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Recent advancements in the production of rhamnolipid biosurfactants by *Pseudomonas aeruginosa*

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Rhamnolipid (RL) biosurfactant which is produced by *Pseudomonas* species is one of the most effective surface-active agents investigated in the literature. Over the years, many efforts have been made and an array of techniques has been developed for the isolation of RL produced strains as well as RL homolog characterization. Reports show that RL productivity by the best-known producer, *Pseudomonas aeruginosa*, is very diverse, from less than 1 gr/l to more than 200 g L^{-1}. There are some major parameters that can affect RL productivity. These are culture conditions, medium composition, the mode of operation (batch, fed-batch and continuous), bioengineering/gene manipulation and finally extraction methods. The present paper seeks to provide a comprehensive overview on the production of rhamnolipid biosurfactant by different species of *Pseudomonas* bacteria. In addition, we have extensively reviewed their potential for possible future applications.

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1. Background

Biosurfactants (BS) as one of the important bio-products have many applications in environmental, food, agriculture, petroleum, paper/pulp, cosmetics, and pharmaceutical industries. These natural compounds, which are produced by microbial cells,[1] have several benefits compared to synthetic counterparts, including great biodegradability, low toxicity, better environmental compatibility, acceptable surface activity at extreme temperatures, pH and salinity, and the ability to be synthesized from renewable feedstocks.[2,3] The main reason behind the current global interest in rhamnolipid production is due to their broad range of advantages as well as potential applications in various industries along with "eco-friendly" characteristics.

Rhamnolipids (RLs), with the glycolipid-type structure, which are produced mainly by *Pseudomonas aeruginosa*, are the most intensively studied biosurfactants.[4–6] This is because of their relatively high surface activities and high yields of production after relatively short incubation periods by a well-understood, easy to cultivate microorganism.

The discovery of RLs dates back to 1946 when an oily glycolipid, named pyolipic acid, was produced by *Pseudomonas pyocyanea* (*P. aeruginosa*) on glucose. L-Rhamnose and b-hydroxydecanoic acid were also reported as its structural units.[7,8] The exact chemical nature of these biomolecules was unraveled by Jarvis *et al.*[9] followed by Edwards *et al.*[10] Since then, extensive investigations have been conducted covering various aspects of RL research. Fig. 1 shows various common structures of RLs.

Rhamnolipid structure varies due to the number of rhamnose moiety and the length and number of carbon chain. In view of the structures, RLs can be described as follows: they are glycosides composed of mainly two parts, first a rhamnose moiety (glycon part) and the second lipid moiety (aglycon part).[11] The rhamnose moiety of RLs is composed of one or two rhamnose moieties linked to each other one which called mono-RLs and di-RLs, respectively. The lipid moiety, however, is composed of hydroxy fatty acid chains (saturated, mono-, or polyunsaturated and of chain length varying from C8 to C16) linked to each other. Abdel-Mawgoud *et al.*[11] studied different structures of RLs and they concluded that variations in the chemical structures of RLs give rise to a large pool of RL homologues that approaches 60 structures. Their glycon and aglycon parts alterations cause homologues differences.

Although RLs have shown many advantages in their applications, there was no mass-production for them up to 2016 due to difficult process and low yield. However, the first company producing RL in large scale was Evonik Industry. They used recombinant *Pseudomonas putida* and butane to rhamnolipid production. Also, German biotech company Biotensidon GmbH is a leading and first company which develop cost-effective process to produce RL in industrial scale (annually 5000 tons). Other companies which are producing RLs in commercial plants are: TeeGene Biotech (UK), AGAE Technologies LLC (USA), Jeneil Biosurfactant (USA), Paradigm Biomedical Inc. (USA), and many Chinese companies such as Shaanxi Pioneer Biotech Company.

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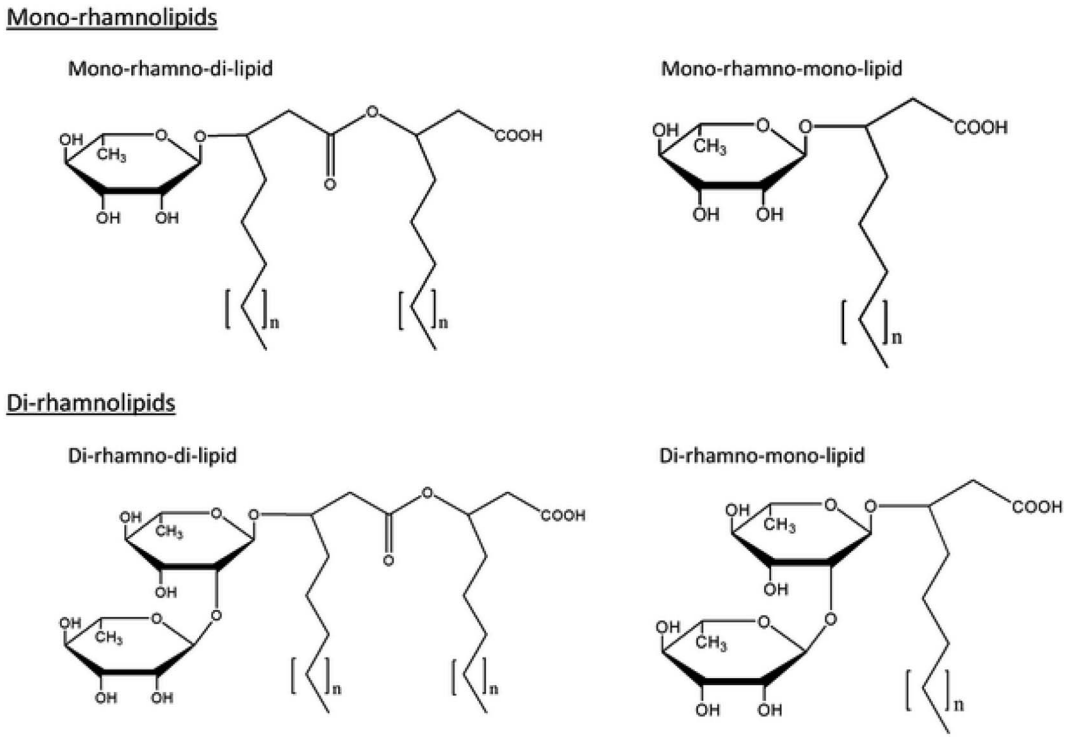

Fig. 1 Common structures of rhamnolipid biosurfactants.

2. Detection and analysis

Detection and analysis of rhamnolipids is a base for better understanding of their different structures and functions. Several methods of analysis with variable precision and different purposes are generally employed which divided into qualitative and quantitative techniques.

2.1. Qualitative methods

2.1.1. Surface tension measurement. The effectiveness of a typical biosurfactant is determined by its ability to lower the surface tension. As it was shown by Busscher *et al.*,[12] to identify any microorganism as a biosurfactant producer, more than 8 mN m^{-1} reductions in surface tension are needed. However, rhamnolipid can reduce the surface tension of water from 72 to less than 30 mN m^{-1}. This is the most accurate method which can prove the presence of rhamnolipids in the culture based on their surface activity. However, it should be noted that in the cultivation of some bacteria, free fatty acids may produce which can also reduce the surface tension of water. So, for confirmation, the BS need to be extracted from the culture.

2.1.2. Methylene blue active substance (MBAS) assay. In order to estimate RL concentration in water, the sample pH is first adjusted to about 2 using HCl and then extracted with chloroform. The chloroform extract is put in contact with a methylene blue solution (freshly prepared) and centrifuged. Using a calibration curve, the absorbance of the chloroform phase is attributed to RL concentrations.[13] The MBAS method is useful, cheap and simple; however, the procedures are rather troublesome and time-consuming. Using chloroform in high doses is another flaw, as this compound is toxic and harmful for humans and the environment.

2.1.3. Drop collapse method. This is a general but effective method that can be used to detect the presence of biosurfactants in the culture broths. The test is performed in a microwell plate with small wells. The collapse of the droplets of the broth in the oil-coated wells can indicate the presence of RL. The large number of studies employed this test to detect RL presence in the culture.[14–16] The drop collapse assay is rapid and easy to carry out, requires no specialized equipment and just a small volume of sample. In addition, it can be performed in microplates. But it displays a relative low sensitivity since a significant concentration of surface active compounds must be present in order to cause a collapse of the aqueous drops on the oil or glass surfaces.

