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Abstract

Pathogenicity of biosurfactant-producing microorganisms is currently raising some health, safety and environmental concerns. As a result, the industrial-scale production and application of biosurfactants as potential alternatives to the synthetic one is still an unachieved task. The production of biosurfactants using nonpathogenic/recombinant strains requires more attention and investigation for some advantages that includes the discovery of non-toxic biosurfactants suitable for all industrial applications, identifying new biosurfactant congeners with better inherent surface-active properties compared to that from pathogens and synthetic ones and the synthesis of biosurfactant without complex metabolic regulations. Although a number of nonpathogenic/recombinant, eco-friendly biosurfactant-producing strains have been documented, there is need for more research in this area focusing especially on improved biosurfactant production by these strains using

	optimisation processes and the discovery of new nonpathogenic/ recombinant strain using molecular techniques for future sustainability.
Keywords (separated by “-”)	Bioremediation - Biosurfactants - Recombinant strains - Rhamnolipids

Production of Biosurfactants Using Eco-friendly Microorganisms

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Abstract

Pathogenicity of biosurfactant-producing microorganisms is currently raising some health, safety and environmental concerns. As a result, the industrial-scale production and application of biosurfactants as potential alternatives to the synthetic one is still an unachieved task. The production of biosurfactants using nonpathogenic/recombinant strains requires more attention and investigation for some advantages that includes the discovery of non-toxic biosurfactants suitable for all industrial applications, identifying new biosurfactant congeners with better inherent surface-active properties compared to that from pathogens and synthetic ones and the synthesis of biosurfactant without complex metabolic regulations. Although a number of nonpathogenic/recombinant, eco-friendly biosurfactant-producing strains have been documented, there is need for more research in this area focusing especially on improved biosurfactant production by these strains using optimisation processes and the discovery of new nonpathogenic/recombinant strain using molecular techniques for future sustainability.

Keywords

Bioremediation • Biosurfactants • Recombinant strains • Rhamnolipids

1 Introduction

Biosurfactants refer to surfactants from microbial origin and can be synthesised by several identified microorganisms including bacteria, yeast and fungi. Originally, majority of the surfactants used in the industries today are petrochemical based, and these surfactants are not only partially biodegradable but are also toxic to living organisms and have contributed to a wide range of environmental hazards. In addition, the production of these petrochemical-based surfactants contributes to the depletion of the world's non-renewable petrochemical resources. It has been reported that the worldwide production of

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P. Thangavel and G. Sridevi (eds.), *Environmental Sustainability: Role of Green Technologies*,
DOI 10.1007/978-81-322-2056-5_11, © Springer India 2015

synthetic surfactants is over 15 million tons per year (Van-Bogaert et al. 2007) that are employed in different industrial sectors ranging from food processing, pharmaceutical, cosmetics, detergent production, environmental cleanup (bioremediation) and enhanced oil recovery. According to Reznik et al. (2010), the worldwide production of synthetic surfactants per annum was 13 million tons in 2008, increased by 2 % in 2009 resulting to annual turnover of US$ 2,433 billion. The market was then expected to experience a continuous growth by 28 % from 2009 to 2012 and thereafter increase approximately by 3.5–4 %. However, biosurfactants have been reported by many researchers to have a variety of advantages over the petrochemical-based or synthetic surfactants. They display excellent surface activity and emulsification properties with very low toxicity and higher biodegradability features. They have also been found to be very effective at low concentrations and over a wide range of environmental conditions such as pH, temperature, salinity, alkalinity and acidity. Other important characteristics of these biomolecules include better environmental compatibility, lower critical micelle concentration, higher selectivity, specific activity and ability to be synthesised from renewable low-cost resources (Desai and Banat 1997; Oliveira et al. 2009; Rahman and Gakpe 2008).

These biosurfactants due to their amphiphilic properties have found potential application in an extremely wide variety of industrial process involving emulsification, foaming, detergency, wetting, dispersing or solubilisation. In addition, biosurfactants are currently being used as antibacterial, antifungal, antiviral, antioxidant, moisturisers and antiradical and stabilising agents in the production of various industries, products ranging from cosmetics, food, pharmaceutical, agriculture and detergents. Their ability to stimulate dermal fibroblast metabolism and support healthy skin physiology has also put them on the front line as potential raw material for cosmetics such as deodorants, facial cosmetics, lotions, eye shadow, skin smoothing and anti-wrinkle and anti-ageing products (Rosenberg and Ron 1999; Banat et al. 2000; Rahman et al. 2002).

The commercial viability of biosurfactants as alternatives to the synthetic surfactants has been greatly hindered due to limiting technical and economic factors including high substrate cost (Mukherjee et al. 2006), low product yield (Maneerat 2005; Thavasi et al. 2011b), product mixtures resulting in high-cost downstream recovery/purification processes (Heyd et al. 2008) and the type of producing strains (Reiling et al. 1986). Most biosurfactant-producing organisms are pathogenic and difficult to handle in large-scale industrial processes (Gunther et al. 2005; Toribio et al. 2010). In this review, after a discussion of the pathogenicity of biosurfactant, producers focus on the state of the art of production of biosurfactants from nonpathogenic eco-friendly organisms and on the heterologous production by recombinant strains.

2 Biosurfactant Production Using Pathogenic Organisms and Health and Safety Issues

The development of biosurfactant production in nonpathogenic organisms is a current challenge that is receiving increased attention in order to avoid pathogenicity and complex metabolic regulations (Dusane et al. 2010) especially in rhamnolipid synthesis by *Pseudomonas aeruginosa* and to screen for novel product spectra (Müller et al. 2012). Application of crude biosurfactants from pathogens in industrial and environmental applications is largely unacceptable especially in the cosmetics, health and food sectors due to the potential presence of toxins and pigments (Nicas and Iglewski 1985).

2.1 Rhamnolipids

Rhamnolipids are widely studied glycolipid biosurfactants synthesised in large quantities by different strains of *P. aeruginosa*. As biosurfactants, rhamnolipids play an important role for the producing organism, displaying useful physico-chemical, physiological (Zhong et al. 2007)

and antimicrobial functions on other living systems (Bergstrom et al. 1946; Abalos et al. 2001). [AU3] Excellent emulsifying properties relative to synthetic surfactants make rhamnolipids viable alternatives due to their efficacy at low concentrations and their inherent biodegradability (Ron and Rosenberg 2002; Calvo et al. 2009; Rahman et al. 2003), for example, the bioremediation of hydrocarbon-polluted sites (Thavasi et al. 2011a; Jorfi et al. 2013) and in enhanced oil recovery (Amani et al. 2013). However, the industrial-scale application of *P. aeruginosa* strains for rhamnolipid production is an unrealistic task: this organism is an opportunistic human pathogen (Rahman et al. 2010) and is responsible for infectious diseases in immune-compromised individuals (Goethals et al. 2001). Addressing these safety issues renders that *P. aeruginosa*-derived biosurfactant is uneconomical (Ochsner et al. 1995; Tuleva et al. 2002) and unsafe for industrial processes.

