

Review Article

A review on microbial surfactants: production, classifications, properties and characterization

Abstract: Surfactants are a surface-active group of molecular compounds with hydrophobic and hydrophilic moieties in one single molecule that distributes themselves between two immiscible fluids, reduce surface/interfacial tensions and cause the solubility of non-polar compounds in polar solvents. Besides surface and interfacial activities, they display properties such as solubilization, detergency, lubrication, emulsification, stabilization and foaming capacity. Microbiologically derived surfactants are called biosurfactants. They are produced as either metabolic products or as the surface chemistry of an actual cell. The employment of screening techniques such as surface tension measurements, drop collapse test, oil spreading assay, emulsification index (%EI24), CTAB/methylene blue agar plate test and strain characterization. Others are analytical techniques including liquid chromatography-mass spectroscopy, thin layer chromatography, high-performance liquid chromatography, Fourier transform infrared spectroscopy, nuclear magnetic resonance, fast atom bombardment-mass spectrometry and electrospray ionization-mass spectrometry. These have led to the identification of biosurfactant producing microorganisms, properties and characterization of biosurfactants. Therefore, this review tends to provide the current knowledge of the screening techniques and chromatography/spectroscopic tools employed to study biosurfactants. Results from a detailed study of these tools can unveil new surfactant producing microorganism, decipher chemical diversity and multifunctional properties of biosurfactants critical for applications in diverse industrial sectors.

Keywords: Biosurfactants; chromatography; screening techniques, spectrometry; surface tension

1 Introduction

Surfactants are surface-active group of molecular compounds with hydrophobic and hydrophilic moieties in one single molecule and tend to distribute themselves between two immiscible fluids, reduce surface/interfacial tensions (ST/IFT) and cause the solubility of non-polar compounds in polar solvents [1,2]. They display properties such as solubilization, detergency, lubrication; have stabilizing and foaming capacity [3,4]. Surfactants are either produced chemically or biologically. The biologically derived surfactants are known as biosurfactants (BSs) since they are produced from living entities especially microorganisms. These molecules are produced as metabolic products or the surface chemistry of the cells themselves [5]. Majorly, BSs are produced from aerophilic microbes in aqueous media with carbon source feedstock such as hydrocarbons, carbohydrates, fats and oil which are mostly from bacteria genera (*Pseudomonas*, *Bacillus* and *Acinetobacter*), fungi genera (*Aspergillus*, *Fusarium*) and yeast (*Candida*, *Pseudozyma*) [6]. The most common BSs are rhamnolipids, surfactins, sophorolipids, emulsans, mannosylerythritol lipids. These surface-active compounds play a physiological role for the benefit of the BSs producing microorganisms to grow on water-immiscible substrates, ensure exponential biomass increase, exhibit antimicrobial activities against possible predators, make them survive inhospitable environmental conditions, virulence and cell desorption for survival [7] The physiological roles differ with the class a particular biosurfactant belongs to.

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40 Broadly, biosurfactants are grouped into low and high molecular weight (LMW and HMW) biosurfactants.
41 The LMW-BSs lower ST and ITF while the HMW-BSs are more of emulsion-stabilizing agents. Glycolipids,
42 lipopeptides and phospholipids belong to the LMW biosurfactants while the HMW biosurfactants are particulate and
43 polymeric [8,9]. Based on chemical composition, biosurfactants are grouped into glycolipids (rhamnolipids,
44 sophorolipids, trehalolipids, mannosylerythritol lipids), lipopeptides (surfactin, lichenysin, iturin, fengycin,
45 serrawettin), fatty acids/phospholipids/neutral lipids (phosphatidylethanolamine, spiculisporic acid), polymeric
46 biosurfactants (emulsan, alasan, biodispersan, liposan) and particulate biosurfactants (vesicles, whole-cell) [10-12].
47 This classification is made possible by chromatographic and spectroscopic studies. Studies conducted with these
48 analyses had proven the hydrophobic moiety of BSs to be a long-chain fatty acid while the hydrophilic component
49 could either be alcohol, amino acid, carbohydrate, phosphate, carboxyl acid or cyclic peptide [13].