2.1.4. Cetyltrimethylammonium bromide (CTAB) agar test. In this method, an insoluble complex is formed by mixing the anionic RL and appear in methylene blue agar. Forming a dark blue halo around the colony in methylene blue agar can be considered as RL-producing strains. However, this method is not as reliable as other methods, and it sometimes may lead to wrong results. The CTAB agar assay is a comfortable screening method, but it is specific for anionic biosurfactants. Different culture conditions can be applied directly on the agar plates, *e.g.*, different substrates or temperature. Furthermore, it could be transferred to liquid culture conditions. The disadvantage is that CTAB is harmful and inhibits the growth of some microbes.

2.1.5. Oil spreading test (OST). The assay was developed by Morikawa *et al.*[17] For this assay, 10 μL of crude oil is added to the

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surface of 40 mL of distilled water in a Petri dish to form a thin oil layer. Then, 10 μL of culture or culture supernatant is gently placed on the center of the oil layer. The presence of biosurfactant can be proved by forming a clearing zone and the surface activity is reported by its diameter. Generally, there is a linear correlation between the quantity of pure biosurfactant and clearing zone diameter. OST is a very rapid and simple test that can show the presence of rhamnolipid in the culture of *P. aeruginosa*. However, this method cannot say anything about the type of produced biosurfactant.

2.2. Quantitative methods

2.2.1. Spectrophotometric methods. Orcinol test is a widely used methodology to determine RL concentration. In this technique, the orcinol reagent (orcinol in sulfuric acid) is prepared immediately before use. The mixture of the sample and the reagent as the reaction mixture, was well stirred, warmed, and then kept at room temperature. The absorbance, consequently, is measured and the concentration of RL is estimated by using a standard curve.[18] However, one problem with this approach is that the results may vary with the proportion of mono- to di-RLs in the culture.

2.3. Chromatographic methods

2.3.1. Thin layer chromatography (TLC). Thin layer chromatography (TLC) is a practical method to show the presence of compounds such as lipids, peptides, and carbohydrates. Briefly, in this method, chloroform is used as a solvent, and a small quantity of the RL is dissolved in and then applied on TLC plate. Once dried, the plate is developed in a solvent system (methanol : chloroform). One of the plates put into a jar saturated with iodine vapors to detect lipids as yellow spots and another plate is sprayed evenly with the anthrone reagent and placed in an oven to detect the presence of rhamnose as blue-green spots.[19]

2.3.2. High-performance liquid chromatography (HPLC). It is a reliable method for rhamnolipids analysis. C8 or C18 reverse-phase columns with a water/acetonitrile gradient are the major components in this method. Pinzon *et al.* used HPLC technique to quantify rhamnolipid species in rhamnolipid mixtures produced by *P. aeruginosa* on corn oil.[20] In another study, the mixture of two distinct rhamnolipid spots (R_f values 0.28 and 0.37) was tested for purity by HPLC. The determined retention times of the compounds (4.118 and 4.5 min) proved the presence of di-rhamnolipid and mono-rhamnolipid.[14] Using a standard solvents, in HPLC analysis, the exact structure of rhamnolipid could be determined.

2.3.3. Liquid chromatography coupled to mass spectrometry. Mass spectrometry (MS), is a powerful tool, which can provide helpful information to identify the composition of elements. One of the efficient and reliable methods to analyze the RL synthesized by *P. aeruginosa* is Liquid Chromatography Coupled to Mass Spectrometry (LC/MS). The retention time along with its mass spectral signature are two important factors used for rhamnolipid identification.[21,22] Déziel *et al.* used this method to characterization of RL produced by *P. aeruginosa* strain.[22] Furthermore, Lotfabad *et al.* have indicated that surface active agents produced by *P. aeruginosa* MR01 are RL using LC/MS of extracted biosurfactant.[23]

2.4. Spectroscopic methods

2.4.1. Fourier-transform infrared spectroscopy (FTIR). It is a useful tool for the rapid analysis of RL which indicates their functional groups. This technique is based on the IR absorption bands namely hydroxyl, ester, and carboxylic groups in rhamnolipids. Abbasi *et al.* used this method to evaluate the purified mono-rhamnolipid and di-rhamnolipid biosurfactants produced by *P. aeruginosa* MA01.[24] The test clearly indicated that the functional groups appeared in FTIR spectra were similar to those which form part of rhamnolipids and proved the presence of rhamnolipid. It should be noted that, we cannot determine the exact type of BS with FTIR method. We can just compare the functional groups with other well-known biosurfactants to estimate the structure. FTIR method should be used beside other techniques such as NMR and chromatographic methods as complementary tests.

2.4.2. Nuclear magnetic resonance (NMR). This analysis is a simple method measuring the absorption of radio frequencies for various atoms exposed to a magnetic field and can provide very detailed information on the chemical environment of atoms (the proton and 13C) within a molecule.[25] Tahzibi *et al.* used NMR method in order to test the rhamnolipids produced by a *P. aeruginosa* mutant (*P. aeruginosa* PTCC1637) and its parent strain.[26] The results of the NMR test indicated that the glycolipids produced by the *P. aeruginosa* PTCC 1637 are identical to those by wild type strain. In another study, the rhamnolipid produced by *P. aeruginosa* MR01 was characterized by NMR spectroscopy. The results clearly revealed that the sample had the molecular structure of rhamnolipid 1 (RL1) and rhamnolipid 2 (RL2) which are two major types of rhamnolipids yielded by *P. aeruginosa* species.[23] NMR itself cannot give us much information on complete structure. It should be used along with other techniques such as FTIR and chromatographic methods.

All the mentioned methods can be used in different condition based on researchers' requires for detection and analysis as some of them are suitable for structural studies and not for quantification purposes. However, using both type of methods can be helpful to provide useful information for more accurate study.

3. Fermentation condition

In recent years, there are so many studies on the production of rhamnolipid biosurfactants, their characterizations, and evaluation of different aspects of these compounds. Due to a large number of researches performed in this field, different culture compounds and conditions are employed to produce rhamnolipid. Optimization plays an important role to improve rhamnolipid production. The solubility of a carbon source, the type of feeding, pH, temperature, aeration rate, dissolved oxygen, cell density and capability for removing the product *in situ* are

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Table 1 Growth composition and condition as well as extraction method of biosurfactant in different studies

Strain	Source	Substrate and growth condition	Extraction procedure	Ref.
P. aeruginosa S6	Oil-containing wastewater	- Nutrient medium - 165 rpm - 30 °C - pH 7.5 - 48 h	- Centrifugation and acidification	98
P. aeruginosa P20	Institute of Medical Research (IMR), Malaysia	- Mineral slat medium - Carbon source: 1% (v/v) crude oil - 150 rpm - 40 °C - 7 d	- Centrifugation and solvent extraction	42
P. aeruginosa DR1	Rice rhizosphere	- Mineral slat medium with different concentrations - 30 °C - Harvesting every 24 h till 120 h	- Centrifugation, acidification, and extraction with chloroform–methanol - Column chromatography for further purification	99
P. aeruginosa MA01	Spoiled apples	- Culture medium (g L^{-1}): $NaNO_3$ 3.0, KH_2PO_4 0.25, $MgSO_4 \cdot 7H_2O$ 0.25, yeast extract 1.0 and soybean oil 10 - 200 rpm - 30 °C - 7–10 d	- Acid precipitation and solvent extraction method	24
P. aeruginosa LBI	Petroleum-contaminated soil	- Mineral slat medium - 30 °C - 86 h	- Centrifugation and absorption chromatographic column filled with a polystyrene resin	100
P. aeruginosa M14808	High magneto-gravitational environment	- Culture medium (g L^{-1}): $NaNO_3$ 3.0, KH_2PO_4 2.0, K_2HPO_4 1.0, $MgSO_4 \cdot 7H_2O$ 0.50, KCl 0.1, $CaCl_2 \cdot 2H_2O$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.01, yeast extract 0.01, vegetable oil 40, 0.05 mL trace element solution containing - 220 rpm - 30 °C - pH 7.0 ± 0.2 - 7 d	- Centrifugation and solvent extraction using chloroform–methanol (2 : 1, v/v)	101
P. aeruginosa ATCC 9027	American Type Culture Collection	- PPGAS medium - 250 rpm - 37 °C - pH 7.2	- Centrifugation, acidification, and extraction with chloroform–ethanol (2 : 1) three times	102
P. aeruginosa #112	Crude oil sample obtained from a Brazilian oil field	- Different culture media containing corn steep liquor (10%, v/v) and sugarcane molasses (10%, w/v), supplemented with olive mill wastewater at concentrations between 5% and 25% (v/v) - 180 rpm - 37 °C - pH 7.0	- Centrifugation and adsorption chromatography	103
P. aeruginosa strain-PP2	Soil contaminated with lube oil and distillery spent wash	- Crude whey - 150 rpm - 30 °C - pH 7.0 - 96 h	- Centrifugation	104
P. aeruginosa PA1	NM[a]	- Culture medium (g L^{-1}): $NaNO_3$ 1.0, KH_2PO_4 3.0, K_2HPO_4 7.0, $MgSO_4 \cdot 7H_2O$ 0.2, 0.5% yeast extract, peptone 0.5%, and 3% glycerol - 170 rpm - 30 °C - 168 h	- Centrifugation, using reverse osmosis process, and purification using purifying using a chloroform/methanol/culture medium mixture	105

