates rhlR and RhlR-regulated genes, including rhamnolipids, even under low AHL levels [80]. In addition, in the last few years many other factors related to the QS system have been identified and reported to directly or indirectly influence rhamnolipid production.

3.5. Stationary phase and quorum sensing-related factors

Stationary phase is a physiological state frequently related to nutrient scarcity. Rhamnolipids is also associated with physiological roles in the uptake of poorly accessible substrates and is often associated with bacterial response to nutrient-deficient environments. The production is repressed in exponential phase and low cell density while is activated in stationary phase and high cell densities. This environmental regulation is related to several factors that directly or indirectly control transcription and post-transcription levels of expression.

The production in stationary phase is related to factors which contribute to the production in this phase and with other factors that inhibit the production in the exponential phase. The main factor that contributes to the rhamnolipid production in stationary phase appears to be the *rhl* system. As mentioned, the *rhl* system activates the *rmlBDAC* and *rhlAB* operons when complexed to C4-HSL [78, 79]. This QS system is activated in high cell densities, so it represents a direct link between high cell population and rhamnolipid production activation.

The second factor that guarantees the rhamnolipid production on stationary phase is related to a posttranscriptional regulation. It has been shown that most QS-regulated genes are not induced before the stationary growth phase, even when exogenous acyl-HSL signals are present in early growth phases [81]. These findings clearly indicate the other factors are involved in the expression of QS-regulated genes. The posttrancriptional regulator GidA was

shown to primarily modulate the expression of RhlR and RhlR-dependent genes in *P. aeruginosa*. In *gidA* mutants, the levels of *rhlR* mRNA are similar to the levels in wild-type, whereas *rhlA* mRNA levels were significantly decreased, suggesting that the rhamnolipid production is controlled via posttranscriptional modulation of RhlR levels by GidA [82].

In relation to factors that inhibit the rhamnolipid production in the exponential phase, two regulators can be identified. The first of them is the QscR factor. It has been demonstrated that the *rhlAB* operon is present in the subset of genes repressed by QscR in the exponential phase [83]. This factor is a luxR-homologue protein that integrates the QS regulatory network and controls a distinct but overlapping regulon with the *las* and *rhl* systems [83]. QscR forms inactive heterodimers with LasR and RhlR in low AHL levels, while in increased AHL concentrations, QscR-AHL complexes are formed, and LasR-3OC12-HSL and RhlR-C4-HSL interactions occur, as a result of the dissociation of the heterodimer [84]. This can explain the effect of the QscR over the *rhlAB* operon. The second regulator is the DksA protein. DksA overexpression was shown to reduce *rhlI* and *rhlAB* expression in *P. aeruginosa*, while *dksA* mutants have higher *rhlI* expression [85]. It was demonstrated that DksA is required for *rhlAB* translation in *E.coli* heterologous expression, but does not affect its transcription [85]. DksA synthesis reaches its maximum during the exponential growth phase and it is posttranscriptionally downregulated during the late exponential and stationary growth phase [86]. This evidence indicates that DksA inhibits rhamnolipid production by repressing C4-HSL production.

Beyond the factors related to the growth phase, an important factor related to the QS systems which influences rhamnolipid production in *P. aeruginosa* is the *Pseudomonas* quinolone signal (PQS). PQS is involved in a complex regulatory network of QS connecting systems *las* and *rhl*. While PQS directly activates the *rhl* system in a *las* independent manner [87, 88], PQS synthesis is driven by *pqsABCDE* operon, which in activated by PqsR (MvfR), a LysR-type regulator. PqsR is activated by *las* QS system and repressed by *rhl* QS system, evidencing a complex network of regulation [89]. PQS was shown to be related to rhamnolipid production in different ways. Firstly, PQS production occurs in the late logarithmic phase and reaches its maximum in the late stationary phase [87, 88], with a similar profile to that of rhamnolipid biosynthesis. It was shown that *pqsR* and *pqsE* mutants have reduced rhamnolipid production, even when exposed to wild-type levels of C4-HSL [78]. This indicates a direct participation of PqsE, PqsR and/or PQS in rhamnolipid synthesis. Also, it has been hypothesized that PqsR is essential for rhamnolipid production, since PQS does not overcome the absence of *pqsR* in the regulation of phz1 operon, also involved in *rhl*-dependent phenotype [74]. As PqsR controls PQS production and rhamnolipid production is abolished in the absence of PQS signaling [87], it is possible that this effect is indirect. Rhamnolipid regulation also requires PqsE, which is involved in bacterial response to PQS and PqsR [74]. An alternative hypothesis is the regulation of rhamnolipid production by the LasR/RhlR ratio, known to modulate pqsR expression.