50 These components of the biosurfactants elicited in the supernatant are subjected to different surfactant
51 activity tests which include hemolytic activity test, ST measurements, drop collapse test, oil spreading assay,
52 emulsification index (%EI₂₄), CTAB/methylene blue agar plate test and strain characterization [14-18]. In an
53 optimum environmental condition (including availability of carbon and other nutrients sources) a competent
54 microorganism can yield enough biosurfactants in a bioreactor which can be extracted by several available options
55 (acetone precipitation, ethanol precipitation, acid precipitation, solvent extraction technique, etc.) which can be
56 purified by either dialysis/lyophilization, thin layer chromatography or isoelectric focusing [19-22]. Purified
57 biosurfactants can be characterized using nuclear magnetic resonance (NMR), liquid chromatography-mass
58 spectroscopy (LC-MS), high-performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy
59 (FT-IR) [19,23-25]. The employment of these techniques has led to the identification of novel biosurfactant
60 producing microorganisms and uncommon biosurfactants as reported in scientific literature. Therefore, the purpose
61 of this review is to provide current knowledge on biosurfactant-producing microorganisms, the screening techniques
62 and the chromatography/spectroscopic tools employed to study biosurfactants.

63 64 **2 Biosurfactants**

65 Biosurfactant is a portmanteau word that means surfactant from biological origin. Surfactant represents a
66 group of molecular compounds made up of tensio-active agents incorporated with both hydrophobic and hydrophilic
67 components that reduce ST and ITF by being distributed at the adjoining point of the two immiscible fluids, thereby
68 causing the solubility of one of the fluids in the other [2,18]. Their ability to reduce ST and IFT in most cases is
69 accompanied by detergency, lubrication, solubilisation and phase dispersion [4]. Therefore, from a technical point of
70 view, biosurfactant represents surface-active agents which are either metabolic products produced by cells or the
71 surface chemistry of the actual cells [5] with an amphiphilic property that enable them to shape micelles that collect
72 at the interface between fluids of varying polarities with ultimate reduction of pressure and interfacial tension
73 pressure [26]. They are different from synthetic surfactants in that they are non-toxic, biodegradable, specific and
74 tolerant to extreme conditions [9]. They are majorly produced by aerophilic microorganisms in aqueous media with
75 either carbohydrates or hydrocarbons or fats, and oils as carbon source feedstock. These BSs are mostly from
76 bacteria, fungi and yeast [27,6] though plants and humans also produce biosurfactant [28]

77 Genera of *Pseudomonas*, *Bacillus* and *Acinetobacter* dominate the literature space of biosurfactants
78 production [4]. Notable genera among the yeasts
79 are *Torulopsis*, *Pseudozyma*, *Saccharomyces*, *Rhodotorula* and *Kluyveromyces* [29-31]. For the
80 fungi, *Aspergillus*, *Ustilago*, *Fusarium*, *Trichoderma*, *Penicillium* [32-37] are well reported in research publications.
81 A summary list of some species of bacteria, yeast and fungi that produce biosurfactants are listed in Table 1.

