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Rhamnolipid Biosurfactants Produced by *Pseudomonas Species*

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ABSTRACT

Surfactants are chemical products widely used in our daily life in toothpaste and other personal hygiene and cosmetic products, and in several industries. Biosurfactants are surfactants of biological origin that can be produced by microorganisms and have many advantages, such as low toxicity and high biodegradability, compared to synthetic counterparts. Unfortunately, high production costs limit the use of biosurfactants. Low-cost production is the most important factor for biosurfactants to be able to compete in the global market place. This review presents general information on rhamnolipid biosurfactant produced by Pseudomonas species, as well as on their production and applications. In addition, industrial products and their wastes used for rhamnolipid production are reviewed in detail based on recent studies.

Key words: application of rhamnolipid, biosurfactant, *Pseudomonas* spp., rhamnolipid

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INTRODUCTION

Surfactants, or surface-active agents, are compounds that reduce the surface and interfacial tension between liquids or a liquid and a solid. Surfactants are chemically synthesized amphiphilic compounds containing both hydrophobic and hydrophilic groups. Biosurfactants are surfactants of biological origin, produced by microorganisms, and an increasing emphasis has been given to them recently due to their outstanding features, such as low toxicity, biodegradability, selectivity, and specific activity at extreme conditions (temperature, pH, salinity, etc.). However, production costs of biosurfactants prevent them from competing with their synthetic counterparts. The use of cheaper substrates is the most important production factor in this competitive environment, and therefore current biosurfactant studies have been focused on increasing the yield and reducing the cost of production. Biosurfactants can be produced from industrial wastes, which means that cheaper production is possible. Besides, using wastes reduces their polluting effects on nature. Biosurfactants are categorized, mainly on the basis of their chemical composition, into glycolipids, lipopeptides, fatty acids, polysaccharide–protein complexes, peptides, phospholipids, and neutral lipids (Cooper and Goldenberg 1987). Rhamnolipids are the best known glycolipid biosurfactants and effective compounds with one or two molecules of β-hydroxydecanoic acid (Desai and Banat 1997). These surfactants of biological origin are largely produced by *Pseudomonas* spp. *Pseudomonas aeruginosa* produces two forms of rhamnolipids, mono- and di-rhamnolipids in liquid culture,. Rhamnolipids with one sugar molecule are defined as mono-rhamnolipids, while those with two sugar molecules are defined as di-rhamnolipids. Some *Pseudomonas* species produce only mono-rhamnolipids, while others produce both. Jarvis and Johnson first reported rhamnolipid production by *P. aeruginosa* in 1949. They incubated *P. aeruginosa* in a nutrient medium containing 4% peptone and 3% glycerol in a shaking flask at 30 °C for four–five days and isolated 2.5 g/L of the product. The first patent (US 4,628,030) for rhamnolipid production was received by Kaeppeli and Guerra-Santos (1986) for their study conducted with *P. aeruginosa* DSM 2659. Rhamnolipids are predominantly produced by *P. aeruginosa*, and the other *Pseudomonas* species that have been reported to produce rhamnolipids are *P. chlororaphis* (Gunther et al. 2005), *P. putida* (Wittgens et al. 2011; Nanganuru and Korropati 2012), *P. fluorescens* (Abouseoud et al. 2008; El-Amine Bendaha et al. 2012), *P. nitroreducens* (Onwosi and Odibo 2012), and *P. alcaligenes* (Oliveira 2009).Rhamnolipids reduce the surface tension of water from 72 $mN \cdot m^{-1}$ to below 30 $mN \cdot m^{-1}$ and the interfacial tension of the water/oil system from 43 $mN \cdot m^{-1}$ to about 1 $mN \cdot m^{-1}$. In addition, they have many environmental applications, such as the enhancement of oil recovery, degradation of hydrocarbons, and removal of metals from soil (Bordoloi and Konwar 2008; Amani et al. 2010; Das and Chandran 2011).

In the last three decades, considerable research has been conducted on the production and application of rhamnolipids. This review aims to provide information on the industrial products used for rhamnolipid production by *Pseudomonas* spp. and the factors affecting this process.

FACTORS AFFECTING PRODUCTION OF RHAMNOLIPIDS

As with all biosurfactants, the composition and yield of rhamnolipid depends on the culture conditions as well as the producer strain. There are a number of studies in the literature about effects of various factors on rhamnolipid production, especially on yield. The carbon and nitrogen source, the amount of ions used in the medium and the culture conditions, such as pH, temperature, and agitation, influence the quality and quantity of rhamnolipids.