The cultivation of *P. aeruginosa*, a Gram-negative bacterium belonging to the taxonomical class of Gammaproteobacteria and the family Pseudomonadaceae, has been well studied, and the genome of strain PAO1 has been fully sequenced and annotated. The organism is found in various habitats including water, soil, plants and air, is resistant to a variety of antibiotics (Tummler et al. 1991) and is the main causative organism for cystic fibrosis and nosocomial infections. The pathogenicity of *P. aeruginosa* in cystic fibrosis was confirmed by Kownatzi et al. (1987) who discovered rhamnolipids of up to 8 µl/ml in the sputum of *P. aeruginosa*-colonised cystic fibrosis patients. Read et al. (1992) also showed the presence of 65 µl/ml rhamnolipid in the secretions of a lung removed from a cystic fibrosis patient. The cytotoxic and haemolytic effects of crude rhamnolipids from other rhamnolipid producers have been demonstrated by researchers (Häussler et al. 1998, 2003; Rahman et al. 2010). Rhamnolipids have been shown to be heat-stable haemolysins having haemolytic activity on [AU4] various erythrocyte species (Fujita et al. 1998; Johnson and Boese-Marrazzo 1980) and cytotoxic at high concentration to immune cells (Bjarnsholt et al. 2005; Jensen et al. 2007).

Rhamnolipids from *P. aeruginosa* stimulate the release of allergy and inflammatory mediators from the mast cells such as histamine, serotonin and 12-hydroxyeicosatetraenoic acid (Bergmann et al. 1989; McClure and Schiller 1992; König et al. 1992; Cosson et al. 2002; Andrä et al. 2006). Furthermore, rhamnolipids have been associated with several dysfunctions in the lungs and the entire respiratory tract. These include inhibition of ciliary function, damage to bronchial epithelium, alteration of respiratory epithelial ion movement and induction of the release of mucus conjugates from human bronchial mucosa, and very recently it has been reported that rhamnolipid synthesis is an important prerequisite for the invasion of *P. aeruginosa* into human respiratory epithelial cells (Stutts et al. 1986; Graham et al. 1993; Fung et al. 1995; Zulianello et al. 2006). *P. aeruginosa* has been classified as biosafety level 2 organism due to the health and safety issues associated with the organism as well as the cytotoxic properties of their rhamnolipid species.

These safety concerns inhibit the economic viability of large-scale rhamnolipid production from *P. aeruginosa*. Such viability is further compromised by the involvement of complex quorum sensing and transcriptional mechanisms (Soberón-Chávez et al. 2005). Furthermore, the purification and treatment of the rhamnolipid yield from *P. aeruginosa* are also costly at the industrial scale, and it is important to note that rhamnolipids produced from *P. aeruginosa* fermentation cannot be considered as safe raw materials in the production of food, pharmaceutical and cosmetics products. Thus there is need to explore for new nonpathogenic natural rhamnolipid producers. A survey of biosurfactants produced by pathogenic strains is presented in Table 1 together with their pathogenesis in humans, plants and animals.

Rhamnolipids are also produced by *Burkholderia* species; some of these organisms such as *B. cepacia* have been identified as human pathogens. This organism capable of producing a

t1.1 **Table 1** Biosurfactant production by pathogenic bacterial strains

t1.2 Biosurfactants	Pathogenic producers	Pathogenicity	References
t1.3 Rhamnolipids	*Pseudomonas aeruginosa*	Cystic fibrosis, respiratory infections, multidrug resistant	Thavasi et al. (2011b), Jorfi et al. (2014), Kownatzi et al. (1987)
t1.6	*Burkholderia cepacia*	Lung infection, pneumonia	St Denis et al. (2007), Yalçin and Ergene (2010)
t1.8	*Burkholderia pseudomallei*	Melioidosis	Andrea et al. (2008), Howe et al. (2006)
t1.10	*Burkholderia plantarii*	Fire blight disease of apple and pear trees	Hörmann et al. (2010), Mitchell and Teh (2005)
t1.12	*Burkholderia glumae*	Rot of rice grains and seedlings	Urakami et al. (1994), Costa et al. (2011)
t1.14	*Renibacterium salmoninarum*	Bacterial kidney disease of salmonid fish	Christova et al. (2004), Wiens et al. (2008)
t1.16 Glycolipids	*Pantoae agglomerans*	Bacteraemia, wound infection, arthritis, infection of soft tissues bones and joints in children	Vasileva-Tonkova and Geshava (2007), Jacobucci et al. (2009), Koo et al. (2006)
t1.20	*Nocardia otitidiscaviarum*	Pulmonary nocardiosis, brain abscess, actinomycetoma	Vyas and Dave (2011), Pelaez et al. (2009), Chi et al. (2013)
t1.22	*Alcaligenes faecalis*	Post-operative endophthalmitis, peritonitis	Kaliaperumal et al. (2006), Kahveci et al. (2011), Bharali et al. (2011)
t1.25 Trehalose mycolates	*Acinetobacter calcoaceticus* IMV B-7241	Urinary tract infections, respiratory tract infections, post-operative infections	Pirog et al. (2013), Pal and Kale (1981)
t1.28 Phospholipids	*Klebsiella pneumoniae*	Haemorrhage/necrosis, upper and lower respiratory tract infections, osteomyelitis, UTI, diarrhoea, wound infection, meningitis, bacteraemia, septicaemia, ankylosing spondylitis	Jamal et al. (2012), Rashid and Ebringer (2007), Ryan and Ray (2004), Limbago et al. (2012)
t1.35 Lipopeptides	*Serratia marcescens*	Urinary tract infections, nosocomial bacteraemia, meningitis, endocarditis	Ibrahim et al. (2013), Anyanwu et al. (2010), Körner et al. (1994)
t1.38	*Inquilinus limosus* KB3	Cystic fibrosis, multidrug-resistant lung infections	Saimmai et al. (2013), Wellinghausen et al. (2005), Hayes et al. (2009)
t1.41 Heteropolysaccharides	*Cronobacter sakazakii*	Bacteraemia, meningitis, necrosis, enterocolitis, contamination of infant formula	Lai (2001), Jain et al. (2012), CDC (2002)