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Table 1 (*Contd.*)

Strain	Source	Substrate and growth condition	Extraction procedure	Ref.
P. aeruginosa BYK-2 KCTC 18012P	The southern sea of Korea	- Basal salts medium - Fish oil and urea as the carbon source and nitrogen source - 180 rpm - 25 °C - 40 h	- Centrifugation	48
P. aeruginosa HAK02	Urban wastes of the Kahrizak site in the south of Tehran	- Culture medium (g L^{-1}): sunflower oil (Nina, a local company) 20, $NaNO_3$ (Merck, C 99.5%) 5, KH_2PO_4 (Merck, 99.5–100.5%) 0.2, and $MgSO_4 \cdot 7H_2O$ (Merck, C 98.0%) 0.2 - 180 rpm - 30 °C - 24 h	- Centrifugation, acidification, and extraction using ethyl acetate (99.5%)	4
P. aeruginosa SP4	Petroleum-contaminated soil in Thailand	- Culture medium (g L^{-1}): $NaNO_3$ 0.5, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, KCl 0.1, and $FeSO_4 \cdot 7H_2O$ 0.01	- Centrifugation, acidification, and extraction using a solvent (2 : 1 CH_3Cl–C_2H_5OH)	106
P. aeruginosa SP4	Petroleum-contaminated soil in Thailand	- Mineral medium + palm oil - 200 rpm - 37 °C - 22 h	NM	107
P. aeruginosa CPCL (GQ241355)	A petroleum contaminated site located in Chennai	- Mineral medium - pH 7.0 ± 0.2 - 48 h	- Acidification, extraction using $CHCl_3$: CH_3OH (2 : 1), and concentrating by a rotary evaporator	75
P. fluorescens PMMD3	The biofilm formed on metal coupons at Ennore port, Chennai (India)	- Minimal salt medium - Paraffin as carbon source - 35 °C	- Acidification and extraction using equal volume of chloroform and ethanol (2 : 1) mixture	108
P. aeruginosa CPCL	A petroleum-contaminated soil, Chennai (India)	- 180 rpm - 1 month under aerobic condition		
P. aeruginosa O-2-2	The Ocean University of China	- Culture medium (g L^{-1}): soybean oil 80, KH_2PO_4 4.0, K_2HPO_4 6.0, $NaNO_3$ 3.0, NaCl 1.1, KCl 1.1, $MgSO_4 \cdot 7H_2O$ 0.2, anhydrous $CaCl_2$ 0.2, anti-foam 1 mL L^{-1}, trace elements solution 5 mL L^{-1} - 180, 350 and 500 rpm - 30 °C - pH 7.0 - 96 h	- Centrifugation, acidification, and extraction using an equal volume of $CHCl_3/CH_3OH$ (2 : 1) - Concentrating by a rotary evaporator	29
P. cepacia CCT669	The culture collection of the André Tosello Research and technology Foundation in the city of Campinas	- Mineral medium - 200 rpm - 27 °C - pH 7 - 120 h	- Centrifugation, acidification - Concentrating by a rotary evaporator	109
P. aeruginosa ATCC 10145	NM	- Liquid medium - Hexadecane (2% v/v) as the carbon source - 300 rpm (Shaker) - 600 rpm (fermenter) - 28 °C	- Centrifugation, acidification, and extraction using hexane	110
P. aeruginosa 57RP	Hydrocarbon contaminated soil	- Iron-limited mineral salts medium (MSM) supplemented with 2% (w/v) mannitol - 200 rpm - 30 °C	- Centrifugation and filtration - Adding an internal standard (hydroxyhexadecanoic acid)	111
P. aeruginosa ICP70	Oily sludge	- Culture medium (per dm^{-3} of drinking water): glycerol, 30.5 cm^3; $MgSO_4$, 0.1 g; K_2HPO_4, 7 g; KH_2PO_4, 3 g; $(NH_4)_2SO_4$ - 140 rpm - 305 K - pH 6.5–7.0	- Thioglycolic acid method	112

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Table 1 (*Contd.*)

Strain	Source	Substrate and growth condition	Extraction procedure	Ref.
P. aeruginosa ATCC 9027	American Type Culture Collection	- Mineral salts medium - 200 rpm - 37 °C - pH 7.0 - 96 h	- Centrifugation, acidification, and extraction using 9 : 1 ratio of chloroform to methanol - Concentrating by rotoevaporation - Chromatography	113
P. aeruginosa 6k11	Soil contaminated with crude-oil (Talara, Peru)	- Mineral salt medium - 140 rpm - 37 °C - pH 6.8 - 250 hours	- Centrifugation, acidification, and extraction using ethanol and chloroform	34
P. aeruginosa PAO1	NM	- BM2 minimal medium - 170 rpm - 37 °C - 6 and 24 h	- Solvent extraction - Freezing and using subsequent phase separation	36
P. aeruginosa PA1	Brazilian petroleum exploring environment	- Culture medium (g L^{-1}): $NaNO_3$ 1.0, KH_2PO_4 3.0, K_2HPO_4 0.7, $MgSO_4 \cdot 7H_2O$ 0.2, yeast extract 5.0, peptone 0.5, and glycerol 30 - 170 rpm - 30 °C - pH 7.0 - 24 h	- Centrifugation and acidification	114
P. SWP-4	WCO-contaminated sludge samples	- Culture medium (g L^{-1}): NH_4NO_3 2, NaCl 5, KH_2PO_4 1, K_2HPO_4 1, $MgSO_4 \cdot 7H_2O$ 0.3, $FeSO_4 \cdot 7H_2O$ 0.1, $CaCl_2$ 0.1, and WCO 20 - 150 rpm - 35 °C - 1 d	- Centrifugation, acidification, and extraction using ethyl acetate	115
P. aeruginosa PAO1	M. Foglino, Marseille, France	- PPGAS medium - 37 °C - pH 7.2	- Identifying and quantifying rhamnolipids using LC-MS	35
P. aeruginosa DN1	Petroleum contaminated soil	- BPLM supplemented with palm oil as the carbon source and sodium nitrate as the nitrogen source - 7 d	- Centrifugation, acidification, and extraction using ice-cold 2 : 1 chloroform and methanol ethyl acetate	116
P. aeruginosa ATCC 9027	The ATCC collection	- Mineral base - Oleic acid as the carbon source - Sodium nitrate as the nitrogen sources - Phosphoric acid as the phosphorus sources - 150 rpm - 30 °C - 24 h	- Centrifugation and acidification - Adsorption chromatography	117
P. aeruginosa NITT 6L	NM	- Culture medium (g L^{-1}): glucose 40, sodium nitrate 3.5, magnesium sulphate 0.2, $FeSO_4$ 0.003, K_2HPO_4 5, NaCl 0.1. - 200 rpm - 37 °C - pH 7.0 - 144 h	- Solvent extraction using chloroform : methanol (2 : 1 v/v)	118
P. aeruginosa ATCC 9027	American Type Culture Collection	- Culture medium (g L^{-1}): $MgSO_4$ 0.2, NaCl 1, KCl 1, $CaCl_2$ 0.04, as well as corn oil 4.5% (v/v), H_3PO_4 (85%) (5 mL L^{-1}), and 1 mL L^{-1} of trace element solution - 260 rpm - pH 7.0	- Centrifugation	119