Carbon source

Many microorganisms synthesize biosurfactants using different carbon sources. Studies indicate that the yield of a biosurfactant varies depending on the carbon source and the nutrient medium (Robert et al. 1989; Bodour et al. 2003; Soberon-Chavez et al. 2005). Crude oil, glucose, sucrose, and glycerol have been reported as good carbon sources for biosurfactant production (Guerra-Santos 1984; Desai and Banat 1997). Carbon sources used in biosurfactant production can be divided into three categories, including carbohydrates, hydrocarbons, and vegetable oils. Water-soluble carbon sources,

such as glycerol, glucose, mannitol, and ethanol, have been recommended for rhamnolipid production by *Pseudomonas* spp. (Santa Anna et al. 2001; Silva et al. 2010).

Nitrogen source

Nitrogen is an essential component for microbial growth and enzyme production for fermentation processes and hence an important factor for biosurfactant production. Different nitrogen sources have been used for the production, such as peptone, urea, ammonium sulfate, ammonium nitrate, sodium nitrate, meat extract, and malt extract. Robert et al. (1989) observed that nitrate was the best nitrogen source for the biosurfactant production by *Pseudomonas* strain 44T1. Venkata Ramana and Karanth (1989) reported that nitrogen limitation caused increased biosurfactant production by *P. aeruginosa.* Guerra-Santos et al. (1986) determined that the maximum rhamnolipid yield was obtained after nitrogen limitation, at a C:N ratio of 16:1 to 18:1. Syldatk et al. (1985) showed that nitrogen limitation also changed the composition of the biosurfactant produced. Onwosi and Odibo (2012) reported a production level of 4.39 g/L of rhamnolipids for *P. nitroreducens* with sodium nitrate as a nitrogen source, and the observed nitrogen source efficiency was in the order of sodium nitrate > yeast extract > urea. According to many studies, sodium nitrate was more effective than ammonium sulfate and urea for rhamnolipid production (Guerra-Santos et al. 1986; Santa-Anna et al. 2001; Jeong et al. 2004; Rashedi et al. 2005). Sodium nitrate has also been reported as the best nitrogen source for rhamnolipid production by *P. aeruginosa* (Wei et al. 2005; Prieto et al. 2008; Wu et al. 2008). According to Aboseoud et al. (2008), ammonium nitrate was the best nitrogen source for rhamnolipid production by *P. fluorescens*. Consequently, the results related with nitrogen sources vary depending on the *Pseudomonas species* used in the studies so nitrogen source should be selected according to the *Pseudomonas species* which will be used in the study. But generally it can be said that sodium nitrate and ammonium nitrate are best source for rhamnolipid production.

Culture conditions

pH: A wide variety of culture conditions have been tested for biosurfactant production to obtain large quantities of the product of interest. According to Guerra-Santos et al. (1984), the maximum rhamnolipid yield was obtained in the pH range from 6.0 to 6.5, and the yield sharply decreased above pH 7.0. Mata-Sandoval et al. (2001) reported in their study with *P. aeruginosa* UG2 that neutral culture conditions (pH 7.0) increased the average production levels of all rhamnolipid species by 25% compared to their production at slightly acidic conditions (pH 6.25).

Temperature: The optimum temperature ranges have been identified to be 30–37 °C in a study by Wei et al. (2005) and 30–35 °C by Sahoo et al. (2011) with *P. aeruginosa*. Chen et al. (2007) reported the optimum temperature to be 37 °C based on their investigation of temperatures between 30 and 42°C.

Agitation: The agitation speed and method applied during the incubation are important in the production of a biosurfactant to ensure oxygen transfer from the gas phase to the aqueous phase. Pimienta et al. (1997) reported that orbital shaking is more effective than lateral shaking. In studies with flasks, shaking speeds have been varied between 120 and 220 rpm. Wei et al. (2005) tested agitation speeds in the range between 50 and 250 rpm in their study with *P. aeruginosa* strains isolated from petrochemical waste waters and observed a better result at 200 rpm. As well as microorganisms , carbon and nitrogen sources, optimization of the culture medium and conditions is the significant parameter to increase rhamnolipid yield. Additionally, according to Banat et al. (2010), recombinant and mutant producer strains may give high yields of biosurfactants and can be an important step to their economical production.

APPLICATIONS OF RHAMNOLIPIDS

Environmental applications of rhamnolipids

Petroleum-based products are the major source of energy for industry and daily life. Oil spills into the environment are a main cause of water and soil pollution, and they can result in both immediate and long-term environmental damage. Biodegradation is a process including decomposition of organic material and removal of petroleum and other hydrocarbon pollutants from the environment by microorganisms, and it is cheaper than other remediation technologies (Das and Chandran 2011). Chemically synthesized surfactants have been used for enhanced oil recovery (EOR) and for oil spill clean-ups for decades. However, because of

their toxicity and resistance to degradation, chemical surfactants can cause serious environmental problems (Mulligan 2005). Biosurfactants have been tested for EOR and were demonstrated to be effective in microbial enhanced oil recovery (MEOR), where their lower toxicity and biodegradability represent advantages. MEOR is a technique that can recover the residual oil using microorganisms or their products (Bordoloi and Konwar 2008; Amani et al. 2010). Rhamnolipids have been found to be effective compounds for MEOR, and they can change physical and chemical properties of crude oil and stimulate oil–water interactions that improve oil recovery (Amani et al. 2010, 2013; Amani 2015).