82

83 **3 Factors affecting biosurfactant production**

84 **3.1 Biosurfactant production**

85 Production of biosurfactants starts from sampling location. There are three commonly reported
86 environmental media that are commonly sampled which are stressed soil, stressed aquatic environments or pristine
87 environmental media [15-17] However, the most interesting results emanate from ecologically compromised
88 environments, mostly with hydrophobic compounds. Examples of sites where biosurfactants producing microbes
89 have been isolated are diesel polluted soil; [14] in an oilfield; [38] from extreme environments; [16] from an oil
90 reservoir; [39] in an automobile garage; [40] from sea harbor [41] and unpolluted soil. Media used for the isolation
91 of the microbes are mostly mineral salt media (MSM) and incorporated with organic substrates as the sole carbon
92 source and nutrient broth. The carbon source mostly used is hydrophobic [41-43] however; cheap renewable
93 hydrophilic carbon sources are currently used. Nwachi *et al.* [44] Glucose is the sole carbon source in MSM.
94 Competent microorganisms are inoculated into a broth media having suitable carbon, nitrogen and in a controlled
95 environment within optimum conditions. Biosurfactant (BS) production can be done through laboratory-scale or on
96 large scale (fermentation). A laboratory-scale production of BSs is illustrated in Fig. 1.

97

98 **3.2 Factors affecting biosurfactant production**

99 **3.2.1 Nutrient factors and salt concentration**

100 **Carbon sources** do influence the quantity and quality of BS production [45]. Crude oil, diesel, sucrose, glucose,
101 glycerol are good carbon sources for BS production [7] However, researchers are now focusing attention on the use
102 of wastes to cut down the cost of downstream processing.

103 **Nitrogen** is a limiting nutrient and it is essential in the formulation of medium for BS production because it is very
104 critical for microbial growth, protein and enzyme syntheses. Yeast, meat and malt extracts, urea, peptone,
105 ammonium sulphate, nitrate and sodium nitrates are common nitrogen sources used in BS production [46,7]

106 **Phosphate** is also very important for the growth of microorganisms. It is usually provided in triphosphates form.
107 Maqsood and Jamal [47] reported that cultivation of gram-negative bacterium on ethanol with a low phosphate
108 concentration yielded a maximum concentration of rhamnolipids. A mutant strain of *P. aeruginosa* (mutation caused
109 by N-methyl-N-nitrosoguanidine) in a study produced 10 times more of rhamnolipid in comparison to the parental
110 strain at 200 rpm/37°C [48].

111 **Salt concentration** is expected to influence BS production since cellular activities of microorganisms are affected
112 by salt concentration [49]. Md [7] noticed in their study that some biosurfactant products were not affected by salt
113 concentrations up to 10% (weight/volume), though a slight reduction in the critical micelle concentration (CMC)

114 value was detected. A range of 1-10% of NaCl concentration has been proven to have an optimal influence
115 on *Pseudomonas aeruginosa*, which produces rhamnolipids [49,50]. Table 2 shows the optimum conditions under
116 which some microorganisms produce maximum yield of BSs.

117

118 3.2.2 Environmental factors

119 Environmental factors are extremely important because it affects the characteristics and output of BS. To
120 obtain an appreciable yield of BS, it is vital to optimize the bioprocess as the product may be susceptible to changes
121 in pH, temperature, agitation speed or aeration.

122 **Temperature** between 25-37 °C influences the growth of biosurfactant producing organisms [51]

123 A **pH** of culture medium around 8 has been reported to ~~accompany~~ enhance the best production of biosurfactants
124 [31] However, Jagtap *et al.* [52] reported the optimal pH to be 7 for most microorganisms. Bacteria tend to do best
125 at alkaline pH while yeast and fungi thrive best in acidic condition, but there are some exceptions. For
126 instance, *Yarrowia lipolytica* experience its optimal growth at pH 8 [53] and *Lactobacillus* spp. thrives at a pH of 6
127 [54].

128 **Incubation periods** also affect biosurfactants production. Auhim and Mohamed51 demonstrated that optimal
129 incubation period for *Azotobacter chroococcum* is 4 days. Fontes *et al.* [53] noted 24 h for *Yarrowia lipolytica* while
130 Bhardwaj *et al.* [36] reported 8 days for *Candida lipolytica*.