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Strain	Source	Substrate and growth condition	Extraction procedure	Ref.
P. aeruginosa PAO1		- 37 °C - 6 d (batch) and 10 d (fed-batch)		
P. aeruginosa AB93066	NM	- Culture medium (g L^{-1}): COFCs 40, $NaNO_3$ 6, $Na_2HPO_4 \cdot 12H_2O$ 1, KH_2PO_4 1, $FeSO_4 \cdot 7H_2O$, 0.01 and $MgSO_4 \cdot 7H_2O$ 0.1 - pH 7.0	- Centrifugation and acidification - Collecting crude RLs by vacuum evaporation	120
P. aeruginosa SG	NM	- Culture medium (g L^{-1}): crude glycerol 60, KH_2PO_4 3.4, $K_2HPO_4 \cdot 3H_2O$ 4.0, $MgSO_4 \cdot 7H_2O$ 0.8, $NaNO_3$ 3.5, KCl 0.5, $CaCl_2$ 0.05, NaCl 0.5 - 200 rpm - pH 6.8 - 37 °C	- Centrifugation, acidification, and extraction using 2 : 1 chloroform/methanol (v/v) - Drying by vacuum rotary evaporation	33
P. aeruginosa E03-40	Soil samples near a biodiesel plant	- Culture medium (g L^{-1}): vegetable oil 100, NH_4Cl 5.72, KH_2PO_4 6.0, NaCl 1.5, $MgSO_4 \cdot 7H_2O$ 0.9, $FeSO_4 \cdot 7H_2O$ 0.1, $CaCl_2 \cdot 2H_2O$ 0.03, $MnCl_2 \cdot 4H_2O$ 0.03, yeast extract 5.0, peptone 5.0, and 2 mL of a trace element solution - 800 rpm - 32 °C - pH 7.0	- Centrifugation and acidification	43
P. aeruginosa MA01	NM	- Culture medium (g L^{-1}): sun flower oil 20, yeast extract 1.0, $NaNO_3$ 3.0, $MgSO_4 \cdot 7H_2O$ 0.25 and KH_2PO_4 0.25 - 200 rpm - 30 °C - 5–6 d	- Solvent extraction - Column chromatography	1
P. aeruginosa (ATCC 10145)	American Type Culture Collection	- Culture medium: 30 to 50% olive oil mill wastewater or whey - 100 or 200 rpm - 30 or 37 °C - PH 7.0 - 96 h	- Centrifugation, acidification, and extraction using ethyl acetate	121
P. aeruginosa ATCC 15692	American Type Culture Collection	- Culture medium (g L^{-1}): $NaNO_3$ 8.0, NaCl 1.0, KCl 1.0, $MgSO_4$ 0.25, $CaCl_2 \cdot 2H_2O$ 0.05, and H_3PO_4 (85%) 5 mL L^{-1}, corn oil 7.5% (v/v) as well as 1 mL L^{-1} of a trace element solution - 12% (v/v) of corn oil (batch culture) and 3% (v/v) of oil every 3 d after 5 d culture (fed-batch fermentation) - 240 rpm - 37 °C - pH 7.0 - 17–20 d	- Centrifugation	44
P. aeruginosa USM AR2	A local crude oil sample	- Culture medium: 0.6% (w/v) yeast extract, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.05% (v/v) Tween 80, and 30 mL diesel oil - pH 5.0 - 27 °C	- Centrifugation	122

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Table 1 (*Contd.*)

Strain	Source	Substrate and growth condition	Extraction procedure	Ref.
P. sp. MIS38	NM	- L broth: 1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl - 27 °C - pH 7.2 - 40 h	- Centrifugation - Concentrating by ultra-filtration - Extraction using an equal volume of hexane	17
P. aeruginosa 57RP	A hydrocarbon-contaminated soil	- Iron-limited mineral salts medium supplemented with 2% (w/v) mannitol - 150 rpm - 30 °C - pH 6.7 - 359 h	- Centrifugation and filtration	21
P. aeruginosa 47T2 NCIB 40044	Oil contaminated soil sample	- Culture medium (g L^{-1}): $NaNO_3$ 5, KH_2PO_4 2.0, K_2HPO_4 1.0, KCl 0.1, $MgSO_4 \cdot 7H_2O$ 0.5, $CaCl_2$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.012, yeast extract 0.01 and 0.05 mL of a trace element solution - 150 rpm - 30 °C - pH - 96 h	- Centrifugation - Adsorption chromatography	73
P. aeruginosa strain ZJU211 (CCTCC M209237)	A heavily oil-contaminated soil	- Culture medium (g L^{-1}): $NaNO_3$ 10.0, NaCl 1.0, KCl 1.0, $CaCl_2 \cdot 2H_2O$ 0.1, KH_2PO_4 6.5, $Na_2HPO_4 \cdot 12H_2O$ 11.0, $MgSO_4$ 0.25, and 2 mL of a trace element as well as crude oil (from the Shengli oil field) 0.4% (w/v), - 300 rpm - 37 °C - 96 h	- Acidification, centrifugation, and extraction by chloroform : methanol (2 : 1, v/v)	79
P. aeruginosa 47T2 NCIB 40044	An oil-contaminated soil sample	- Medium with the following composition (g L^{-1}): $NaNO_3$ 5, KH_2PO_4 2.0, K_2HPO_4 1.0, KCl 0.1, $MgSO_4 \cdot 7H_2O$ 0.5, $CaCl_2$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.012, yeast extract 0.01, and 0.05 mL of a trace elements solution containing (g L^{-1}): H_3BO_3 0.26, $CuSO_4 \cdot 5H_2O$ 0.5, $MnSO_4 \cdot H_2O$ 0.5, $MoNa_2O_4 \cdot 2H_2O$ 0.06, $ZnSO_4 \cdot 7H_2O$ 0.7 - 150 rpm - 30 °C - pH 7.2	- Centrifugation - Adsorption chromatography	82

[a] NM: not mention.

the essential factors for setting up an efficient fermentation condition as these parameters can significantly impact on the rhamnolipid yield.[27]

Researchers have always tried to improve rhamnolipid production by optimization routes. The parameters of fermentation such as the type of feeding of substrates, pH, temperature, aeration rate, dissolved oxygen, cell density and capability for removal the product *in situ*, are essential prerequisites for setting up an efficient fermentation condition as these parameters can significantly impact on the rhamnolipid yield.[28,29]

One of the important factors increasing the rhamnolipid yield is the solubility of carbon source in the culture media. For instance, palm oil and diesel, the insoluble carbon sources, generally produce more rhamnolipids in comparison with water-soluble carbon sources (*e.g.* glucose).[30] Another important parameter is substrate feeding profile.[29] To achieve the optimal microbial growth in lag and growth phase, the pH of the culture

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Table 2 Rhamnolipid production yield from different *P. aeruginosa* strains as well as surface tension (ST) and critical micelle concentration (CMC)

Microorganism	Fermentation mode	Min. ST[a] (mN m^{-1})	CMC (mg L^{-1})	Max. yield (g L^{-1})	Ref.
P. aeruginosa S6	Batch	33.9	50	0.18	98
P. aeruginosa (P20)	Batch	—	—	7.5	42
P. aeruginosa DR1	Batch	30	80	2.8	99
P. aeruginosa MA01	Batch	32.5	10.1	12	24
P. aeruginosa LBI	Batch	24	120	15.8	100
P. aeruginosa S2	Fed-batch	30	—	9.4	49
P. aeruginosa ATCC 9027	Batch	30	40	0.3	123
P. aeruginosa #112	Batch	30	13	5.1	103
P. aeruginosa PA1	Fed-batch	—	—	16.9	124
P. aeruginosa HR	Batch	20	19	4.2	125
P. aeruginosa PA1	Batch	27	25.7	—	105
P. aeruginosa MR01	Batch	28	—	1.4	126
P. aeruginosa SP4	Batch	28–30	150	—	106
P. aeruginosa RS29	Fed-batch	26.3	90	0.80	127
P. aeruginosa SP4	Batch	28–30	120	0.126	107
P. aeruginosa KVD-HR42	Batch	30.14	100	5.09 ± 2.1	128
P. aeruginosa	Batch	19	25–30	16–17	108
P. aeruginosa O-2-2	Fed-batch	—	—	70.56	29
P. aeruginosa AT10	Batch	—	—	18.7	129
P. cepacian CCT6659	Batch	27.57	—	—	109
P. aeruginosa DSM2659	Batch	29	—	1.5	130
P. aeruginosa HR	Batch	20	19	4.2	131
P. aeruginosa ATCC 9027	Batch	—	—	2.6 ± 0.26	113
P. aeruginosa DS10-129	Batch	27.5	10	—	113
P. aeruginosa 6K11	Batch	—	—	3.2904	34
P. aeruginosa (P20)	Batch	—	—	7.5	42
P. aeruginosa DN1	Batch	25.88	50	25.9	116
P. SWP-4	Batch	24.1	27	13.93	115
P. aeruginosa ATCC 9027	Batch	29	30 mg L^{-1}	0.9	132
P. aeruginosa IFO 3924	Batch	—	—	32	133
P. aeruginosa ATCC 9027	Batch	—	—	4.261	134
P. aeruginosa NITT 6L	Batch	27.5	11	7.65	118
P. aeruginosa ATCC 9027	Fed-batch	—	—	43.3	119
	Batch	—	—	61.2	
P. aeruginosa AB93066	Batch	25.3	45	—	120
P. aeruginosa SG	Batch	27.2	60	1.98	33
P. aeruginosa PrhlAB	Batch	≤ 30	80	2.87	
P. stutzeri Rhl	Batch	≤ 30	90	0.87	
P. sp. SWP-4	Batch	22.7	—	6.87	86
P. aeruginosa PA1	Batch	30	60	13.2	85
P. aeruginosa PAO1	Batch	30	—	—	76
P. aeruginosa HAK02	Fed-batch	30	500	240	4
P. aeruginosa HAK02	Batch	30	500	22.5	4
P. aeruginosa ATCC 15692TM	Fed-batch	28	30	150	44
P. aeruginosa YPJ-80	Batch	—	—	4.4	47
P. aeruginosa MTCC 2297	Batch	24.02	—	1.975 ± 0.007	14
P. aeruginosa 47T2 NCIB 400044	Batch	32.8	108	8.1	82
P. aeruginosa FIN2	Batch	28.6	195	—	135
P. aeruginosa EBN-8	Batch	28.5	—	8.5	56