In Banat's (1995) and Reis et al. (2013) review discussing biosurfactant production and possible uses in MEOR and remediation of oil pollution, the following three main strategies were indicated, involving the use of biosurfactants in MEOR: (i) injection of biosurfactant-producing microorganisms into a reservoir through the well, with subsequent multiplication of the microorganisms *in situ* within the reservoir rocks; (ii) injection of selected nutrients into a reservoir to stimulate the growth of indigenous biosurfactant-producing microorganisms; and (iii) production of biosurfactants in bioreactors *ex situ* and their subsequent injection into a reservoir.

Various bacteria from the genus *Pseudomonas*, especially *P. aeruginosa* strains, are the best known bacteria capable of utilizing a number of aliphatic and aromatic hydrocarbons as carbon and energy sources. These bacteria can inhabit contaminated soils and enhance the availability and biodegradation of organic components (Das and Chandran 2011; Kadali et al. 2012; Saikia et al. 2012; Puskarova et al. 2013). Biosurfactants play a dual role in bioremediation by increasing the surface area and the bioavailability of hydrophobic, water-insoluble substrates.

There are a large number of research publications on rhamnolipid effectiveness in biodegradation as well as in cleanup of soils contaminated with gasoline and other hydrocarbons. Originally, Itoh and Suzuki showed in 1972 that hydrocarbon culture media stimulated the growth of a rhamnolipid-producing *P. aeruginosa* strain. Subsequent studies confirmed rhamnolipid effects on hydrocarbon biodegradation (Arino et al. 1998) and indicated that the addition of rhamnolipids enhanced biodegradation of hexadecane, octadecane, and *n*-paraffin in a liquid system and hydrocarbon mixtures in soil. The Exxon Valdez oil spill is a well-known example of biosurfactant use in bioremediation (Harvey 1990).

Zhang and Miller (1997) investigated rhamnolipid effects on octadecane dispersion and biodegradation and showed that octadecane mineralization increased from 5 to 20% within 84 h in the presence of 300 mg/L of rhamnolipids. Van Dyke et al. (1993) demonstrated a 25 to 70% increase in the recovery of hydrocarbons from a contaminated sandy loam soil and a 40 to 80% increase in the recovery of hydrocarbons from a silt loam soil upon use of *P. aeruginosa* rhamnolipids.

Rahman et al. (2003) investigated the biodegradation of *n*-alkanes in petroleum sludge contaminated with 87.4% of oil and demonstrated that with the addition of rhamnolipids, C8–C11, C12–C21, C22–C31, and C32–C40 alkanes were degraded by 100%, 83–98%, 80–85%, and 57-73%, respectively, after 56 days. Several studies reported that rhamnolipids are efficient in the removal/clean-up of heavy metals due to interactions between their polar glycosidic groups and metal ions. Rhamnolipid interactions with organic compounds increase the bioavailability of the latter. Rhamnolipid surfactants have been shown to be effective in reducing oil concentrations in contaminated soils, and their addition at a low concentration (80 mg/L) to a diesel/water system increased biomass growth and diesel degradation (Whang et al. 2008).

Polycyclic aromatic hydrocarbons (PAHs) pollute the environment and are toxic, mutagenic, and carcinogenic compounds. They are emitted to the environment as byproducts of coal processing and by oil spills. The reason for prolonged presence of large-molecular-weight hydrophobic compounds in aquatic environments is their low solubility. Low aqueous solubility of PAHs limits their availability for microorganisms. This poses a potential problem for the bioremediation of an area contaminated with these compounds. Biosurfactants accelerate the utilization of hydrophobic compounds by increasing the solubility of PAHs for their bioremediation (Cameotra and Bollag 2003). Deschenes et al. (1996) reported that rhamnolipids are more effective than sodium dodecyl sulfate in increasing solubilization of PAHs. Daziel et al. (1996) demonstrated in their study that rhamnolipid production is responsible for an increase in the aqueous solubility of naphthalene. Zhang et al. (1997) investigated the effect of two forms of rhamnolipids on the dissolution and bioavailability

of phenanthrene and found monorhamnolipid was more effective than dirhamnolipid for solubilization but phenanthrene within monorhamnolipid micelles was less bioavailable than phenanthrene within dirhamnolipid micelles. So they indicated that the effect of a surfactant on biodegradation is a combination of the solubilizing power of the surfactant and the bioavailability of the substrate within the surfactant micelles. an increase in both solubility and degradation. In another study, it was shown that adding a rhamnolipid producer, *Pseudomonas* spp. DS10-129, increased the bioremediation process in an oil-contaminated soil (Rahman et al. 2002). Similarly, Straube et al. (2003) reported that adding *P. aeruginosa* strain 64 enhanced the bioremediation in a soil contaminated with PAHs and pentachlorophenol. Kumar et al. (2008) reported that a crude biosurfactant from the *Pseudomonas* DHT2 strain isolated from an oil-contaminated soil enhanced the solubility of PAHs in a dose-dependent manner.