131 **Aeration and agitation** are very important factors that influence BS production since they both facilitate oxygen
132 transfer into the culture medium [26]. Adamczak and Bednarski [46] demonstrated that improved yield value of BS
133 (45.5 g/l) was achieved when the air-flow-rate was 1 vvm and the dissolved O₂ concentration was sustained at 50%
134 saturation. Agitation of between 120 rpm to 200 rpm is most common in microbial growth studies [56]. It is safe to
135 conclude that incubation period remains the most unpredictable.

136

137 4 Properties of biosurfactants

138 Biosurfactants properties such as ST reduction, detergency, emulsifying capacity, foaming capacity,
139 stabilizing capacity, low-CMC and solubility, are key in performance evaluation of BS and selection of
140 microorganisms with BS producing potentials [57] Though chemical composition diversity and properties may
141 differ, some properties are common to most of the biosurfactants [58]

142 4.1 Surface and interface activity

143 An efficient BS reduces fluid ST at a lower concentration in comparison to synthetic surfactants or
144 ineffective BS. The CMC of biosurfactants (a measure of effectiveness) ranges from 1-2000 mg/l, whereas IFT
145 (oil/water) and ST are around 1 and 30 mN/m respectively [4]. According to [26] a good surfactant should lower ST
146 of water from 72 to 35 mN/m and the IFT of water/hexadecane from 40 to 1 mN/m. For example, rhamnolipids
147 lower the ST of water and IFT of water/hexadecane to 26 mN/m and 1 mN/m respectively; surfactin from *B.*
148 *subtilis* reduces the ST of water to 25 mN/m while the IFT of water/hexadecane to less than 1 mN/m and

149 sophorolipid from *T. bombycolia* reduces the ST to 33 mN/m and the IFT to 5 mN/m. In general, BSs are more
150 effective and powerful since their CMC are lower than chemical surfactants [26].

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152 **4.2 Tolerance to pH, ionic strength, temperature**

153 Many BSs and their surface activities are not much affected by environmental parameters such as pH and
154 temperature. For example, the lipopeptide produced by *Bacillus licheniformis* JF-2 was tolerant to a temperature of
155 75 °C for up to 140 hours and a pH range of 5-12 [4]. Biosurfactants also tolerate high salt concentrations up to 5
156 times the concentration (2%) that could inactivate synthetic surfactants [21]. A lipopeptide from *B. subtilis* was
157 subjected to different extreme conditions (autoclaving condition (121°C/15 minutes), -18 °C for 6 months, varying
158 pH between 5-11 and 20% NaCl concentrations) without losing its surface activity property [26]. Mukherjee [58]
159 demonstrated that a BS produced by *Arthrobacter protophormiae* withstood a temperature of 30-100 °C and a pH of
160 2 to 12. Since industrial processes pass through extreme pH, temperature, and pressure [2], it is expedient to use
161 biosurfactants in industries that require extreme conditions.

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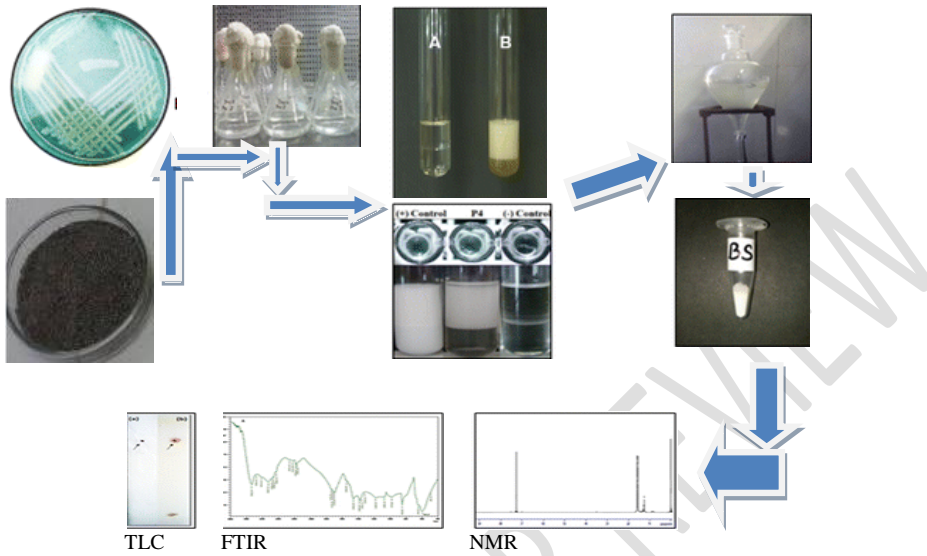
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UNDER PEER REVIEW