[a] The second value is the surface tension of water in the defined condition of the experiments.

media should be adjusted around 7–7.5. In the stationery and death phase of fermentation, the slight acidity (pH 6–6.5) can effectively increase rhamnolipid production. The level of dissolved oxygen is also an effective parameter which leads to higher biosurfactant production.[31]

According to Table 1, medium and culture conditions are different for each strain. Table 2 also shows rhamnolipid production yield from different *P. aeruginosa* strains as well as surface tension and CMC values. Generally, 37 °C is the optimum temperature for *P. aeruginosa* growth; however, they are able to survive at the temperature between 4 °C and 42 °C. They can be stored at a temperature of 4 °C within a week. Moreover, some carbon sources along with nitrate as the

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terminal electron acceptor could be utilized as an anaerobic media for *P. aeruginosa*.[32]

Regarding culture media, LB broth also shows its ability in *P. aeruginosa* growth. A well-growing culture medium, which can be modified to study its impact on *P. aeruginosa* growth and virulence, includes crucial ingredients such as carbon and nitrogen sources.[32]

Zhao *et al.* have studied on three different rhamnolipid producers namely *P. aeruginosa* SG (the wild-type strain) and two recombinant strains, *P. aeruginosa* PrhlAB and *P. stutzeri* Rhl.[33] The growth condition for all three strains was the same (37 °C, 200 rpm for 5 days) and crude glycerol, KH_2PO_4, $K_2HPO_4 \cdot 3H_2O$, $MgSO_4 \cdot 7H_2O$, $NaNO_3$, KCl, $CaCl_2$ and NaCl were the main compounds in the medium. In another study, Hospinal *et al.* worked on *P. aeruginosa* 6k11 isolated from soil contaminated with crude-oil and inoculated in mineral salt medium (MSM).[34] Furthermore, *P. aeruginosa* PAO1 (obtained from M. Foglino, Marseille, France) grown in PPGAS medium employed LC-MS to identify and quantify rhamnolipid from culture supernatants.[35] Schmidberger *et al.* used BM2 minimal medium for *P. aeruginosa* PAO1.[36] In this study, the effects of two changes on gene expression and rhamnolipid production were investigated: adding an extra amount of iron ions (Fe^{3+}) as well as omitting them from BM2 medium and comparing with standard BM2.

Overly, the components of the substrates used for RL production can play an important role in increasing production yield. As carbon is the major component in deriving RLs, using low-cost waste containing sugar as the carbon source such as agricultural residues, whey products, *etc.* can be useful in reducing the cost of production. Also, it is noted that purification process is easier when using sugar (either commercial or waste containing sugar) as the carbon source. Proteins, amino acids, and lipids are also important in RL production which should be considered. Other factors such as type of strain, growth condition, feeding profile, pH, temperature, aeration rate, dissolve oxygen, and fermentation strategy should be optimized to explore the best procedure and suitable condition for industrial production.

4. Extraction and purification

Downstream processing plays an important role to determine the production cost of bioproducts, especially for those with high yields. It often allocates about 60–80% of the overall manufacturing costs. In addition, the difficulty of product extraction leads to the selection of the purification method which is mostly affected by the ionic charge and the metabolite types (intracellular or extracellular).[28,37] Thus, the commercialization of the products necessarily needs the economic downstream producer.[38] The compounds used in the fermentation broth namely salts, amino acids, proteins and *etc.* and their complexity are the main factors in the rhamnolipid purification (downstream process).[28] Table 3 shows extraction methods using in different research works for rhamnolipid production by *P. aeruginosa*.

A few recovery methods for purification of rhamnolipids has been reported in recent years. Foam fraction, adsorption chromatography, ultrafiltration, and ion exchange chromatography are important strategies for rhamnolipid purification.[38] Precipitation with acid or ammonium sulfate is the most common method for purification. This technique is commonly followed by centrifugation and solvent extraction.[39] Anion exchange chromatography is another technique measuring the negative charge of the rhamnolipids at high pH. It is superior to acid precipitation method due to the lower losses.[40] However, having the extracted mixture of rhamnolipid and some fatty acids is the main disadvantage of anion exchange chromatography.[39] In addition, foam fraction is a type of downstream processing method for RLs purification[41] used foam fraction in order to extract surfactin produced from fermentation broth. Shah *et al.* compared four different downstream recovery methods including acid precipitation, zinc sulfate precipitation, ammonium sulfate precipitation, and solvent extraction.[42] Based on the results, the best method for rhamnolipid purification with the highest yield could be solvent extraction. Beuker *et al.* have studied the foam fraction method to purify the rhamnolipids driven from *P. putida* and the highly concentrated rhamnolipids were extracted from fermentation broth.[39]

Another downstream process method is adsorption chromatography developed for rhamnolipids purification and separation. In this method, the purification of rhamnolipid is carried out with normal phase resin and nonpolar solvents. It is claimed that the highest purification of rhamnolipids can be obtained being appropriate for food and medical applications. Moreover, purified rhamnolipids show noticeable antibacterial activity and can be applied in the formulations of cosmetics and skin care products.[37] Invally *et al.* extracted rhamnolipid from fermentation broth using different unit operations.[43] First, ethanol precipitation was used to remove biopolymers, followed by acid precipitation method. The separated rhamnolipids, then, were dissolved in neutral aqueous solution. Finally, calcium precipitation was used to enhance the purity of the product and remove residual impurities. It was shown that the percentage of purification reached around 87% by using this sequence. Moreover, this method has been reported as an eco-friendly technique.

5. Fermentation strategies

Batch, fed-batch and continuous modes are three main strategies for the fermentation process. In the batch cultivation process, all the nutrients required for growth of bacteria and production of desired metabolites are added to the culture medium before cultivation is started, and the product is only discharged from the fermenter at the end of the process. In the continuous microbial fermentation method, all the nutrients are continuously added to the fermenter and the components of the culture medium are removed from the fermenter at the same time in order to maintain a constant culture volume. Fed-batch is clearly similar to semi-batch. This technique is one of the most effective ways to enhance rhamnolipid productivity and yield by feeding more nutrients added to the fermenter