Mulligan (2009) reviewed in detail environmental applications of biosurfactants for an enhanced clean-up of hydrocarbon- and metal-contaminated soils. Wen et al. (2009) have investigated the use of rhamnolipids for bioremediation of soils contaminated with Cd and Zn and reported that rhamnolipids remain in soil long enough to enhance the metal phytoextraction. In a study of Obayori et al. (2009), biodegradative properties of a biosurfactant produced by the *Pseudomonas* sp. LP1 strain were investigated for crude oil and diesel. The authors reported 92.34% degradation of crude oil and 95.29% removal of diesel oil. Gonzini et al. (2010) observed that with the increasing dose of rhamnolipids, the gasoil removal efficiency increased up to 86.7%. Zhang et al. (2011) investigated the effect of rhamnolipids on the remediation of a crude oil- and salt-contaminated soil. They observed a distinct decline in the total petroleum hydrocarbon (TPH) concentration in the soil when using rhamnolipids during a remediation period of 30 days, with a maximum TPH reduction of 86.97%. Wana et al. (2011) investigated the selective adsorption of hexachlorobenzene (HCB) from a rhamnolipid solution by a powdered activated carbon (PAC) and observed that when a 25 g/L rhamnolipid solution was applied the HCB leaching from soils was 55–71% after three cycles of cleaning and the HCB removal by the PAC was nearly 90%. Pacwa-Plociniczak et al. (2014) investigated the *Pseudomonas* sp. P-1 strain isolated from a petroleum-contaminated soil for its bioremediation potential and indicated that the strain had the ability to degrade various hydrocarbons (hexadecane, crude oil, and fractions A5 and P3 of crude oil). In another study conducted by Gudina et al. (2015) with agro-industrial byproducts (corn steep liquor and molasses), rhamnolipids exhibited a better performance in removing oil from contaminated sand compared with two chemical surfactants (Enordet and Petrostep).

Other applications

Antimicrobial activity and anti-cellular effects of rhamnolipids produced by *Pseudomonas* spp. have been described by many authors in the literature. Rhamnolipids showed activity against a large variety of bacteria, including both Gram-negative (*Salmonella typhimurium*, *Escherichia coli*, and *Enterobacter aerogenes)* and Gram-positive bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus cereus*) and fungi (*Phytophthora infestans*, *Phytophthora capsici*, *Botrytis cinerea*, *Fusarium graminearum*, *Mucor* spp., *Cercospora kikuchii*, *Cladosporium cucumerinum*, *Colletotrichum orbiculare*, *Cylindrocarpon destructans*, *and Magnaporthe grisea)* (Abolos et al. 2001; Rodrigues et al. 2006; Lotfabad et al. 2008; Onbasli and Aslim 2009; Sridhar et al. 2013). Yilmaz and Sidal (2005) reported that the antimicrobial activity against Gram-positive bacteria was better than that against Gram-negative bacteria. They explained this fact by the differences in the cell wall structure between Gram-positive and Gram-negative bacteria. It is known that Gram-negative bacteria have the membrane that is hardly permeable to hydrophobic and amphipathic molecules. In more recent years, it has been shown that rhamnolipids have antimicrobial activity against *Listeria monocytogenes* and show a synergistic effect when combined with nisin (a polycyclic antibacterial peptide) (Magalhaes and Nitschke 2013). Additionally, Araujoa et al. (2010) determined that a rhamnolipid inhibited *L. monocytogenes* adhesion and suggested that this surfactant could be explored as a potential agent to control *L. monocytogenes*. Interactions of the rhamnolipid produced by *P. aeruginosa* OBP1 with the cell surfaces of *S. aureus* MTCC 3160 and *Klebsiella pneumoniae* MTCC 618 were studied by Bharali et al. (2013), and rhamnolipid concentrations below the critical micelle concentration (CMC) (“CMC is defined as

the concentration of surfactants above which micelles start to form.) exhibited no significant antibacterial activity. However, upon increasing the rhamnolipid concentration over the CMC, a significant antibacterial activity was observed. Currently, rhamnolipids have been studied to determine their synergistic activities with antibiotics, essential oils, and various other agents (Ganesh et al. 2010; Das et al. 2014; Elouzi et al. 2014; Haba et al. 2014).