3.6. Regulatory factors related to stress conditions

An important environmental condition which highly influences the rhamnolipid production in *P. aeruginosa* is related to stress. Conditions such as nutrient deprivation and nitrogen

exhaustion, even in a QS-independent manner, contribute to an increase in rhamnolipid production. Thus, several regulatory factors have been identified in last years which connect this condition to specific gene regulation patterns. Relevant ones are the sigma factors of RNA polymerase RpoS (σ^S or σ^{38}) and RpoN (σ^{54}).

The sigma factor of stationary phase, RpoS, plays an important role in the response to different stress conditions in *P. aeruginosa*. *rpoS* mutants have been shown to be more susceptible to carbon starvation, heat shock, high osmolarity, low pH, and hydrogen peroxide [90]. RpoS levels increase at the onset of stationary phase and in response to nutrient deprivation, even at low cell densities [91]. RpoS regulon comprises virtually all genes regulated in stationary phase, and has several overlaps with *las* and *rhl* regulons [92]. RpoS is involved in rhamnolipid production by two different manners, which indicates a genetic link between rhamnolipid production and nutrient deprivation and environment stress adaptation. Firstly, the *rhlAB* operon integrates the RpoS regulon and has been shown to be upregulated and partially RpoS-dependent [93]. Secondly, the *rmlBDAC* operon was shown recently to be activated by RpoS, which interacts in a different promoter region than the RhlR-C4HSL [79], which suggests a regulation related to stress in a QS-independent manner. Furthermore, RpoS was shown to be required for swarming motility [94], a phenotype related to HAAs and rhamnolipids [10].

The sigma factor RpoN (σ^{54}) is known to be involved in nitrogen metabolism in bacteria. The rhamnolipid production is largely dependent on nitrogen exhaustion in *P. aeruginosa* [95], and different nitrogen sources can act as inhibitors (e.g. ammonium, glutamine, asparagine and arginine) or activators (nitrate, glutamate, and aspartate) of rhamnolipid production [95, 96]. Moreover, nitrate has been shown to provide the highest yields of rhamnolipid production [7], related to upregulation of glutamine synthase under nitrogen-limiting conditions by RpoN [97]. The RpoN activity on the expression of catabolic pathways is related to CbrA-CbrB and NtrB-NtrC, two-component regulatory systems, which are regulated by nitrogen availability [98]. On the other hand, the activation of rpoN-dependent promoters seems to be CbrB and NtrC-dependent [99]. The *rhlAB* operon and *rhlR* are controlled indirectly and directly, respectively, by RpoN [93], since under nitrogen-limiting conditions, *P. aeruginosa* seems to improve its nitrogen assimilation through ATP-dependent pathways (e.g. glutamine synthase) and the increase of nitrogen uptake via, e.g., upregulating rhamnolipid biosynthesis.

Another important factor that can correlate rhamnolipid production to stress conditions, but also with quorum sensing, is the PQS. PQS is involved in stress response in *P. aeruginosa* and has been reported to be related to oxidative stress, UV irradiation resistance and antimicrobial agents [100]. Rhamnolipids were shown to act in PQS solubilization/ assimilation, and therefore, in its activity as a transcriptional regulator [101]. This clearly indicates the interconnection between rhamnolipid and PQS synthesis.

3.7. Regulatory factors related to virulence

The great adaptability of *P. aeruginosa* to a wide range of habitats is also related to its ability to produce several virulence factors such as pyocyanin, elastases, proteases and rhamnolipids, which provides antimicrobial activity [102], haemolytic activity in human pathogenesis [103]

and promotion of swarming motility [10]. Different regulatory factors that control virulence in *P. aeruginosa* have been shown to activate rhamnolipid production.