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during fermentation process.[4,44,45] The fed-batch process is completely different. In this method, fresh culture medium and substrate are fed to the fermenter without removing the rhamnolipids produced in the fermenter. Therefore, efficient feeding strategy results in a significant increase in rhamnolipid production.[28] Generally, there are two main types of feeding strategy including feedback control mechanism and without feedback control mechanism. A constant and increasing feeding rate are not involving feedback control. In the former, the rate of nutrient feed is constant during the fermentation, therefore, the specific growth rate drops with time by increasing nutrient consumption. In the latter, the specific growth rate is constant during the fermentation due to continuous feeding based on calculating the required feeding rate. In contrast, DO-stat and pH-stat cultivations are fed-batch processes with feedback control.[46] A number of studies have been done to improve rhamnolipid production by different feeding strategies. However, there are a few works studied on the production of rhamnolipid biosurfactant in fed-batch mode. Table 4 shows fermentation modes as well as rhamnolipid production yield from different *P. aeruginosa* strains in different literatures. In one study, the yield of rhamnolipid derived from *P. aeruginosa* YPJ-80 by pH-stat fed-batch has reached reported 4.4 g L^{-1}.[47] Lee *et al.* reported the rhamnolipid concentration of 22.7 g L^{-1} by fed-batch cultivation of *P. aeruginosa* BYK-2 KCTC 18012P with feeding fish oil as a carbon source.[48] In another study, a pH-stat feeding strategy was investigated and the maximum yield of rhamnolipid reached about 6 g L^{-1}.[49] Zhu *et al.* claimed yield of 70 g L^{-1} by pH-stat controlled fed-batch cultivation of *P. aeruginosa* O-2-2.[29] Additionally, Invally *et al.* showed that *P. aeruginosa* E03-40 could produce 55.7 ± 2.6 g L^{-1} rhamnolipids in fed-batch cultivation by using vegetable oil as a carbon source.[43] Bazsefidpar *et al.* reported the highest overall rhamnolipid production of 240 g L^{-1} with the productivity of 0.9 (g L^{-1} h^{-1}) by feeding sunflower oil under tight DO control which showed 4.8-fold improvement compared to the batch cultivation.[4] This is the highest rhamnolipid concentration has been ever reported from *P. aeruginosa* fermentation without genetic manipulation.

In conclusion, an excellent fermentation strategy is critical factor to reduce the cost of production and increase productivity. The knowledge about metabolic pathway can help in selecting the best type of fermentation. In fed-batch fermentation method, the substrate inhibition is controlled, so developing fed-batch fermentation can effectively improve the RL yield as kinetic model for substrate utilization shows. In addition, the effect of nutrient concentration on yield and productivity in fed-batch cultivation is more than batch fermentation process. Type of feeding strategy also depends on the bacterial strains and desired metabolites.

6. Bioengineering

One of the efficient approaches for increasing biosurfactants production is bioengineering. In this context, two main strategies have been targeted to increase rhamnolipids production: (a) genetic engineering and (b) random mutagenesis. Genetic engineering has been widely used to generate a large number of

Table 3 Extraction methods which used in rhamnolipid production by *P. aeruginosa*

Downstream process method	Biosurfactant feature for separation	Advantages	Ref.
Acid precipitation	Insolubility at low pH values	- Low cost - Effective in the recovery of rhamnolipids	38 and 42
Centrifugation	Precipitating due to the centrifugal force	- Efficient in the recovery of crude rhamnolipids - Reusable	38
Ammonium sulfate precipitation	Salting-out of the polymeric or protein rich biosurfactant	- Efficient in polymeric biosurfactants	136
Organic solvent extraction	Dissolving in organic solvents due to the hydrophobic ends	- Effective in the recovery of biosurfactants - Reusable nature	136
Ion exchange chromatography	—	- High purity - Reusability - Fast recovery	136
Adsorption on wood active carbon	Absorption capability with organic solvents	- High pure biosurfactant - Reusable - Capability to recover from continues culture	136
Ultrafiltration	Forming aggregates above the CMC	- Inexpensive - High purity of biosurfactant	137
Foam fractionation	Ability to form foam due to surface activity are able	- Continues recovery from fermentation	138
Adsorption chromatography	Adsorption capability of crude rhamnolipids on normal phase resin	- High quality purified rhamnolipid - Economic method - Low solvents for purification	37

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modified strains, involving in gene expression. Random mutagenesis mostly is generated by UV-irradiation to randomly create fundamental changes in different *Pseudomonas aeruginosa* strains and do not focus on biosynthetic enzymes or genes. Table 5 shows the summary.

6.1. Genetic engineering for enhanced production of rhamnolipids

Genetic engineering or genetic modification is a manipulation of the selected organism's genome by employing biotechnological tools. In genetic engineering, alteration of genetic makeup is being performed through the transfer of genes across and within various species to develop improved or desired organism with a particular trait. The organism developed through genetic manipulation is considered as genetically modified and known as a genetically modified organism (GMO).

In the last few years, many researchers worked on the metabolic engineering strategy to increase the production rate of rhamnolipids.[50] On the other hand, understanding of the biosynthesis and genetic regulation systems of rhamnolipid production may help in the development of mutant strains with increased ability to produce rhamnolipids.[28] The use of metabolic engineering tools may enable the development of bioprocesses that provide the necessary conditions for optimal synthesis of biosurfactants.[51]

3-(Hydroxyalkanoyloxy)alkanoic acid (HAA) and dTDP-l-rhamnose are two important precursors to rhamnolipid biosynthesize which the former is synthesized from β-hydroxydecanoyl-ACP. Due to the similarity of the biosynthesis pathway for both β-hydroxydecanoyl-ACP and dTDP-l-rhamnose in a large number of bacteria, the recombination of strains can be possible, and metabolic engineering can pave the way for constructing non-pathogenic recombinant strains by using different types of genes which are important in the biosynthesis of rhamnolipids. By introducing rhlA, rhlB, and rhlC into the target recombinant strains, both mono- and di-rhamnolipids can be produced.[28]

Cabrera-Valladares *et al.* worked on the production of the mono-rhamnolipids using biosynthesis methods.[52] They used HAAs in a recombinant *Escherichia coli* strain and expressed it into *P. aeruginosa* rhlAB operon. This technique, accordingly, lead to a noticeable increase in rhamnolipid yield. In another study, Cha *et al.* have studied on the replacement of pathogen strains (*P. aeruginosa*) in a heterologous host (*Pseudomonas putida*) in order to produce a safe industrial strain.[53] They used bioengineering techniques as well as the colonized rhlAB rhamnosyltransferase genes and the rhlRI quorum sensing system to produce mono-rhamnolipid in *P. putida* and the rhamnosyltransferase acted as a catalyzer. As the results showed, using this method lead to increase the rhamnolipid yield from 5.18 g L^{-1} (produced by *P. aeruginosa* EMS1) to 6.97 g L^{-1} (produced by *P. putida* 1067 (pNE2)). It should be noted that the rhamnolipid production from non-pathogen *P. putida* is ecologically more feasible than from pathogen *P. aeruginosa*

Table 4 Fermentation methods using in rhamnolipid production

Strain	Maximum yield (g L^{-1})	Substrate	Feeding strategy	Downstream extraction producer	Ref.
P. aeruginosa YPJ-80	4.4	Glucose	Fed-batch-pH-stat	—	47
P. aeruginosa BYK-2 KCTC 18012P	22.7	Fish oil	Fed-batch	Rosenberg method	48
P. aeruginosa S2	6.06	Glucose	Fed-batch-pH-stat	Acid precipitation Solvent extraction	49
P. aeruginosa USM-AR2	2.61	Diesel	Batch	Optimal density (OD_{540})	30
	18.9	Diesel	Fed-batch-pulse-pause feeding of diesel		
	23.6	Diesel	Fed-batch-MSUR-based feeding of diesel		
P. aeruginosa USM-AR2	2.35	Diesel	Batch	Optimal density (OD_{421})	139
	3.13	Diesel	Fed-batch-plus feeding of carbon source		
P. aeruginosa (ATCC 53752)	0.7	Glycerol	Batch	Phenol-sulfuric acid method	140
	4.12	Glycerol	Fed-batch- feeding of glycerol		
P. aeruginosa	55	Soybean oil	Fed-batch- feeding of medium and substrate	Acid precipitation Solvent extraction	141
P. aeruginosa O-2-2	24.06	Soybean oil	Batch	Acid precipitation Solvent extraction	29
	70.56	Soybean oil	Fed-batch-pH-stage-controlled		
P. aeruginosa ATCC 15692	150	Soybean oil	Sequential fed-batch	Acid precipitation Solvent extraction	44
P. aeruginosa E03-40	55.7 ± 2.6	Vegetable oil	Fed-batch- feeding of vegetable oil	Ethanol precipitation Acid precipitation Calcium precipitation	43
P. aeruginosa HAK02	22.5	Sunflower oil	Batch	Acid precipitation	4
	240	Sunflower oil	Fed-batch cultivation under tight DO control	Solvent extraction	

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Table 5 Rhamnolipid production yield using mutation methods