Thanomsub et al. (2007) investigated the chemical structures and biological activities of the rhamnolipids produced by *P. aeruginosa* B189 isolated from a milk factory waste. The culture produced two kinds of biosurfactants. Rhamnolipid A showed significant anti-proliferative activity against a human breast cancer cell line (MCF-7) with a minimum inhibitory concentration of 6.25 µg/mL, while rhamnolipid B showed activity against the insect cell line C6/36 at 50 µg/mL. Also, rhamnolipids have been found to be effective for skin treatment, including wound healing with reduced fibrosis and wrinkle treatment (Piljac and Piljac 2007), thereby showing promise in pharmaceutical applications. Rhamnolipids are emulsifiers and surface-active detergents; therefore, detergent compositions, laundry products, shampoos, and soaps are also their usage areas (Parry et al. 2013).

COST-EFFECTIVE SUBSTRATES FOR RHAMNOLIPID PRODUCTION

Biosurfactant-producing companies have been discussed in detail in Sekhon Randhawa and Rahman's (2014) review. The authors indicated that there are still a very limited number of companies in the field that produce rhamnolipids at a marketable scale. There are six companies, including TeeGene Biotech Ltd. (UK), AGAE Technologies LLC (USA), Jeneil Biosurfactant Co., LLC (USA), Paradigm Biomedical, Inc. (USA), Rhamnolipid Companies, Inc. (USA), and Henkel (Germany), involved in rhamnolipid production. In particular, rhamnolipids are used as cosmetic additives in Japan (Iwata Co., Japan, personal communication). In the same review, the authors mentioned that there are 42 patents and grants obtained on rhamnolipids. Despite their many advantages compared with synthetic surfactants, industrial production of biosurfactants has not been undertaken due to high investment costs. Although there have been studies focusing on low-cost production, the costs continue to remain high. Syldatk and Hausmann (2010) explained the reasons for a limited use of biosurfactants in industry, which include the use of expensive substrates, limited product concentrations, and the availability of only few pure compounds. As with all biosurfactants, there are three main strategies adopted in the world for the cost-effective rhamnolipid production: 1) utilization of rhamnolipid producer strains giving a high yield 2) using a non-expensive substrate and 3) development of a bioprocess including optimized culture conditions.

Using a low-cost material is a possible key to solve the cost problem; however, it is of great importance to select suitable products compatible with cell growth. Various cheap substrates are currently available as a carbon source in industrial biosurfactant production. In the future, waste substrates may become more important, since they are usually less expensive. Additionally, using waste substrates for biotechnological processes is beneficial for the environment.In the literature, there are many studies showing rhamnolipid production using various wastes. The wastes used for rhamnolipid production are shown in table 1.

Table 1: Wastes used for rhamnolipid production

Source	RL (g/L)	References	*Isolate*
Olive oil mill effluent (OOME)	1.4	Mercade 1993;Sidal et al. 2000	*Pseudomonas spp.*
Frying olive oil	2.7	Haba et al. 2000	*Pseudomonas spp.*
	12.47	Zhu et al. 2007	*P. aeruginosa*
Frying coconut oil	2.26	George and Jayachandran 2012	*P. aeruginosa D*
Frying soybean oil	3.3	Lima et al.2009	*P. aeruginosaPACL*
Soapstock,	12	Benincasa et al. 2002	*P. aeruginosa LBI*
Soybean soapstock	11.7	Nitschke et al. 2005	
Molasses	0.24	Patel and Desai 1997	*P. aeruginosa GS3*
	0.04	Rashedi et al.2005,	*P. aeruginosa*
	1.45	Raza et al.2007	
	0.38	Onbasli and Aslim 2009	*Pseudomonas spp.*
Whey	1	Dubey and Juwarkar, 2001	*P. aeruginosa BS2*
	9.2	Praveesh et al., 2011	*Pseudomonas spp.*
Curd whey and distillery waste	2- 0.92	Babu et al.,1996, Dubey and Juwarkar, 2004	*P. aeruginosa BS2*
Sunflower oil wastes	7.3	Benincasa and Accorsini, 2008	*P. aeruginosa LBI*