Vfr, the global regulator of the virulence in *P. aeruginosa*, is a cAMP binding protein that activates the RhlR expression in a LasR-independent manner [78]. It affects the expression of multiple virulence factors downstream in the QS cascade, including rhamnolipid production. Vfr is also involved in RpoS synthesis [104], which has been demonstrated to influence rhamnolipid production acting on *rmlBDAC* and *rhlAB* operons. Another factor related to virulence is the VqsR protein. This is a LuxR homologue that has been shown to modulate the expression of QS genes and others metabolic process, seeming to be a global regulator in *P. aeruginosa* [105]. Rhamnolipid production is reduced in *vqsR* mutants, which can be related to the influence of this factor in the QS system, since most of the VqsR-regulated genes were previously identified as QS-regulated [99, 105]. PtxR, a LysR-type transcriptional regulator, which modulates the production of virulence factors in *P. aeruginosa* by repressing PQS-genes expression [106], is also involved in the biosurfactant production. The rhamnolipid synthesis increase in *ptxR* mutants, which can be related to its modulation of the QS system, since the *ptxR* mutants showed upregulation of *rhlI* and *lasI* genes, as well as the C4-HSL and 3OC12-HSL autoinducers, when compared to the wild type strain [106].

3.8. Regulatory factors related to biofilm formation

Biofilm formation provides several advantages to the bacteria, providing protection that may enhance bacterial survival under environmental stress conditions. Rhamnolipids play a major role in the architecture of biofilms produced by *P. aeruginosa*. The cell detachment of the biofilm structure and the formation of water channels have been shown to be dependent of the rhamnolipids synthesis. It has been reported that rhamnolipid production is related to biofilms in *P. aeruginosa* through AlgR, a regulatory factor related to biosynthesis of alginate [107]. AlgR was shown to be the main repressor of rhamnolipid production within adherent biofilms and during its development, acting as a repressor of the expression of *rhlI* and *rhlAB* [108]. No such effect was reported on planktonic growth so far, therefore, it was hypothesized that AlgR acts through a contact-dependent or biofilm-specific mode of regulation. BqsS-BqsR, a two component system, has also been reported to be related to biofilm formation in P. aerugionosa [109]. In *P. aeruginosa*, bqsS-bqsR mutant showed reduced rhamnolipids production, which might be an indirect regulation, since the production of C4-HSL, as well as PQS, were reduced in this mutant. Although the environmental stimuli that trigger BqsS-BqsR activity are still unknown, evidences support the existence of opposite effects of AlgR and BqsS-BqsR on rhamnolipid production in the context of biofilm formation [109].

3.9. Regulatory factors related to unknown stimuli

P. aeruginosa is a ubiquitous and highly adaptable bacterium, and rhamnolipids play different roles in its adaptation processes. Besides the known environmental stimuli, others are likely to play important role in rhamnolipid production. Some regulatory factors have been identified as involved on rhamnolipids production, however their environmental stimuli are still to be discovered. GacS-GacA is a well characterized two-component system in *P. aeruginosa* that

modulates gene expression through promoting the synthesis of two small RNAs, RsmY and RsmZ. These small RNAs regulate gene transcriptional by modulating the activity of the small RNA-binding protein RsmA [110]. RsmA is a translational regulator and acts by preventing the translation initiation of target RNAs. However, RsmA also activates indirectly gene expression, by acting over repressor factors. GacS-GacA can have opposite effects on rhamnolipids production, evidencing a complex regulation. RsmA represses the QS system, including C4-HSL and 3OC12-HSL production [111], whereas, RsmA acts as an activator of rhamnolipid synthesis, since *rhlB* was downregulated in the *rsmA* mutant [112]. Therefore, it has been hypothesized that RsmA might stabilize *rhlAB* mRNA and/or facilitate its translation initiation. Another global regulator involved in rhamnolipids production is VqsM, an AraC-type transcriptional regulator. VqsM has been shown to modulate the expression of several genes, including QS regulators. *vqsM* mutants show reduced expression of *rhlAB* [113], *rhlR* and *rpoS*, evidencing an indirect regulation upstream of the QS regulatory network. The environmental stimuli for this regulation are also unknown.