Microorganism	Host microorganism	Mutation method	Primary yield (g L^{-1})	Final yield (g L^{-1})	Ref.
P. aeruginosa PAO1	*E. coli*	Genetic engineering	0.227	0.121	52
P. aeruginosa EMS1	*P. putida* 1067	Genetic engineering	5	6.97	53
P. aeruginosa SQ6	*P. stutzeri* Rhl	Genetic engineering	3.12 ± 0.11	4.37 ± 0.14	142
P. aeruginosa 65E12	*E. coli*	Genetic engineering	<0.1	0.85	143
P. aeruginosa (ATCC 10145)	—	Genetic engineering	9.6	13.3	121
P. aeruginosa SG	*P. aeruginosa* PrhlAB *P. stutzeri* Rhl	Genetic engineering	1.98	2.87 0.87	25
P. aeruginosa EBN-8	—	Random mutagenesis	—	8.50	56
P. aeruginosa	—	Random mutagenesis	—	70–120 g L^{-1}	58
P. aeruginosa MM1011	*P. aeruginosa* PTCC1637	Random mutagenesis	1.2	12.5	26

and they are environmentally acceptable as they are not contaminated with toxins and pigments. The introduction of estA into *P. aeruginosa* PAO1 proposed by Wilhelm *et al.* cause an increase in rhamnolipid production.[54]

Similarly, the overexpression of rhlAB in *B. kururiensis* resulted in a noticeable increase in rhamnolipid production (0.78 to 5.76 g L^{-1}). Likewise, by overexpressing rhlC in *P. chlororaphis*, it could produce both mono- and di-rhamnolipid while without using biosynthesis method, the strain was only able to produce mono-rhamnolipid.[55]

6.2. Improving rhamnolipid yield through random mutagenesis

The random mutagenesis technology which has been widely used to enhance microbial production is divided into chemical or radiation treatment. Through this technique, the rhamnolipid yield can be improved, however, it may lessen its productivity after some time.[28] By subjecting the parent strain (*P. aeruginosa* S8) to the best gamma radiation dose, its mutant (*P. aeruginosa* EBN-8) was obtained which showed a better growth on oil refinery wastes and produced 8.5 g L^{-1} rhamnolipids.[56] UV mutagenesis is another type of random mutagenesis technology being able to produce mutants and enhance rhamnolipid production. Husain *et al.* used this method to obtain *P. fluorescens* 29L which could produce mutants on pyrene and pyrene metabolism by *P. fluorescens* 29L is dependent on biosurfactants.[57] In another study, mutagenesis of *P. aeruginosa* by using chemical mutagen could increase the rhamnolipid production from 70 to 120 g L^{-1}.[58] Dobler *et al.* used UV-radiation mutagenesis on a sample driven from soil. As a result, the generated mutant could produce rhamnolipid more than the parent strain.[59]

As a conclusion, although both genetic engineering and random mutagenesis, have been improved the rhamnolipid production, generally there is not a significant change in production. These methods should be employed along with

Table 6 Reported applications for rhamnolipid in literature

Application	Example	Ref.
Bioremediation	Desorption of contaminants from soil	83 and 84
	Impacts on microbial adhesion/microbial mobility	83
	Bioremediation of petroleum	71, 85, 144 and 145
	Bioremediation of pesticides at agricultural fields	83, 146 and 147
	Remediation of oil-contaminated water	148
Pest control	Enhancing the pesticide and agrochemical solubility	83, 147 and 149
	Control plant diseases	78–80 and 150
Oil recovery	Microbial enhanced oil recovery (MEOR)	83, 86 and 151
	Increase amount of recoverable oil aided by rhamnolipid	86, 87 and 149
	Microbial de-emulsification of oil emulsions	71 and 88
	Oil-processing operations	83
Medical use	Low toxicity, biocompatibility and digestibility	71, 74 and 89
	Prevent biofilm formation	76, 149 and 152
	Anticancer agents	149 and 152
Food processing	Improvement in the stability of dough, volume, texture and conservation	72, 153 and 154
	As antimicrobial agent preventing food spoilage	67–71 and 73
Mining processing	Enhanced metal extraction from the mining	71 and 83
Nanoparticles	Nanoparticle synthesis using microemulsion method	91–96 and 155
	Drug delivery	97
UF membranes cleaning	Great potential in industrial application as membrane cleaner	153
Microbial fuel cells	Promoting power density output of microbial fuel cells	156
Cosmetic and pharmacy	High emulsifying activity	82

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other feasible methods to enhance rhamnolipid productivity which represents a challenge and needs further understanding and exploring.

7. Applications

Low toxicity, biodegradability, pore-forming capacities, anti-adhesive, and anti-biofilm formation ability, anti-bacterial activity against a wide variety of bacteria, emulsification and de-emulsification activity are some RLs properties toward their great potential applications in many industries such as oil, cosmetics, special chemical foods, agriculture, medicine, *etc.* The potential applications of rhamnolipids in diverse industries have been presented in Table 6.

There are little publications strictly discussed on toxicity of biosurfactants. Biosurfactants are commonly considered as low- or non-toxic. Selected data on biosurfactants toxicitycab found on literature.[60–62] Biosurfactants in comparison with synthetic surfactants pose haemolytic activity to human erythrocyte lower than cationic surfactants (CTAB, TTAB, BC) and anionic SDS. They do not pose detrimental effect to heart, lung, liver and kidney and interfere in blood coagulation in normal clotting time.[60–62]

The efficiency of surfactants and biosurfactant is expressed by some parameters mainly the critical micelle concentration (CMC) and emulsification index (E24). The CMC is the concentration limit of a biosurfactant after which the addition of more biosurfactant will not cause the surface tension to be further reduced. A biosurfactant with a low CMC is more efficient in lowering surface and interfacial tensions than a biosurfactant with a high CMC. Biosurfactant CMCs range from 1–200 mg L^{-1} (ref. 63) and are 10–40 fold less than that of synthetic surfactants.[64] The CMC of surfactin has been reported to be as low as 21 mg L^{-1}, while that of rhamnolipids has been reported to be around mg L^{-1}.[24] Also, Ferhat *et al.* showed a higher emulsification index than synthetic surfactants such as SDS, Tween 20, and Tween 80.[65]

Some of noticeable applications of RLs in the food industry are mostly due to their emulsification ability, antibacterial activity.[66] Furthermore, they can act as preventive agents against contamination, food spoilage factors, and the transmission of diseases. The formation of biofilms is another concerning issue in the food industry. One effective solution is the pre-conditioning of surfaces, using biosurfactants, which can be helpful in avoiding adhesion.[67,68] An investigation by ref. 69 showed the inhabitation ability of rhamnolipid produced by *P. aeruginosa* strain against both Gram-positive and Gram-negative bacterial strains, namely *E. coli*, *B. subtilis*, *S. aureus*, and *S. epidermidis*. In another study, the rhamnolipids produced by *P. aeruginosa* PAO1 could rapidly disrupt *B. bronchiseptica* biofilms on polystyrene.[70] Furthermore, emulsions are an integral part of the food industry playing an important role in the quality of products such as mayonnaise, butter, cream, margarine, salad dressing, chocolates and hotdogs.[71] On the other side, de-emulsification is breaking a stable emulsion and can be an effective process in the food industry especially when related to fat and oil products. RLs may stabilize (emulsify) or destabilize (de-emulsify) the emulsion. A recent invention by Van Haesendonck *et al.* have clearly investigated the effect of a sufficient amount of rhamnolipid on the stability of dough and texture of bakery products.[72] In another study, Haba *et al. P. aeruginosa* 47T2 NCIB 40 could produce a rhamnolipid which showed acceptable results as an emulsifier.[73] Finally, RLs are able to improve food quality by preserving them from contamination due to their antibacterial activities. Moreover, they could serve as a source of L-rhamnose having substantial potential in high-quality flavor compounds.

Biofilm is a complex community of microorganisms, and its formation is an important problem for many industries. It can be produced by microorganisms such as bacteria and fungi. Removing of biofilms are difficult since are resided within a polysaccharide and/or protein matrix. The resistance of biofilm to antimicrobial agents is becoming a global issue.[74,75] Rhamnolipid as a natural surfactant regulated by quorum sensing has the inhibitory effect on biofilm formation.[76] Davey *et al.* studied on the behavior of the purified rhamnolipid on cell-surface (on polyvinylchloride plastic) and cell–cell interaction (on pellicles of wild-type *P. aeruginosa* cells).[77] As the results showed, biofilm formation on polyvinylchloride plastic significantly reduced by increasing in rhamnolipid concentration.

Moreover, adding rhamnolipid caused the disruption of cell-to-cell interactions. Wood *et al.* found that *P. aeruginosa* supernatants had a significant inhibitory effect on sulfate reducing bacteria (SRB) biofilm formation, which is the main reasons for metal corrosion in oil wells and drilling equipment.[74]

Another important application of rhamnolipid biosurfactants is in agriculture since they have shown their inhibitory effects against plant pathogens.