164 Table 1. Biosurfactant producing species of bacteria, fungi and yeast

Genus	Phylum/Division	Class	Species	Note
	Bacteria			
<i>Pseudomonas</i>	Proteobacteria	Gammaproteobacteria	<i>P. aeruginosa</i>	Gram negative, rod-shaped, an opportunistic pathogen, versatile
<i>Bacillus</i>	Firmicutes	Bacilli	<i>B. subtilis</i>	Gram positive, rod-shaped, found commonly in soil, tolerant
<i>Acinetobacter</i>	Proteobacteria	Gammaproteobacteria	<i>A. calcoaceticus</i>	Non-motile, gram negative coccobacillus, commensal in humans
<i>Rhodococcus</i>	Actinobacteria	Actinobacteria	<i>R. erythropolis</i>	Nonsporulating, non-motile, gram positive, hydrocarbon degrader
<i>Mycobacterium</i>	Actinobacteria	Actinobacteria	<i>M. aurum</i>	Non-pathogenic, fast-growing saprophytic mycobacterium
<i>Serratia</i>	Proteobacteria	Gammaproteobacteria	<i>S. marcescens</i>	Gram negative, rod-shaped opportunistic pathogen
<i>Corynebacterium</i>	Actinobacteria	Actinobacteria	<i>C. kutscheri</i>	Gram negative, pathogenic to rodents but can exist as free living
<i>Nocardia</i>	Actinobacteria	Actinobacteria	<i>N. amarae</i>	Slow-growing, often gram positive, alkyl benzenes degrader
<i>Lactobacillus</i>	Firmicutes	Bacilli	<i>L. casei, jensenii</i>	Probiotic found in gut, have wide pH and temperature range
<i>Arthrobacter</i>	Actinobacteria	Actinobacteria	<i>A. paraffineus</i>	A soil microorganism, Gram-positive obligate aerobes
	Fungi			
<i>Aspergillus</i>	Ascomycota	Eurotiomycetes	<i>A. flavus</i>	Ubiquitous mold few of which causes illness in humans
<i>Fusarium</i>	Ascomycota	Sordariomycetes	<i>F. proliferatum</i>	Filamentous fungi widely found in soil and associated with plants
<i>Penicillium</i>	Ascomycota	Eurotiomycetes	<i>P. crysogenum</i>	Industrially important fungi and usually the most abundant in soil
<i>Ustilago</i>	Basidiomycota	Ustilaginomycetes	<i>U. maydis</i>	Smut fungi parasitic to grasses
<i>Trichoderma</i>	Ascomycota	Sordariomycetes	<i>T. viride</i>	Many species are opportunistic avirulent plant symbionts
<i>Mucor</i>	Zygomycota	Zygomycetes	<i>M. mucedo</i>	Ubiquitous filamentous fungi that causes mucormycosis
<i>Rhizopus</i>	Zygomycota	Zygomycetes	<i>R. oryzae</i>	Ubiquitous filamentous fungi that seldom causes serious infections
<i>Phoma</i>	Ascomycota	Dothideomycetes	<i>P. complanata</i>	A dematiaceous filamentous fungus found in plants and soil
<i>Curvularia</i>	Ascomycota	Dothideomycetes	<i>C. clavata</i>	A facultative pathogen of many plants and common in soil
	Yeast			
<i>Candida</i>	Ascomycota	Ascomycetes	<i>C. albicans</i>	Yeast found in soil/humans and when overgrown causes disease
<i>Saccharomyces</i>	Ascomycota	Ascomycetes	<i>S. cerevisiae</i>	Brewer's or baker's yeast important in food production
<i>Pseudozyma</i>	Basidiomycota	Ustilaginomycetes	<i>P. rugulosa</i>	Environmental yeast that rarely cause diseases
<i>Yarrowia</i>	Ascomycota	Saccharomycetes	<i>Y. lipolytica</i>	A yeast that can use unusual carbon sources
<i>Rhodotorula</i>	Basidiomycota	Microbotryomycetes	<i>R. babjevae</i>	An environmental yeast that acts as an opportunistic pathogen
<i>Kluyveromyces</i>	Ascomycota	Saccharomycetes	<i>K. marxianus</i>	A probiotic yeast with industrial applications
<i>Aureobasidium</i>	Ascomycota	Dothideomycetes	<i>A. pullulans</i>	A yeast-like fungi that is ubiquitous, isolated as saprophytes
<i>Geotrichum</i>	Ascomycota	Saccharomycetes	<i>G. cadidum</i>	A ubiquitous filamentous yeast-like fungi
<i>Galactomyces</i>	Ascomycota	Saccharomycetes	<i>G. geotrichum</i>	A yeast used as moisturizing agent with antioxidant effect
<i>Apiotrichum</i>	Basidiomycota	Tremellomycetes	<i>A. loubieri</i>	An anamorphic basidiomycetous yeast genus