Agro-Industrial Wastes

Agro-industrial wastes contain high amounts of carbohydrates and lipids and hence can be used as a rich carbon source for microbial growth. These wastes include plant oil extracts and wastes, distillery and whey wastes, olive oil mill effluents, cassava flour and its wastewater, and sugar cane and beet molasses. Among the agro-industrial waste products, molasses has been examined by many researchers. Molasses is a sweet, dark brown, concentrated syrup byproduct of the sugar cane and beet processing industries, which has a high sucrose concentration in the range of 50–55% by weight. Initially, Patel and Desai (1997) reported rhamnolipid production by *P. aeruginosa* GS3 using molasses and corn steep liquor as the carbon and nitrogen sources. Then, many other researchers followed the trend. Thus, Rashedi et al. (2005) investigated the possibility of using soy molasses as an inexpensive source for rhamnolipid production. They reported rhamnolipid production rates at molasses concentrations of 2, 4, 6, 8, and 10% to be 0.00065 g/L, 4.556 g/L, 8.94 g/L, 8.85 g/L, and 9.09 g/L, with the rhamnolipid/biomass yield ratios of 0.003g, 0.009g, 0.053g, 0.041g, and 0.213g, respectively. Raza et al. (2007) obtained the maximum rhamnolipid yield of 1.45 g/L after 96 h of incubation of a *P. aeruginosa* EBN-8 mutant on 2% blackstrap molasses. Similarly, Onbasli and Aslim (2009) used molasses in their study conducted with *P. luteola* B17 and *P. putida* B12 and obtained the maximum rhamnolipid yield after a 12-h incubation with 5% sugar beet molasses. Molasses distillery wastewater was investigated by Li et al. (2011), as an unconventional substrate for rhamnolipid production by *P. aeruginosa* GIM32, and 2.6 g/L of rhamnolipids was obtained after a 64-h incubation. Gudina et al. (2015) obtained in their study the highest biosurfactant production yield of 3.2 g/L using a culture medium containing corn steep liquor (10%, v/v) and molasses (10%, w/v).

The availability of agro-industrial wastes is usually locally confined and access difficulty to these wastes at large enough quantities is a handicap for large-scale production of biosurfactants. Additionally, agro-industrial wastes have variable components so the actual concern is sustainability of same wastes with same ingridients for production.

Dairy and distillery industry by-products

Large quantities of whey, including curd whey, whey waste, and cheese whey, are easily available as a substrate for microbial production of surfactants (Dubey and Juwarkar 2001, 2004; Praveesh et al. 2011; Dubey et al. 2012). Whey is the most important byproduct of the dairy industry, and the liquid contains up to 75% of lactose and 15% of protein in dry matter, as well as vitamins

and minerals. Whey waters constitute the major part of the total pollution load in the dairy industry, and whey disposal is still an important environmental problem. Production of rhamnolipids by *P. aeruginosa* using whey as a carbon source has been investigated by Dubey and Juwarkar (2001), and a 1 g/L rhamnolipid yield could be achieved. In recent years, Colak and Kahraman (2013) have conducted a study using cheese whey and olive oil mill wastewater and obtained the highest rhamnolipid yield from whey in cultures grown at 37 °C and 100 rpm, reaching 9.6 and 13.3 g/L within 72 h for a *P. aeruginosa* strain and its recombinant derivative, respectively.

Oil and oil processing wastes

Several vegetable oils and wastes from the oil processing industry have been used for the production of microbial surface-active compounds. The oils used for rhamnolipid production are listed in table 2.

Table 2: Oils used for rhamnolipid production

Source	RL (g/L)	References	Isolate
Olive oil		Robert et al., 1989	*P. aeruginosa* 44T1
	0.8	Abouseoud et al., 2008	*P. fluorescens*
	0.12	El- Amine Bendaha et al.,2012	*P.aeruginosa P.B:2*
		Moussa et al., 2014	*P.fluorescens P.V:10*
	0.19		*P. aeruginosa* TMN
Sunflower oil	4.9	Benincasa et al., 2002	*P. aeruginosa* LB1
	39	Müller et al., 2010	*P. aeruginosa*PAO1
	3	Rikalovic et al., 2012	*P. aeruginosa* san-ai
		Xia et al., 2012	*P. aeruginosa WJ-I*
	0.187	Peter and Singh, 2014	*P.fluorescens*
Safflower oil	2.98	Rahman et al., 2002	*P.aeruginosa* DS10-129
Soybean oil	4.31	Rahman et al.,2002	*P.aeruginosa* DS10-129
	1.42	Prieto et al., 2008	*P. aeruginosa* LBM10
		Abdel-Mawgoud et al., 2009	*P. aeruginosa Bs20*
	0.437	Peter and Singh, 2014	*P.fluorescens*
Rapeseed oil	45	Trummler et al., 2003	*P. aeruginosa* DSM 2874
Fish oil	17	Lee et al., 2004	*P. aeruginosa* BYK-2KCTC
Palm oil	2.91	Thaniyavarn et al., 2006	*P. aeruginosa* A41
	0.289	Peter and Singh, 2014	*P. fluorescens*
Canola oil	17-24	Sim et al., 1997	*P.aeruginosa* UW-1
Babassu oil	0.2	Santa Anna et al., 2001	*P.aeruginosa* PA1
Brazilian nut oil, passion fruit oil	9.9 9.2	Costa et al., 2006	*P. aeruginosa LBI*

Vegetable oils, such as soybean oil, corn oil, canola oil, and olive oil are major sources for the highest production of rhamnolipids. First, Mercade et al. (1993) used a vegetable oil from the distillation process and found it to be effective for rhamnolipid production by *Pseudomonas* strains. Thaniyavarn et al. (2006) investigated different oils as carbon sources for rhamnolipid production by *P. aeruginosa* A41 isolated from seawater in the Gulf of Thailand and determined the yields of the biosurfactant to be 6.58, 2.91, and 2.93 g/L with olive oil, palm oil, and coconut oil, respectively. Although they obtained the highest yield with olive oil, the authors indicated that the biosurfactant obtained from palm oil performed best in lowering the surface tension of the medium. In the study of