The first report on the rhamnolipid insecticidal activity was by Kim *et al.*[78] They reported the ability of rhamnolipid produced by *P.* sp. EP-3 against Green Peach Aphid (*Myzus persicae*). In another study, the antifungal activity of rhamnolipids against seven plant pathogens has been surveyed. The results showed the high-level ability of rhamnolipid derived from *Pseudomonas aeruginosa* ZJU211 against two *Oomycetes*, three *Ascomycota*, and two *Mucor* spp. fungi.[79] Similarly, rhamnolipid synthesized by *P. aeruginosa* DS9 were reported to show their antifungal ability against *F. sacchari* causing pokkah boeng disease.[80]

Since the chemical synthesis of surfactants reveals adverse effects on people's health, *P. aeruginosa* derived RLs play a noticeable role in cosmetic and pharmacy industries due to their emulsifying ability, solubilizing biodegradability, low toxicity, and detergency properties which can guarantee the cosmetics and drug delivery system safety. High emulsifying activity is the basis of the texture consistency of health care and cosmetic products such as antacids, acne pads, contact lens solutions, deodorants, and *etc.*[81] Furthermore, the emulsifying ability of rhamnolipids synthesized by *Pseudomonas aeruginosa* 47T2 NCBIM 40044 was evaluated by Haba *et al.*[82] Different types of oils were tested and it was found that only using linseed oil along with RL47T2 led to the formation of the strong and stable emulsion.[82]

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Another application for RLs is their feasibility in bioremediation of heavy metals in soil or other media owing to their effect on the oil-water interface. It, consequently, enhances the degradation of such compounds in the environment. Because of rhamnolipid anionic nature, they widely use in removing heavy metals such as Ni and Cd from soils.[83] Juwarkar *et al.* showed the ability of rhamnolipid biosurfactant by *P. aeruginosa* BS2 for bioremediation of multi-metal contaminated soil (Cr, Pb, Cd, Ni, and Cu).[84] As a result of conducting column experiment, the feasibility of using rhamnolipid was proved although it was different for different metals. In another study, Santa *et al.* have concluded that rhamnolipid biosurfactant (extracted from *P. aeruginosa* PA1) had the ability to remove oil contamination from sandy soils.[85]

In recent years, many investigations have been carried out on the RL applications in oil recovery. RLs mostly have shown their feasibility in petroleum due to their high surface activity. They have been widely employed for heavy crude oil biodegradation and microbial enhanced oil recovery (MEOR). Lan *et al.* have indicated that the increase in rhamnolipid produced by *P.* sp. SWP-4 successfully could reduce the viscosity of crude oil and efficiently could enhance oil recovery.[86] Li *et al.* showed the ability of rhamnolipid synthesized by *P. aeruginosa* (P-1) to increase the oil recovery by 11.2% and decreased crude oil viscosity by 38.5%.[87] In another study, it was shown that rhamnolipid could recover over 98% of crude oil from the wastes using the demulsification process.[88]

Another potential application of rhamnolipids is devoted to biomedicine. Due to their antimicrobial activity against a wide range of bacterial, they are a safe alternative for the recovery of different illnesses. Thanomsub *et al.* evaluated the ability of rhamnolipid (derived from *P. aeruginosa* B189) isolated from milk factory waste in breast cancer therapy and insect cell line.[89] Two rhamnolipids namely Rha–Rha–C_{10}–C_{10} and Rha–Rha–C_{10}–C_{12} produced by the mentioned strain showed inhibition activity in the spread of breast cancer at its minimum inhibitory concentration (MIC) (6.25 and 50 μg mL^{-1}). However, the crude RL extract showed no antimicrobial activity. Also, successful treatment was reported by Piljac *et al.* for Decubitus Ulcer using di-rhamnolipid ointment.[90] The wound was completely healed after 48 days while there was no evidence of improvement by the standard drugs.

In recent years, using rhamnolipids as an agent having the ability of the molecular self-assembly is a promising trend in nanotechnology. RLs could alter their self-assembled structures owing to their carboxylic acid on the headgroups. Consequently, they can be used in synthesizing nanoparticles and microemulsions,[91] and they were named the natural green biosurfactants.[92] Xie *et al.* investigated the effect of using rhamnolipid biosurfactants in silver nanoparticles stabilization in the liquid phase in the reverse micelles.[92] The uniformity of obtained nanoparticles was analyzed and proved by the TEM and AFM. Moreover, in another study Xie *et al.* studied the difference between W/O and O/W microemulsion in a rhamnolipid/*n*-butanol/water/*n*-heptane system.[93] In another research, microemulsion technique was employed to synthesize the nickel oxide nanoparticles by using, *n*-heptane, water, and rhamnolipids as biosurfactant. Besides, the effect of increasing pH on the size of nanoparticles was investigated. It was found that the nanoparticles were completely spherical in shape and in pH values of 11.6, 12.0, and 12.5, the size of nanoparticles was 86 ± 8 nm, 63 ± 6 nm and 47 ± 5 nm, respectively.[94] Narayanan *et al.* used a novel method for synthesizing nanoparticles in aqueous condition by rhamnolipids.[95] They have indicated the rhamnolipids ability as capping agents for capping ZnS nanoparticles and then they were evaluated by FT-IR, SAXS, HR-TEM to prove the formation of uniform nanoparticles. Furthermore, Farias *et al.* investigated the formation of silver nanoparticles using rhamnolipid produced by a strain of *P. aeruginosa* UCP0992 in a low-cost medium using microemulsion method.[96] The size of the formed nanoparticle was about 1.13 nm and it could be stabilized for at least 3 months without adding passivator. Also, TEM was used to confirm the uniformity of particles.

Furthermore, improvement in making nanoparticles leads to the advancement in drug delivery. Recent researches have indicated that rhamnolipid nanoparticles have potential in imaging and nanomedicine. The only report on the application of rhamnolipid produced by *P. aeruginosa* in the intravenous injection of rhamnolipid nanoparticles for photodynamic therapy released by Yi *et al.*[97] In this study, the rhamnolipid nanoparticles were prepared by pheophorbide having about 136.1 nm diameter and high water solubility. The results showed that after the injection of the loaded nanoparticle to SCC7 tumor-bearing mice model, tumor growth was prevented.

8. Conclusion

Generally, surfactants are surface active agents using to reduce surface tension between two surfaces in different industries. However, these surfactants are mostly allergic. They are not biodegradable and in some cases they can be toxic. All of these negative properties cause the researcher find a way to solve the problems and drive surfactants from various microorganisms named biosurfactant. Rhamnolipids and sophorolipid are two main group of biosurfactant. They not only can noticeably reduce the surface tension activity, but also they are biodegradable and environmentally friendly products. They also can decrease surface tension more than chemical surfactant at the same CMC.

Introducing new products to the market is always a challenge. This is especially true for bio-materials such as rhamnolipids. Although every bio-products like biosurfactants exhibit many advantages over chemically synthesized counterparts, there are some bottlenecks for using them in commercial scale. They are not toxic in contact with human skin. However, it should be noted that they are toxic to microbial growth in certain concentrations. Scientists use this advantage of RLs for antimicrobial applications. Also, they are easily degraded in the environment by bacteria and other microscopic organisms; hence they are not considered a threat to the environment. In comparison with chemically synthesized surfactants, they show great surface activity great surface activity with very low CMC. In addition, waste and cheap raw materials (such as waste oil)

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which are available in large quantities can be used as the raw material to produce RLs. It can enhance economic efficiency in producing them. RLs can be efficiently used in bioremediation of contaminated soil, biodegradation, and detoxification of industrial effluents, preparation of industrial emulsions, and control of oil spills.

Despite the numerous advantages of RLs, there are still some challenges for their commercial production and application. The main issue is the high production cost. Many researchers tried to overcome this problem by optimization of culture condition and utilization of waste substrates. Due to the high cost of downstream processes (mainly RL purification), there is difficulty in obtaining pure substances. High purity compound is required and necessary in pharmaceutical, food and cosmetic applications. Another important factor for commercial production of RLs is high productivity strains of bacteria. As most of the bacteria used in the experiments display low productivity, they are not suitable for industrial purpose and economic production. In the other side, the mechanism of RL biosynthesis is not well understood. It seems that RL represents secondary metabolite regulation. So, more studies are needed to find the exact mechanism to design a liable and economic process for industrial purpose.

Conflicts of interest

There is no conflicts to declare.

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