biosurfactant exhibiting a surface tension value of 45.7 mN m^{-1} (Yalçin and Ergene 2010) has been associated with lung infections and pneumonia in humans of all ages (St Denis et al. 2007). Similarly, *B. pseudomallei*, a biosurfactant producer (Howe et al. 2006), is a facultative intracellular pathogen, although the mechanism of its intracellular survival is yet unknown. This pathogen is the causative agent of melioidosis, an infectious disease endemic to Southeast Asia, northern Australia and tropical and subtropical regions (Andrea et al. 2008). The di-rhamnolipids from *B. pseudomallei* have been found to exhibit serious cytotoxic effects on macrophages non-phagocytic and phagocytic cell lines (Sierra 1960; Kharazmi et al. 1989; Johnson and Boese-Marrazzo 1980;

Fujita et al. 1988). Di-rhamnolipids have also been reported to be cytolytic to human monocyte-derived macrophages and can inhibit the phagocytic response of macrophages even at low concentration.

Burkholderia plantarii DSM 9509 has been demonstrated to be the most prominent rhamnolipid producer. According to Hörmann et al. (2010) and Walter (2009), *B. plantarii* DSM 9509 produced Rha2-C_{14}-C_{14} congeners that reduced the surface tension of water to 29.4 mN m^{-1} with critical micelle concentrations (CMC) between 15 and 20 mg L^{-1}. The maximum rhamnolipid concentration during 0.5 L bioreactor cultivation was 45.75 mg L^{-1}. However, this organism is a plant pathogen responsible for the fire blight disease of apple and pear trees, and the di-rhamnolipid from *B. plantarii* is an endotoxin (Mitchell and Teh 2005). Costa et al. (2011) also showed rhamnolipid concentration of 1 g L^{-1} in *B. glumae* AU6208 at 34 °C, 2 % canola oil and 100 mM urea, but this organism is toxic to rice grain and seedlings.

Renibacterium salmoninarum is a diplobacillus fastidious bacterium identified as potent biosurfactant producer on n-hexadecane as a carbon source (Christova et al. 2004). It is also interesting to know that this bacterium produces the two typical rhamnolipid as in *P. aeruginosa* and has the potential of being useful in natural degradation of hydrophobic pollutants due to its high cell hydrophobicity. Despite these promising characteristics, this organism has raised a significant ecological concern. It causes a haemorrhagic infection known as bacterial kidney infection (BKD) or salmonid kidney disease in young salmonid fish (Wiens et al. 2008).

2.2 Glycolipids and Trehalose Mycolates

Glycolipid production by other known pathogens has been reported. The Antarctic facultative anaerobe *Pantoea agglomerans* is a biosurfactant producer with hydrocarbon compounds (hexane, kerosene and paraffin) as growth substrates (Vasileva-Tonkova and Gesheva 2007; Jacobucci et al. 2009). *Pantoea* sp. has also been reported as producers of high levels of exopolysaccharides with maximum production of 21 g L^{-1} in 24 h on glucose fructose and sucrose. However, *Pantoea* sp. have recently been consistently linked with infections in humans (wound, blood, soft tissue, bone and urinary tract infections) and plants (Da Baere et al. 2004; Fullerton et al. 2007; Kratz et al. 2003). These organisms have also been associated with bacteraemia outbreaks in many patients through their contact with contaminated cotton pledgets (Koo et al. 2006; Silvi et al. 2013).

Production of glycolipid biosurfactant has been reported for *Nocardia otitidiscaviarum*, a marine strain. The biosurfactant possessed increased cell hydrophobicity, making it very potent for bioremediation of oil-polluted sites (Vyas and Dave 2011). Recently, these bacterial species have been identified as causative agents in a couple of clinical infections ranging from pulmonary nocardiosis, brain abscess and actinomycetoma (Pelaez et al. 2009; Chi et al. 2013).

Alcaligenes faecalis has been reported by Bharali et al. (2011) as a producer of biosurfactant exhibiting excellent surface activity using different hydrocarbon substrates. However, *A. faecalis* is a known pathogen infecting human and domesticated birds such as chickens and turkeys (Simmons et al. 1981). Kaliaperumal et al. (2006) reported *A. faecalis* as the causative agent in post-operative endophthalmitis in human eye resulting in symptoms such as swelling of the eyelid, redness and permanent loss of vision. In addition, Kahveci et al. (2011) have reported a link between the developments of peritonitis to contamination of catheters by *A. faecalis*. The pathogenic mechanism of this microbe is largely unknown.

Rosenberg et al. (1989) identified *Acinetobacter calcoaceticus* A2 as a producer of an extracellular anionic surfactant referred to as biodispersan or emulsan which is a high molecular weight biosurfactant. Pirog et al. (2009) have also reported the production of low molecular weight biosurfactant by *A. calcoaceticus* IMV B-7241 which was confirmed to be trehalose mycolates by enzymatic studies. *A. calcoaceticus* has been described as a commensal in humans but is also

responsible for many clinical infections as an opportunistic pathogen. These infections include urinary tract infection, pneumonia, respiratory tract infection and post-operative infections (Pal and Kale 1981).

2.3 Phospholipids and Lipopeptides

Biosurfactant production by a prominent member of the *Klebsiella* genus known as *Klebsiella pneumoniae* has been identified. According to Jamal et al. (2012), *Klebsiella pneumoniae* WMF02 showed increased biosurfactant production after medium optimisation with a maximum yield of 85 g L^{-1} and surface tension reduction of 25.70 mN m^{-1} compared to the non-optimised medium (36.2 mN m^{-1}). This organism has shown the promising potential of a good biosurfactant producer, but its application will involve certain health and safety risks. *K. pneumoniae* is a significant human pathogen causing different destructive effects to the human upper and lower respiratory tracts if inhaled which includes pneumonia and bronchitis. Other notable infections caused by this organism include diarrhoea, wound infections, osteomyelitis, meningitis, bacteraemia and septicaemia (Ryan and Ray 2004). Recently, these bacterial strains have been reported as resistant to antibiotics especially carbapenem antibiotics (Limbago et al. 2012). They have also been associated with a chronic inflammatory spinal and large joint arthritic condition known as ankylosing spondylitis (AS) affecting young-aged males (Rashid and Ebringer 2007).