4. Economic aspects of biosurfactant production

Several microorganisms are known to produce biosurfactants that can vary in structure and chemical composition. These variations are dependent on the producing microorganism, raw matter used for fermentation and conditions of fermentative process [17]. According to recent data, global biosurfactants market was worth USD 1,7 bi in 2011 and is expected to reach USD 2,2 bi in 2018, based on a growth rate of 3.5% per annum. The global biosurfactants market volume is expected to reach 476,512.2 tons by 2018, due to increasing demand from the Asia, Africa and Latin America, which should account for 21 % of it [114].

The number of publications related to identification, optimization of production process and better understanding of the metabolic pathways has increased in recent years [5]. Many biosurfactants and their production processes have been patented, but only some of them have been commercialized [6]. Some examples of products based on biosurfactants that are available in the market are shown in Table 2.

Besides the research efforts, the cost for biosurfactant production is approximately three to ten times more than the cost to produce a chemical surfactant. Biosurfactants are typically produced by microorganisms growing in hydrocarbons as a carbon source, which are usually expensive increasing the production cost [6]. In addition, the downstream cost, low productivity and intense foaming formation during the biosurfactant production currently is a barrier for an economically viable production of biosurfactant [3, 6]. Therefore, most researches have been focusing on increasing the production yield, reducing raw material cost and developing oxygenation strategies to reduce foaming formation [4, 6, 61]. Several approaches have been applied in order to improve biosurfactant productivity such as optimization of growth conditions (e.g., growth medium, temperature, pH, oxygenation, fermentation phases), genetic modifications (mutation, gene knockout or amplification, altered regulation), com-

Biosurfactant	Origin	Supplier	Application
BioFuture	Bacterial rhamnolipid	BioFuture Ltd, Ireland	Bioremediation of contaminated soil with hydrocarbon
EC-601	Bacterial rhamnolipid	Ecochem Ltd, Canada	Dispersive agent of water-insoluble hydrocarbons
EC-1800	Bacterial consortium	Ecochem Ltd, Canada	Cleans up oil spills in soil, sand and gravel
EC-2100W	Bacterial consortium	Ecochem Ltd, Canada	Degrades hydrocarbon based compounds in waste water treatment plants, lagoons, storage tanks, sumps and other aqueous environments
JBR products	Microbial rhamnolipid	Jeneil Biosurfactant Co., LTC, USA	Used in different industries, such food and agro-industrial markets
Petrosolv	Bacterial unknown	Enzyme Technologies Inc, USA	Oil removal; oil recovery and processing
Surfactin	*Bacillus subtilis*	Sigma-Aldrich Co. LLC, USA	Antifungal, antibacterial and antitumor activities

Table 2. Examples of commercialized products containing biosurfactants

bined omics analysis (genomics, transcriptomics, proteomics, metabolomics, fluxomics) and computational modeling [115].

5. Strategies for improvement of biosurfactant production

5.1. Optimization of medium composition

Rhamnolipids are mainly produced by Pseudomonas species, such as *Pseudomonas aeruginosa*. This bacterium produces rhamnolipids as secondary metabolite and their production coincides with the stationary growth phase [65, 95, 116]. Rhamnolipids can be produced using varies carbon sources, such as vegetable oils (e.g. olive, soy and corn), glucose, glycerol and n-alcanes [7]. The carbon and nitrogen sources are important factors in the production of these biosurfactants and have great influence their production cost and considerable efforts have been done towards the use of agro-industrial byproducts and renewable resources as substrates in the production process [3].

Studies have been shown that use of inexpensive substrates, such as crude or waste materials, dramatically affects the production costs of biosurfactants [3]. Different waste substrates have been used for rhamnolipid production, such as fatty acids from soybean oil refinery wastes [117], glycerin from biodiesel production waste [118] and sunflower-oil refinery waste [119]. Nevertheless, the potential of rhamnolipids production from renewable resources is so far not

fully exploited. According to Henkel et al [3], use of waste substrates in the production processes is likely to increase its influence on the field of microorganism-based production, since they are usually cheaper, maximize the utilization efficiency regarding the overall production process and makes the process more environmentally friendly.