El-Amine Bendaha et al. (2012), *P. fluorescens* P.V:10 and *P. aeruginosa* P.B:2 isolated from a soil contaminated with hydrocarbons (kerosene, kerosene and diesel, and olive oil) were investigated for rhamnolipid production. Nutrient broth supplemented with olive oil was determined to be the best medium for rhamnolipid production. As much as carbon sources used for production, method optimization is the important point for obtaining the best yield. Ji et al (2016) indicated that maximum production of the RLs was obtained after optimization of the culture conditions, with a 6.85-fold increase in the yield of the RLs, up to 12.6 g/L with olive oil, relative to the yield before optimization.

The other carbon source for rhamnolipid production is sunflower oil. Benincasa et al. (2002) obtained 4.9 g/L and 12 g/L rhamnolipid from *P. aeruginosa* LBI strain using sunflower oil and sunflower oil soapstock, respectively with shaking flask method but they indicated that rhamnolipid concentration with soapstock achived 15.9 g/L when biorector was used for production. It is known that more rhamnolipid amounts are obtained with batch bioreactor cultivation process in production. Similarly Müller et al.(2010) in their study conducted with *P.aeruginosa* PAO1 used sunflower oil as a carbon source and batch bioreactor cultivation method and they obtained 39 g/L rhamnolipid after 90 h cultivation. Trummler et al. (2003) achieved best results and product yields up to 45 g/L with rapeseed oil using fed batch process.

There are limited numbers of studies in the literature on other *Pseudomonas* species, except *P. aeruginosa using oils*. Rhamnolipid production by *P. alcaligenes* using palm oil was reported by Oliveira et al. (2009). Peter and Singh (2014) obtained the highest rhamnolipid yield for *P. fluorescens* with soybean oil (0.437 g/L), followed by coconut oil (0.299 g/L), palm oil (0.289 g/L), mustard oil (0.233 g/L), sunflower oil (0.187 g/L), and olive oil (0.108 g/L).

Additionally to the oils, oil process wastes are alternative for rhamnolipid production. Nitschke et al. (2010) used soybean oil soapstock as an alternative source for production of rhamnolipid by *P. aeruginosa* LBI strain. The rhamnolipids obtained in the study were characterized in terms of their chemical structure. The authors concluded that soybean oil soapstock could be used as an alternative low-cost substrate for rhamnolipid production. In addition to vegetable oils and oil process wastes, some studies investigated effects of waste frying oils on rhamnolipid production. Haba et al. (2000) used waste frying sunflower and olive cooking oils for rhamnolipid production by *P. aeruginosa* 47T2 and obtained 2.7 g/L of rhamnolipids. Rhamnolipid production by *P. aeruginosa* ATCC 9027 with waste frying oil as a sole carbon source was studied by Luo et al. (2013) using the response surface method. The maximum rhamnolipid production was 8.5 g/L within 72 h. Benincasa and Accorsini (2008) obtained 7.5 g/L of rhamnolipids from *P. aeruginosa* LBI using a fermentation medium composed of acidic wastewater and soapstock from a sunflower-oil process. Colak and Kahraman (2013) examined rhamnolipid production using olive oil mill wastewater and whey in their study conducted with a *P. aeruginosa* strain and its recombinant derivative containing the *Vitreoscilla* hemoglobin gene. They obtained higher rhamnolipid yields with whey, which reached 9.6 and 13.3 g/L after a 72-h incubation of the wild-type and recombinant strains, respectively.

Mixture of carbon sources have been used in some studies in the literature for increasing rhamnolipid production. Thus, Camilios Neto et al. (2011) investigated rhamnolipid production using a solid-state cultivation method with different carbon sources and obtained the best rhamnolipid production, 45 g/L of the impregnating solution used, with a 50:50 (m/m) mixture of sugarcane bagasse and corn bran, supplemented with a solution containing 6% (v/v) each of glycerol and soybean oil.

Other substrates for rhamnolipid production

In addition to the products described above, other substrates were also used for rhamnolipid production in some studies. These sources are listed in Table 3.