Serratia marcescens is a multidrug-resistant pathogen causing infections in different hosts ranging from plants, animals and humans. This bacterium produces a lipopeptide biosurfactant known as serrawettin (Dusane et al. 2011). The biosurfactant is very potent and has been reported in literature to reduce the surface tension of water from 72 to 37 mN m^{-1}, emulsifying kerosene and diesel with a maximum emulsion index of 72 % and 40 %, respectively (Wei et al. 2004). Ferraz et al. (2002) also reported biosurfactant production by *S. marcescens* strains. The biosurfactant reduced the surface tension of the culture medium from 64.54 to 29.57 mN m^{-1}, while the crude biosurfactant reduced the surface tension of water from 72 to 28.70 mN m^{-1}. However, this bacterium cannot stand the chance of being used for industrial-scale production of biosurfactant due to its pathogenicity. Most importantly, serrawettin biosurfactant is a virulence factor, and *S. marcescens* strains have been linked with a of number infections including nosocomial infections bacteraemia, urinary tract infections, meningitis and endocarditis (Anyanwu et al. 2010; Körner et al. 1994). According to a report by the Centers for Disease Control in the USA, *S. marcescens* was confirmed as the main causative organism in the Alabama hospital outbreaks that happened in 2011 affecting 19 patients with 10 deaths.

Saimmai et al. (2013) identified a Gram-negative bacterium *Inquilinus limosus* KB3 capable of producing lipopeptide biosurfactant using palm oil cake as a carbon source. The biosurfactant reduced the surface tension of water from 72 to 25.5 mN m^{-1} with a maximum yield of 5.13 g L^{-1} and CMC at 9 mg L^{-1}. The genus *Inquilinus* was first defined in 2002 based on the molecular analysis of 51 unknown cystic fibrosis isolates. This group of organisms is unrelated to *P. aeruginosa* and *B. cepacia. Inquilinus limosus* has been reported as a multidrug-resistant pathogen and has been isolated from the lungs of cystic fibrosis patients (Wellinghausen et al. 2005; Hayes et al. 2009).

2.4 Heteropolysaccharides

Jain et al. (2012) reported the production of a heteropolysaccharide biosurfactant by an alkaliphilic bacterium, *Cronobacter sakazakii*, formerly known as *Enterobacter sakazakii*. The biosurfactant comprised of total sugars (733 %), reducing sugars (1,464 %), protein (119 %), uronic acid (1,598 %) and sulphate (6,015 %). The monosaccharide moieties in this biosurfactant were revealed by GC-MS as glucose (14 %), mannose (24 %), galactose (14 %), xylose (20 %) and arabinose (19 %). The extracted biosurfactant from this bacterium efficiently emulsified aliphatic

and aromatic hydrocarbons forming stable emulsions in the presence of xylene, cyclohexane, cyclooctane, toluene, carbon tetrachloride, dichloromethane, cottonseed, jojoba and groundnut oil at 1 mg mL^{-1}. The inherent properties of the biosurfactant make it a potential candidate for bioremediation of oil and hydrocarbons. However, *Cronobacter sakazakii* is a Gram-negative pathogenic bacterium (Lai 2001), reported as the cause of meningitis bacteraemia, necrosis and enterocolitis in infants. Infections have been associated with the use of powdered infant formula contaminated with the bacterium (Bowen and Braden 2006; CDC 2002).

The industrial application of pathogenic biosurfactant producers is clearly problematic based on the pathogenic effects of the organisms and their products on humans and animals. Additionally, some of these pathogens exhibit multidrug resistance and have recorded high mortality rates from their associated diseases. Therefore, they are now considered as potential biological warfare agents and as such are not potential biological models for industrial biotechnological processes (Walter 2009). For this combination of security and health reasons, there is a need to develop industrial processes based on nonpathogenic biosurfactant-producing strains.

3 Biosurfactant Production Using Nonpathogenic Organisms

Recently, due to the health and safety issues associated with some biosurfactant-producing strains, research towards identifying new nonpathogenic producers and biotechnological production of biosurfactants using recombinant/mutant strains has been given more attention. The advantages of using nonpathogenic organisms may include production of biosurfactants with various congener species, including the possibility of biosurfactants with less or no cytotoxic effects on living cells, and disconnection of their synthetic pathways from complex mechanisms such as quorum sensing in rhamnolipid synthesis. Nonpathogenic natural producer strains and their biosurfactant types identified to date have been reported (Table 2).

3.1 Rhamnolipids

Novel natural producers of rhamnolipids identified as *P. clemancea* nov. and *P. teessidea* nov. have been reported by Rahman et al. (2010). Rhamnolipid production can be achieved with *Pseudomonas* sp. other than *P. aeruginosa.* *Pseudomonas fluorescens* is a nonpathogenic Gram-negative, rod-shaped bacterium that has been found to produce biosurfactants as well as useful enzymes (cellulase, pectinase), self-defence factors (hydrogen cyanide), siderophores (pyochelin, pyoverdine), antibiotics (pyrrolnitrin, pyoluteorin) and 2,4-diacetylphloroglucinol, a molecule that can break down plant-derived carbohydrates, enhance host immune mechanisms and inhibit phytopathogens and bacteriophages (Stover et al. 2000; Paulsen et al. 2005). This organism has been reported as a degrader of certain environmental pollutants such as styrene, TNT and polycyclic aromatic hydrocarbons and as a result has been employed in many bioremediation processes.