Nitrogen source, C/N ratio and mineral salts are also important variables to achieve improved productivity of rhamnolipid [7]. Currently, the main nitrogen source used to promote rhamnolipid production is nitrate [5]. Interestingly, in contrast with nitrate, ammonium has been shown to prevent rhamnolipid production [120-122]. Growth limiting conditions are known to promote rhamnolipids production, as well as other secondary metabolites. High carbon and nitrogen (C/N) ratio [7, 121] and carbon and inorganic phosphorous (C/P) [96] have been shown to increase rhamnolipid productivity. Also, limiting concentration of multivalent ions such as Mg, Ca, K, Na, and trace element salts increase rhamnolipid yield [121]. The growth conditions that influence rhamnolipid overproduction with *P. aeruginosa* have been intensively studied. The mainresearches on this field can be divided into (a) batch cultivations under growth-limiting conditions, (b) agitated flasks and bioreactors cultivation, which include continuous cultivation, fed-batch strategies and controlled aeration systems, (c) downstream processing and (d) biochemical and molecular strategies, aiming to improve strain capability to produce these biosurfactants [5].

5.2. Cultivation strategies and aeration systems

Cultivation strategies described for rhamnolipid and other biossurfactants include shake flask, batch, fed-batch, continuous, and integrated microbial/enzymatic processes [5]. Most of these studies involve the optimization of culture conditions in shake flasks, due to its simplicity, whereas bioreactors provides high control over the relevant parameters, such as oxygen transfer and pH during all the cultivation process, and allows upscaling studies [123, 124]. Bioreactor is often applied in continuous or fed-batch fermentation in biosurfactant production. Both alternatives have been proposed to be more economically viable than simple batches, due to the suppression of several steps regarding the inoculum preparation, sterilization and finalization of production process by carrying out the production for long periods [124].

One of the main limitations for the biosurfactant production in bioreactor is the intense foaming formation when the solution is aerated and agitated caused by the surfactant [6]. Particularly with *P. aeruginosa*, high foam formation is further increased by the presence of extracellular proteins, which results in increased production cost. Mechanic foam breakers are not very efficient, and chemical antifoams agents can alter the quality of the product and the pollutant potential of the final effluent of the bioreactor [6]. Several strategies have been proposed to overcome this limitation using. For example, non-dispersive devices have been successfully applied to promote the oxygen transfer from the gas phase to the liquid phase without dispersion of these phases (Santa Anna et al, 2004). This process has been described by Gruber et al (1993) and a similar process, used in alcoholic fermentations, has been patented by L'Air Liquide (Cutayar et al, 1990). Although this oxygenation process is promising for the use in bioreactors, the manual control of the oxygenation was shown difficult and even inefficient. Recently, Kronemberger et al [6] developed a non-dispersive oxygenated device

controlled by a programmable logic controller (PLC), which allows setting the dissolved oxygen concentration in a process for rhamnolipid production by *P. aeruginosa*. The better control over dissolved oxygen also allowed the demonstration that rhamnolipid production by *P. aeruginosa* is partially dependent of the dissolved oxygen concentration in the medium. Moreover, the relationship between rhamnolipid production and oxidative stress has been recently studied and proteins related to oxidative stress pathways and rhamnolipid production by *P. aeruginosa* was identified [125].

5.3. Downstream processing

Comparable to the selection of cheap raw materials and cultivation strategies, reduction of downstream costs is an essential factor towards the establishment of an economic process. However, the purification costs will depend on the employed strain, the rhamnolipid mixtures produced and the application of the product, since for some industrial applications a high purity grade will not be necessary and thus reducing purification costs [5]. For some industrial applications it is proposed that downstream costs can account for approximately 60% of the total production cost [126].

Conventional methods for recovery of biosurfactants have been used, such as acid precipitation, solvent extraction, crystallization, ammonium sulfate precipitation and centrifugation [126]. More recently, other methods for biosurfactants recovery have been reported, including foam fractionation, ultrafiltration and adsorption-desorption on polystyrene resins and ion exchange chromatography. These procedures take advantage of biosurfactant properties, such as their surface activity or their ability to form micelles or vesicles, and are particularly applicable for large-scale continuous recovery of extracellular biosurfactants from culture broth. In addition, these methods can operate in continuous mode for recovering biosurfactants with high level of purify [4].

Recovery techniques that promote high level of purify often require application of solvents (e.g.,acetone, methanol and chloroform), which are toxic in nature and harmful to the environment [4]. Rhamnolipids are usually precipitated by acidification and extracted using ethyl acetate; extraction of sophorolipids is normally carried out with n-hexane, while for trehalolipids, the preferred solvent is a mixture of chloroform and methanol. Although these techniques are already well established for lab-scale applications, their cost does not allow scaling-up for industrial production of biosurfactants. Thus, the research effort has been directed towards the development of low-cost extraction and purification procedures, avoiding the use of hazardous and costly organic solvents [13].