Table 3: Other substrates used for rhamnolipid production

Source	RL (g/L)	References	Isolate
Kefir	11.7	Kaskatepe et al., 2015a	*P. aeruginosa* ATCC 9027
Fish meal	12.3	Kaskatepe et al., 2015a	*P. aeruginosa* ATCC 9027
Pulps of sunflower, hazelnut and barley	6.7- 8.5- 9.2	Kaskatepe et al., 2015b	*P.putida and P.pachastrella*
n-hexadecane, Paraffin oil	0.13- 0.26	Santa Anna et al., 2001	*P.aeruginosa* PA1
Glycerol	0.69	Santa Anna et al., 2001	*P.aeruginosa* PA1
Orange fruit peelings	9.18	George and Jayachandran, 2009	*P. aeruginosa* MTCC 2297

Santa Anna et al. (2001) investigated the rhamnolipid production by *P. aeruginosa* PA1 using different carbon sources, including *n*-hexadecane, paraffin oil, glycerol, and babassu oil, and indicated that the best results were obtained with glycerol. Glycerol can be obtained from renewable substrates, including hydrolysis of triglycerides from animal fats and vegetable oils of different purities, and can also be produced by petrochemical processes. Crude glycerol was used in studies, but unfortunately for trading utilization its prices are relatively high (Kosaric and Sukan 2015). Priya and Usharani (2009) investigated the effects of vegetable oil, kerosene, petrol, and diesel on biosurfactant production by *P. aeruginosa* and indicated that diesel was the best carbon source for the production. George and Jayachandran (2009) used various cost-effective waste materials, such as orange and lime peelings, carrot peel waste, coconut oil cake, and banana waste for rhamnolipid production by *P. aeruginosa* MTCC 2297 and found that the orange peel was the best substrate generating 9.18 g/L of rhamnolipid biosurfactants.

In our previous study (Kaskatepe et al. 2015a), rhamnolipid production by *P. aeruginosa* ATCC 9027 was investigated using fish meal, which is a fish oil factory waste with high protein and mineral contents, and kefir, which is a fermented milk drink containing lactose, casein, albumin, fat, and good amounts of elements such as calcium, magnesium, phosphorus, fluorine, and selenium. As a result, 11.7 and 12.3 g/L of rhamnolipids were obtained from kefir and fish meal, respectively, after seven-day incubation at 35 °C and 150 rpm. In literature survey, we found no study using fish meal, but found two using fish oil. Lee et al. (2004), while studying *P. aeruginosa* BYK-2 KCTC 18012P strain, used fish oil (25 g/L) as carbon source and obtained17 g/L rhamnolipid. On the other hand, Prieto et al. (2008) used soybean- and fish oil in their study. They added 40 g/L from each oils in the basal medium and reported 0.94 g/L rhamnolipid production in soybean oil but noted less amount of rhamnolipid in fish oil. Fish meal, for being produced from unprocessed fish waste and low cost, can be a more suitable source for rhamnolipid production. In another study, we tried to increase the rhamnolipid production by *P. putida* and *P. pachastrellae* strains by formulating different media using pulps of barley, hazelnut, and sunflower. The best media for rhamnolipid production were determined to be the barley pulp (9.2 g/L) for *P. pachastrellae* and the hazelnut (8.5 g/L) and sunflower (6.7 g/L) pulps for *P. putida* (Kaskatepe et al. 2015b).

FUTURE PERSPECTIVES OF RHAMNOLIPID

As it seen in the studies , the main aim is increase the rhamnolipid production with low-cost, for this purpose, different raw material, wastes, production methods and culture conditions are used in studies and the results are vary according to these variable factors. Despite the all studies and promising features of rhamnolipids, the economics of their production is a major problem for commercialization. There is still no economically technology for purifying rhamnolipids at industrial scale and also accessing cheap substrate is a barrier for low-cost production. Kosaric and Sukan (2015) have drawn attention to important issues. The

authors indicated that the local availability of substrates plays an important role in the calculations of the production price because some resources are only generated at very few production sites.

Furthermore, some resources may not be present in large enough quantities to sustain large-scale production. According to the authors, the second major factor is substance purity. When using industrial wastes or other non-pure substrates, such as a feedstock, the main medium composition needs to be investigated. It is possible to define a typical composition; however, it varies among different batches. Thus, the process requires advanced control for standardization.

Some of the important criteria that need to be considered for production on industrial scale are as follows; the need for cost effective raw material and supply raw material with same composition , potential microorganisms with enhanced production capacity, economical production technologies and purification methods. Interdisciplinary research approaches in combination with the technologies of large-scale fermentation and genetic engineering by taking into consideration these parameters are significant to claim rhamnolipid as the commercial product of future.

CONCLUSIONS

Rhamnolipids have a big potential, especially in environmental applications for the remediation of contaminated soils due to their biodegradability and low toxicity and in medical fields due to their antimicrobial activities. Rhamnolipids are an alternative to synthetic surfactants, but their industrial use is still limited because of high costs. Low-cost production and discovery of novel rhamnolipid-producing strains characterized by better yields are the most important keys for rhamnolipids to have a corner on the global market of surfactants. Multidisciplinary research needs to be focused on discovery of novel strains or obtain new strains with genetic engineering, accessing cheap substrates and economical production technology. More studies should be carried out to improve low cost effective production media and process.

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