The production of a thermostable rhamnolipid biosurfactant with excellent foaming and emulsifying stability by *P. fluorescens* was reported by Abouseoud et al. (2007). The biosurfactant was stable at 100 °C and retained its positive effect on surface tension (34–30 mN m^{-1}) even at high pH values. Stoimenova et al. (2009) isolated from industrial wastewater *P. fluorescens* HW-6 capable of producing rhamnolipid biosurfactants at relatively high levels on various carbon substrates including hexadecane, vegetable oil, mineral oil and glycerol. The culture supernatant of the strain exhibited a reduction in surface tension to 28.4 mN m^{-1}. Vasileva-Tonkova et al. (2006) also reported production of rhamnolipid biosurfactant by *P. fluorescens* HW-6 at concentrations of 14–20 g L^{-1} on hexadecane and interfacial tension of 35 mN m^{-1}, possessing a low critical micelle concentration value of 20 mg L^{-1}. Recently, a

t2.1 **Table 2** Biosurfactant production using nonpathogenic bacteria

t2.2 t2.3 Biosurfactants	Microbial strains	Physico-chemical characteristics	References
t2.4 Rhamnolipids	*Pseudomonas clemancea* nov.	Mesophilic	Rahman et al. (2010)
t2.5	*Pseudomonas teessidea* nov.	Mesophilic	Rahman et al. (2010)
t2.6 t2.7 t2.8	*Pseudomonas fluorescens*	Mesophilic	Abouseoud et al. (2007), Vasileva-Tonkova et al. (2006)
t2.9	*Pseudomonas chlororaphis*	Mesophilic	Gunther et al. (2005)
t2.10	*Pseudomonas putida* BD2	Mesophilic	Janek et al. (2013)
t2.11	*Burkholderia thailandensis*	Mesophilic	Dubeau et al. (2009)
t2.12	*Enterobacter asburiae*	Mesophilic	Hošaková et al. (2013)
t2.13	*Thermus aquaticus*	Thermophilic	Řezanka et al. (2011)
t2.14	*Meiothermus ruber*	Thermophilic	Řezanka et al. (2011)
t2.15 t2.16	*Enterobacter hormaechei* PTCC 1799	Mesophilic	Rabiei et al. (2013)
t2.17	*Tetragenococcus koreensis*	Halophilic	Lee et al. (2005)
t2.18	*Pseudoxanthomonas* sp. PNK-04	Mesophilic	Nayak et al. (2009)
t2.19 Glycolipids	*Streptococcus thermophilus* A	Thermophilic	Rodrigues et al. (2006a, b)
t2.20 t2.21	*Lactococcus lactis* 53 *L. acidophilus*	Mesophilic	Rodrigues et al. (2006a, b), Tahmourespour et al. (2011)
t2.22 t2.23	*Lactobacillus coryniformis* sp. *torquens* CECT 25600	Mesophilic	Gudiña et al. (2011)
t2.24 t2.25	*Lactobacillus paracasei* sp. *paracasei* A20	Mesophilic	Gudiña et al. (2011)
t2.26	*L. plantarum* A14	Mesophilic	Gudiña et al. (2011)
t2.27	*Leuconostoc mesenteroides*	Mesophilic	Gudiña et al. (2011)
t2.28	*Lactobacillus delbrueckii*	Mesophilic	Thavasi et al. (2011a)
t2.29 t2.30 Trehalose lipids	*Rhodococcus erythropolis* IMV Ac-5017, *Rhodococcus* sp.	Mesophilic	Pirog et al. (2013), Mutalik et al. (2008)
t2.31	*R. erythropolis* ATCC 4277	Mesophilic	Pacheco et al. (2010)
t2.32	*R. ruber* Z25	Mesophilic	Zheng et al. (2009)
t2.33 t2.34 Lipopeptides/phospholipids	*Bacillus subtilis* DM-03, *Bacillus subtilis* DM-04	Mesophilic	Das and Murkherjee (2007)
t2.35 t2.36	*B. subtilis* PT2	Mesophilic	Pornsunthorntawee et al. (2008)
t2.37 t2.38	*B. subtilis* LB5a	Mesophilic	Nitschke and Pastore (2006)
t2.39	*B. subtilis*	Mesophilic	Cooper et al. (1981)
t2.40	*B. lentus*, *B. firmus*	Mesophilic	Ibrahim et al. (2013)
t2.41	*Bacillus licheniformis*		Biria et al. (2010)
t2.42	*Selenomonas ruminantium*	Mesophilic	Saimmai et al. (2013)
t2.43	*Brevibacterium aureum* MSA13	Mesophilic	Kiran et al. (2010)
t2.44	*Corynebacterium kutscheri*	Mesophilic	Thavasi et al. (2007)
t2.45 t2.46	*Corynebacterium alkanolyticum* ATCC 21511	Mesophilic	Crosman et al. (2002)
t2.47 Heteropolysaccharides	*Halomona* sp. BS4	Halophilic	Donio et al. (2013)
t2.48	*Halomonas* sp. TG89	Halophilic	Gutierrez et al. (2012)
t2.49	*Halomona eurihalina*	Halophilic	Calvo et al. (2002)

rhamnolipid exhibiting high antimicrobial activities on both Gram-positive and Gram-negative pathogenic strains – *Listeria monocytogenes*, *Staphylococcus aureus*, methicillin-resistant *S. aureus*, *E. coli*, *Salmonella typhimurium* and *Candida albicans* – has been reported to be produced from *Pseudomonas fluorescens* MFS03 (Govindammal and Parthasarathi 2013). The biosurfactant was effective as a surface and emulsifying agent and could have potential applications in the bioremediation of hydrocarbon-polluted sites. Rhamnolipid production was also reported for nonpathogenic *P. chlororaphis* (Gunther et al. 2005) and *P. putida* BD2 (Janek et al. 2013).

Dubeau et al. (2009) reported production of 0.4–1.5 g L^{-1} rhamnolipid by *Burkholderia thailandensis* at 34 °C with 4 % glycerol or canola oil in nutrient broth. *B. thailandensis* is a Gram-negative, mesophilic bacterium closely related to *B. pseudomallei* but rarely causes infections in humans or animals (Wuthiekanun et al. 1996; Smith et al. 1997; Lertpatanasuwan et al. 1999). The lethal inoculum size for *B. thailandensis* is approximately 1,000 times higher than that for *B. pseudomallei* (Joost Wiersinga et al. 2008). This organism does not require biosafety level 3 conditions and has no restriction on the use of antibiotics or resistance markers for its genetic manipulation. The organism is not considered as a biosecurity threat and therefore is a potential industrial tool (Haraga et al. 2008; Glass et al. 2006). Recently, Hošaková et al. (2013) identified *Enterobacter asburiae* as nonpathogenic rhamnolipid producers. Rezanka et al. (2011) reported on three novel rhamnolipid-producing organisms and were identified as *Thermus* sp., *Thermus aquaticus* and *Meiothermus ruber*. These organisms have been categorised as biosafety level 1 organisms and are not pathogenic to humans. Pantazaki et al. (2010) have reported the simultaneous production of polyhydroxyalkanoates (PHAs) and rhamnolipids by a nonpathogenic thermophilic bacterium *Thermus thermophilus* HB8 (DSM 579) cultivated in mineral salt medium at 75 °C using glucose and sodium gluconate as sole carbon sources. Other new nonpathogenic rhamnolipid producers include *Enterobacter hormaechei* PTCC 1799 (Rabiei et al. 2013), *Tetragenococcus koreensis* (Lee et al. 2005) and *Pseudoxanthomonas* sp. (Nayak et al. 2009).