Rhamnolipid produced by *P. aeruginosa* is often secreted with other virulence factors and studies have been conducted to better understand the molecular mechanisms related to rhamnolipid production. Ideally, rhamnolipids should be produced with minimum presence of other virulence factors (such as pyocianin, elastase and protease), which may allow the use of crude biosurfactant with the suppression of purification stage for environmental applications and bioremediation [125].

5.4. Metabolic and cellular strategies for strain improvement

Rational metabolic and cellular engineering approaches have been successfully applied to improve strain performance in several cases of biotechnological production of small-volume and high-value bioproducts. However, such attempts are limited to the manipulation of only selected group of genes encoding enzymes and regulatory proteins, which are selected using available information and research experience [115]. Recent advances in high-throughput experimental techniques supported by bioinformatics (genomic, transcriptomic, proteomic, metabolomic) have resulted in rapid accumulation of data, providing a basis for in-depth understanding of biological processes. Although our ability to integrate these data is currently limited, the information obtained with these approaches, together with experimental observations *in vivo* and predictions of modeling and simulation *in silico*, can provide solutions to understand the features and functions of biological systems [115].

Despite the advances described, optimization of rhamnolipid production has been performed with non-genetically modified and randomly mutated strains of *P. aeruginosa* [5]. The genomic and proteomic approaches for biotechnology is still very limited, despite the genus Pseudomonas being extensively studied due to its versatility and ability to environmental biodegradation of contaminants [73]. In *P. aeruginosa*, studies are related predominantly with clinical interests, many of them aimed at understanding the *quorum sensing* system in this bacterium. Recently, a comparative proteome analysis of *P. aeruginosa* PA1 was done investigating the differences of the intracellular proteome after the cultivation of rhamnolipid producing and non-producing cells [11]. Rhamnolipid production was either induced or suppressed by variation of media conditions and a total of 21 differentially expressed proteins could be identified by MALDI-TOF/TOF mass spectrometry. Under the rhamnolipid favoring conditions, proteins involved in the response to oxidative stress, secretion pathways and quorum sensing were mainly differentially expressed. Pacheco et al [125] treated *P. aeruginosa* with hydrogen peroxide to trigger its oxidative stress response, and the proteome profile was analyzed. There were identified 14 differentially expressed proteins between samples that were treated and not treated with peroxide. Several of these proteins are potentially involved in the rhamnolipid production/ secretion pathway and oxidative stress. The production of elastase, alkaline proteases and rhamnolipids seems to be regulated in a similar way, which makes it necessary to develop molecular strategies that maximize the production of rhamnolipids over other virulence factors.

A system-wide analysis of growth and rhamnolipid production regulation has the potential to reveal several unknown interactions between genes, proteins and metabolites, thus facilitating rational process engineering strategies [127], either with *P. aeruginosa* strains or heterologous hosts [8]. According to Muller and Hausmann [8], the main goals of such approaches with *P. aeruginosa* would be: (a) metabolic engineering and further strain improvement to enhance the metabolic fluxes towards the product, (b) removal/alteration of autologous genes to avoid byproduct formation and (c) introduction of heterologous genes for the use of alternative substrates and enhanced metabolism. Thus, the integration of engineering and biology is possible and profoundly needed in order to achieve high yields and low costs on biosurfactant production. High-throughput "omics" analysis, predictive computational

modeling or simulation and experimental perturbation can be combined to generate new knowledge about the cellular physiology and metabolism, in order to design strategies for metabolic and cellular engineering of organisms.

6. Conclusion

In conclusion, the production of the better known biosurfactant, rhamnolipid, can be linked to several regulatory factors that respond to environmental inputs such as population density, nutrient availability and diverse stresses. Rhamnolipids production is highly controlled by multilayered regulatory systems and better knowledge of the effect of environmental stimuli can greatly improve their commercial production. On the other side biosurfactant application in processes such as washing, biorremadiation and MEOR, requires a thoroughly research on a case-by-case basis taking in account the physical chemical properties of biosurfactants and the environmental conditions.

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