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171 Fig. 1. Schematic presentation of a laboratory-limited biosurfactant production procedure: sampling to isolation to
172 growth in MSM to screening of extraction and purification of biosurfactants to characterization of extracted
173 biosurfactant. Adapted from Singh and Tiwary51

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Table 2. Optimum conditions for key biosurfactant producing bacteria, yeast and fungi

Biosurfactant producer	Carbon source	Nitrogen source	Temperature	pH	Reference
<i>Pseudomonas aeruginosa</i>	Glycerol and other water-soluble carbons	Sodium and ammonium nitrates	30 -37 °C	7-8	Ikhwani <i>et al.</i> [49], Maqsood and Jamal [47]
<i>Bacillus subtilis</i>	Glucose (40 g/l) in the presence of activated carbon	Urea (6 g/l)	37 °C	7	Mnif <i>et al.</i> [59], Asad <i>et al.</i> 60, Yeh <i>et al.</i> [56]
<i>Acinetobacter calcoaceticus</i>	Hydrocarbons	Sodium nitrate (2 g/l)	25 – 33 °C	8	Ohadi <i>et al.</i> [61], Vigneshwaran <i>et al.</i> [62]
<i>Lactobacillus fermentum</i>	Lactose	Peptone/ meat extract	25 – 37 °C	6	Satpute <i>et al.</i> [63], Gudina <i>et al.</i> [64]
<i>Candida lipolytica</i>	Glucose and canola oil (10% each)	Yeast extract and ammonium nitrate	27 °C	5	Bhardwaj <i>et al.</i> [36]
<i>Saccharomyces cerevisiae</i>	Galactose and fructose	Peptone water 94.0 mg/100ml	32 °C	4-11	Salari <i>et al.</i> [65]
<i>Pseudozyma antarctica</i>	Soybean oil	Yeast extract and urea	30 °C	-	Bhardwaj <i>et al.</i> [36]
<i>Penicillium spp</i>	Soybean oil (20 g/l)	Yeast extract (30 g/l)	35 °C	9	Sena <i>et al.</i> [66]
<i>Fusarium spp</i>	Ethanol acetate/methanol (5;1)	-	40 °C	5-9	Qazi <i>et al.</i> [67]