3.2 Glycolipids

The *Streptococcus thermophilus* bacterium is used widely in the dairy industries for the production of yogurt and cheese and is considered as beneficial to health since it aids digestion of dairy products in lactose-intolerant individuals (Kiliç et al. 1996; Hutkins 2002; Taylor and Mitchell 2007). According to Busscher et al. (1997), a biosurfactant released from *S. thermophilus* was used in the control of fouling of heat-exchanger plates in pasteurisers because it inhibited the colonisation of other thermophilic and pathogenic strains of *Streptococcus* responsible for fouling. Two probiotic bacteria *Lactococcus lactis* 53 and *S. thermophilus* A have been identified as producers of cell-bound biosurfactants at the stationary growth phase in the presence of lactose and cheese whey as carbon substrates (Rodrigues et al. 2006a, b). A protein-containing biosurfactant from *Lactobacillus acidophilus* has also been demonstrated to reduce the adhesion and biofilm formation of *Streptococcus mutans* on glass slides (Tahmourespour et al. 2011), indicating that the treatment of teeth with this biosurfactant may be an alternative dental control for biofilm development and adhesion of the pathogens on teeth.

Similarly, Gudiña et al. (2011) investigated the production of cell-bound and excreted biosurfactant by three lactobacilli strains and *Leuconostoc mesenteroides* strains. The lactobacilli strains were *L. coryniformis* sp. *torquens* CECT 25600, *L. paracasei* sp. *paracasei* A20 and *L. plantarum* A14. The studies revealed a decrease in surface tension of the culture broth for all the strains after 72 h incubation and the surface tension ranged from 1.4 to 6.4 mN m^{-1}. The highest excreted biosurfactant rate was recorded for *L. paracasei* sp. *paracasei* A20 with surface tension of 6.4 mN m^{-1}. However, the level of cell-bound biosurfactant was found to be higher than that of excreted molecules for all the strains. This is in contrast to other microorganisms

[AU3]

such as *Pseudomonas* and *Bacillus* that primarily excrete biosurfactant into their medium. Biosurfactant production has also been reported for nonpathogenic *L. delbrueckii* by Thavasi et al. (2011a). This organism showed maximum glycolipid production of 5.35 mg L^{-1} at 144 h incubation with peanut oil cake as substrate, but higher production was recorded after the stationary phase of growth. This was thought to be due to the release of cell-bound biosurfactant in the early stationary phase. In addition, *L. delbrueckii* could maximally degrade 61.25 % crude oil in the presence of fertilisers; hence the organism is both a biosurfactant producer and a hydrocarbon degrader. Lactobacilli are probiotics and nonpathogenic organisms designated "Generally Recognized as Safe" (GRAS) by the American Food and Drug Administration (FDA). The organism's ability to utilise lactose instead of glucose via an alternative metabolic pathway for biosurfactant synthesis has proven them as ideal hosts for biotechnological techniques especially metabolic engineering towards large-scale production of cell-bound biosurfactants (Rodrigues et al. 2006a, b). Although the biosurfactant yield by these organisms is low, optimisation of culture conditions may improve their production.

Rhodococcus sp. are a group of aerobic, nonsporulating, Gram-positive bacteria that can be found in a wide range of environments. They are usually considered as experimentally advantageous due to their high growth rate and simple developmental cycle. These organisms are effective for the degradation of aromatic hydrocarbons, production of bioactive steroids, bio-desulphurisation of fossil fuel and bioconversion of waste products to valuable compounds (McLeod and Eltis 2008). Strains of these species have been reported as biosurfactant producers including *R. erythropolis* IMV Ac-5017 (Pirog et al. 2013) and *Rhodococcus* sp. MTCC 2574 (Mutalik et al. 2008). Furthermore, Pacheco et al. (2010) have identified *Rhodococcus erythropolis* ATCC 4277 is a producer of a biosurfactant exhibiting excellent enhanced oil desorption from an oil shale. *Rhodococcus ruber* Z25 identified by 16 rDNA sequencing produced cell growth-associated biosurfactants on n-hexadecane with maximum yield of 13.34 g L^{-1} at 44 h (Zheng et al. 2009). *Rhodococcus* spp. produce surface-active trehalose lipids, and reports have shown that these surface-active compounds present interesting physico-chemical and biological properties. Trehalose lipids can significantly reduce the surface tension of water from 70 mN m^{-1} to 30.8 mN m^{-1} (Mutalik et al. 2008) and can form microemulsions (Zaragoza et al. 2013). In addition, trehalose lipids have been reported to inhibit protein kinase activity in vivo (Isoda et al. 1997) and induce the cell lysis of *E. coli* IEM-1 as well as the vegetative and spore cells of *Bacillus subtilis* BT-2 (Pirog et al. 2013). They have also been found useful in soil bioremediation and microbial enhanced oil recovery (Philip et al. 2002; Bell et al. 1998). This group of bacteria is considered as an ideal host for biotechnological production of non-toxic biosurfactant on industrial scales because of their genetic and catabolic diversity (associated presumably with their large chromosome and three large linear plasmids (van der Geize and Dijkhuizen 2004; McLeod et al. 2006). These organisms are usually nonpathogenic with only two species *R. fascians* and *R. equi* (Goethals et al. 2001) identified as plant and animal pathogens as well as causing infections in immune-compromised individuals. Nonpathogenic biosurfactant producers and their biosurfactants are shown in Table 1.2.

3.3 Lipopeptides and Phospholipids

Biosurfactants other than glycolipids produced by nonpathogenic organisms have been documented in literature. *Bacillus subtilis* is a ubiquitous Gram-positive rod-shaped bacterium, found commonly in water, soil and air, and contributes to nutrient cycling in the environment. This organism is industrially useful as it is one of the most widely used bacteria in the production of enzymes (amylases, proteases, inosine, ribosides and amino acids) and speciality chemicals including biosurfactants (Erikson 1976). This bacterium

has also been shown to produce a variety of antibacterial (Katz and Demain 1977) and antifungal (Korzybski et al. 1978) compounds including difficidin and oxydifficidin with wide spectrum of antibiotic activities against aerobic and anaerobic bacteria (Zimmerman et al. 1987). It has been used as a fungicide because it has the inherent ability to colonise root systems and to inhibit the growth of fungal plant pathogens (Kimura and Hirano 1988; Loeffler et al. 1986). Although *B. subtilis* has been associated with outbreaks of food poisoning (Gilbert et al. 1981; Kramer et al. 1982) and human infections especially in hospitalised patients with surgical wounds, breast cancer and leukaemia (Logan 1988), the exact nature of its involvement in infections has not been established. However, a literature review by Edberg (1991) revealed that *B. subtilis* does not produce significant quantities of extracellular enzymes or virulence factors that would predispose it to cause infection. In addition, Ihde and Armstrong (1973) reported that *B. subtilis* is an organism with low virulence. The organism has therefore been classified as neither a human (Edberg 1991) nor plant (Claus and Berkeley 1986) pathogen. According to the National Institutes of Health (NIH) guidelines for research involving recombinant DNA molecules (US Department of Health and Human Services 1986) and the European Federation of Biotechnology guidelines, *B. subtilis* is considered a class 1 containment agent, and their industrial use in fermentation processes presents low risk of adverse effects to human health and environment. *Bacillus subtilis* produces an effective and active cyclic lipopeptide biosurfactant known as surfactin (Cooper et al. 1987; Peypoux et al. 1999). Das and Mukherjee (2007) reported the production of lipopeptide surfactants by two strains *Bacillus subtilis* DM-03 and *Bacillus subtilis* DM-04 on potato peels using both submerged and solid-state fermentation techniques. Production of biosurfactant with enhanced surface tension reduction of 26 mN m^{-1} and lower CMC of about 25 mg L^{-1} has been reported for *B. subtilis* PT2 strain (Pornsunthorntawee et al. 2008). Similarly, *B. subtilis* LB5a decreased surface tension to a minimum value of 26.6 mN m^{-1} with CMC of 33 mg L^{-1} (Nitschke and Pastore 2006). Cooper et al. (1981) had earlier reported production of lipopeptide biosurfactants by *B. subtilis* strains with a minimum surface tension of 25 mN m^{-1} and a CMC of 25 mg L^{-1}. Surfactin has been demonstrated as one of most effective biosurfactants because of its high surface activity, efficiency in bioremediation and in situ microbial enhanced oil recovery (Mulligan 2005; Awashti et al. 1999; Besson and Michel 1992). Therefore, the production of biosurfactants from *B. subtilis* has the potential for large-scale bio-industrial development. Nonpathogenic lipopeptide-producing bacteria identified as *Bacillus lentus* and *B. firmus* have also been reported by Ibrahim et al. (2013) and Joshi et al. (2013).

Bacillus licheniformis is an important producer of lipopeptide biosurfactants (Biria et al. 2010) and has been used in industrial fermentation processes for over a decade for the production of several enzymes, antibiotics and special chemicals (Gherna et al. 1989; Eveleigh 1981). Although these *Bacillus* species have been reported to be associated with human infections, these occurred only in immunosuppressed individuals following trauma and other predisposing factors. Therefore, according to the Biotechnology Program under the Toxic Substances Control Act under the US EPA (1997), *B. licheniformis* is classified as a nonpathogen and is not toxigenic. Furthermore, the National Institutes of Health and European Federation of Biotechnology guidelines have also placed this organism as a class 1 containment agent (Frommer et al. 1989). Nonpathogens other than *Bacillus* sp. such as *Selenomonas ruminantium* (Saimmai et al. 2013) and *Brevibacterium aureum* MSA13 (Kiran et al. 2010) have also been identified for the production of lipopeptide biosurfactants.

Corynebacterium sp. is Gram-negative, catalase-positive, rod-shaped facultative anaerobic bacterium. Most members of this genus are nonpathogenic and industrially useful as they are known for the synthesis of amino acids, nucleotides and enzymes, for the bioconversion of steroids and for the degradation of hydrocarbons and are used in cheese ageing (Seidel et al. 2007; Natsch et al. 2005). The production of glycolipopeptide and

phospholipid biosurfactants has been reported for two nonpathogenic species such as *C. kutscheri* and *C. alkanolyticum* ATCC 21511, respectively (Thavasi et al. 2007; Crosman et al. 2002).

3.4 Heteropolysaccharides

Halomonas are a group of Gram-negative, non-sporing bacteria usually found in many different extreme water and soil environments, mainly saline, hypersaline or alkaline ecosystems. They belong to the family of Halomonadaceae and are classified as Gammaproteobacteria currently made up of 10 genera. They are considered to be nonpathogenic (von Graevenitz et al. 2000) and are potential industrial microbes due to their ability to synthesise microbial exopolysaccharides. Levan and mauran are examples of microbial polysaccharides from *Halomonas* spp. with a considerable market due to their exceptional performance at extreme industrial conditions (Poli et al. 2009; Llamas et al. 2006.) Although a few strains have been associated with certain human infections and contamination in a dialysis centre (Stevens et al. 2009), several *Halomonas* species have been classified as nonpathogens and also identified as biosurfactant producers. According to Donio et al. (2013), *Halomonas* sp. BS4 isolated from solar salt works has produced a heteropolysaccharide biosurfactant that suppressed the proliferation of mammary epithelial carcinoma cells by 46.77 % at 25 µL concentration. In addition, the pure biosurfactant exhibited antibacterial activity on *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pyogenes* and *Salmonella typhi* and antifungal activity on *Aspergillus niger*, *Fusarium* sp., *Aspergillus flavus* and *Trichophyton rubrum*. The biosurfactant was also found to display antiviral activity on white spot syndrome virus at high percentage (60, 80 and 100 %) and effectively suppressed the pathological effect of the virus. Gutierrez et al. (2013) have also shown the significance of exopolysaccharide (EPS) produced from marine *Halomonas* sp. TG89 in trace metal biogeochemical cycling. Calvo et al. (2002) have also documented the production of EPS from *Halomona eurihalina*. This research revealed that EPS from *Halomonas* contained high levels of K, Ca, Mg and several trace metals such as Zn, Cu, Fe and metalloid Si and has the specific ability to bind Ca, Si, Fe, Mn, Mg and Al in marine sediments. Furthermore, the growth of marine diatom *Thalassiosira weissflogii* was enhanced in the presence of purified EPS or to marine sediments exposed to EPS, indicating that the trace metals bound to the EPS become biologically available for the diatoms to utilise for growth. This bacterium therefore has the potential for the biotechnological development of safe antimicrobial and anticancer drugs as well as eco-friendly environmental products.

4 Production of Biosurfactant by Recombinant Strains

Pathogenicity may be avoided by the expression of biosurfactant production in heterologous microbial strains and mutants. Rhamnolipids can be synthesised by nonpathogenic heterologous strains provided with the rhamnosyltransferase genes *rhlA*, *rhlB and rhlC*. According to Cha et al. (2008), nonpathogenic heterologous *P. putida* 1067 (pNE2) expressing the *rhlABRI* gene from pathogenic *P. aeruginosa* EMS1 cultured in the mineral salt medium for 7 days in the presence of 2 % soybean oil as the sole carbon source exhibited rhamnolipid productivity that was greater than that of *P aeruginosa* EMS1. Scope remains for optimisation of the production conditions potentially leading to increased biosurfactant production making this organism useful for industrial biosurfactant production. Furthermore, Ochsner et al. (1995) investigated the heterologous expression of *rhlAB in P. fluorescens*, *P. putida* and *E. coli*. The results showed 0.25 g L^{-1}, 0.6 g L^{-1} and no rhamnolipid, respectively, for the organisms, but Wang et al. (2007) have reported rhamnolipid production in *E. coli* BL21 with the expression of *rhlAB* operon (Table 3).

In another study, *rmlBDAC* operon involved in dTDP-L-rhamnose biosynthesis was introduced into *E. coli* W3110 in addition to *rhlAB* operon, and a total final concentration of 120.6 mg L^{-1} mono-rhamnolipid was achieved using glucose as

t3.1 **Table 3** Recombinant biosurfactant producers

t3.2 Biosurfactants	Microbial strains	References
t3.3 Rhamnolipids	*Pseudomonas putida* 1067(PNE2)	Cha et al. (2008)
t3.5	*Pseudomonas fluorescens*	Ochsner et al. (1995)
t3.7	*E. coli* W3110, *E. coli* HB101	Cabrera-Valladares et al. (2006)
t3.10	*P. putida* KT2440	Wittgens et al. (2011)
t3.12	*Burkholderia kururiensis* KP23(T)	Tavares et al. (2013)

carbon substrate (Cabrera-Valladares et al. 2006). Similarly, recombinant *E. coli* HB101 was also detected to produce 52 mg L^{-1} rhamnolipid with oleic acid as substrate (Cabrera-Valladares et al. 2006). A successful development of a non-pathogenic host capable of producing mono-rhamnolipids using glucose as substrate has been reported by Wittgens et al. (2011). In this study, the synthesis of mono-rhamnolipid independent from biomass formation was done using *P. putida* KT2440 expressing the *rhlAB* genes from *P. aeruginosa* PA01. A sevenfold increase in the final rhamnolipid concentration from 0.22 to 1.5 g L^{-1} was recorded after genetic optimisation of the strain. The engineered strain exhibited some advantages compared to rhamnolipid production in *P. aeruginosa*. Firstly, these strains are nonpathogenic and can appropriately substitute the opportunistic pathogenic *P. aeruginosa*. Secondly, rhamnolipid production in the recombinant strains was void of the complex quorum sensing regulation. However, the production rate in the recombinant strain was about two thirds of that usually obtained in optimised fermentation with *P. aeruginosa* although there remains the possibility of increasing the production rate by increasing the availability of activated rhamnose in the medium. More importantly, compared to *P. aeruginosa*, it is the utilisation by the recombinant strains of glucose instead of hydrophobic substances. Glucose as a carbon substrate is relatively cheap and is applied in many biotechnological processes (Blank et al. 2008). Furthermore, these recombinant strains capable of substituting *P. aeruginosa* can utilise hydrophilic substances, reaching a maximum rhamnolipid concentration in medium after 1–2 days. This is compared with the wild-type strain of *P. aeruginosa* PA01 that although it exhibits higher rhamnolipid production, it requires 1–3 days to reach their maximal levels. This reduction in process time exhibited by recombinant strains enhances the space-time yield for the process (Wittgens et al. 2011). According to this investigation, the engineered *P. putida* KT2440 is the second recombinant strain featuring the highest recorded space, time, and yield, therefore making this strain a potential industrial tool for biotechnological rhamnolipid synthesis.

Burkholderia kururiensis KP23(T), a trichloroethylene-degrading, nitrogen-fixing and plant growth-promoting bacterium, has been reported to produce rhamnolipids, and when genetically engineered with two biosynthetic enzymes from *P. aeruginosa* RhlA and RhlB, rhamnolipid production increased by sixfold compared to wild-type strain. Rhamnolipids produced by the engineered strains were mainly mono-RL as opposed to wild-type strains *B. kururiensis* and *P. aeruginosa* that predominantly produce di-RLs. This organism is a promising biosurfactant producer with potential environmental and biotechnological application especially due to its nonpathogenicity and compatibility with metabolic engineering procedures (Tavares et al. 2013).

5 Conclusion

The development of biosurfactant production in nonpathogenic organisms is a current challenge that is receiving increased attention in order to avoid pathogenicity and complex metabolic regulations. Most biosurfactant-producing organisms are pathogenic and difficult to handle in large-scale industrial processes. Pathogenicity of biosurfactant producers is key factor to prevent large-scale production; therefore, the nonpathogenic eco-friendly organisms need to be explored further, and a library of collections is important for the future development in this area of study.

Acknowledgment The authors wish to thank the Commonwealth Commission for the financial support for this project.

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Author Queries

Chapter No.: 11 0002193698

Queries	Details Required	Author's Response
AU1	Please check if edit to sentence starting "Excellent emulsifying properties…" is okay.	
AU2	Please provide details for Fujita et al. (1998), Jorfi et al. (2014), and Gutierrez et al. (2012) in the Reference list.	
AU3	Please fix 'a' or 'b' for the citation Rodrigues et al. (2006) here and in similar instances.	
AU4	Please check if edit to sentence starting "This is compared with the…" is okay.	
AU5	Please cite Heyd et al. (2007) in the text.	
AU6	Please provide publisher's name and location in Abouseoud et al. (2007).	
AU7	Please provide complete details for Hutkins (2002) and Joshi et al. (2013)	
AU8	Please provide editor name(s) for Korzybski et al. (1978).	
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