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186 **4.3 Biodegradability and low toxicity**

187 Microbial surfactants, like other microbiologically derived compounds, can be easily degraded unlike the
188 synthetic surfactants so they can be applied in bioremediation, biosorption and waste management [68,69]. Though
189 very little data are available that give credence to BS toxicity, they are generally accepted as non-toxic, proving they
190 can be used in health-related industry [26] The latter author disclosed that Corexit (a synthetic anionic surfactant) is
191 ten times more lethal than rhamnolipids through the use of LC₅₀ test against *Photobacterium phosphoreum*. This
192 signifies that Corexit is far more toxic than rhamnolipids and possibly the comparison will be so between synthetic
193 surfactants and microbial surfactants. A comparative study between biosurfactant from *P. aeruginosa* and a popular
194 industrial synthetic surfactant revealed that the synthetic surfactants are toxigenic and mutagenic unlike the
195 biosurfactants- [26].

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197 **4.4 Physiological properties**

198 Microbial surfactants are secreted either extracellularly or attached to parts of cells during growth on
199 hydrophobic substrates. Biosurfactants:

200 I) Allow microbes to grow on water-immiscible substrates by reducing the surface tension at the interface, thereby
201 making substrates/nutrients soluble for uptake, which is necessary for metabolism.

202 II) Ensure exponential biomass increase needed by microorganisms by way of making soluble hydrocarbons as
203 carbon substrates and energy source, and also utilizing the biosurfactants themselves.

204 III) Modify bacterial cell surface properties. Kaczorek [70] highlighted some of the effects of biosurfactants on
205 bacterial cells which include among other things alteration in biomorphology, cell surface hydrophobicity, surface
206 functional groups, and electrokinetic potential.

207 IV) Exhibit antimicrobial activities towards various microorganisms. Yuliani *et al.* [71] demonstrated
208 that *Bacillus subtilis* C19 produced lipopeptide that had selective antimicrobial effects against *Candida albicans*.
209 Biosurfactants also dissolve cell surface structure by their detergency property.

210 V) Impart stability under hostile environmental conditions, virulence and in cell desorption when organisms need to
211 find new habitats for survival [26].

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214 **5 Basic analysis in biosurfactant study**

215 **5.1 Biosurfactant screening methods**

216 Isolates (or supernatants) that exhibit good growth are subjected to various biosurfactants activity tests
217 (Table 3) which include hemolytic activity test, surface tension measurements, drop collapse test; [17,72] oil
218 spreading assay; [18] emulsification activity; [73] emulsification index (%EI₂₄) [74] CTAB/methylene blue agar
219 plate test [14], Penetration assay for high throughput screening [25], and molecular characterization [16]
220 Biosurfactants producing microorganisms are characterized through this order of steps: cultural isolation,
221 purification of isolates, DNA extraction, a polymerase chain reaction (PCR) of DNA, sequencing and phylogenetic
222 analysis [16] Each of these steps is described in Table 4.

223 Table 3. Description of the basic analysis carried out in biosurfactants study

Biosurfactant activity test	Method	Criterion for inference
Hemolytic activity	Inoculate isolates on blood agar medium (5% of fresh human blood) and incubate at 28 °C for 48-72 hours. The hemolytic activity will be assessed based on α , β and γ type hemolysis to ensure preliminary conformation on biosurfactant activity	If agar under the colony is dark and greenish (α -hemolysis); yellow and transparent (β -hemolysis) or remain unchanged then (γ -hemolysis)
Drop collapse test	Drops of oil placed on a slide and then add 10 μ l of the supernatant by piercing the drop using micropipette without disturbing the dome shaped of the oil.	If the drop collapsed within 1 minute then the test is considered to be positive for the drop collapse test.
Oil spreading test	Add 40 μ l of distilled water into a Petri dish followed by the addition of 20 μ l of diesel oil to the surface of water then 10 μ l of supernatant dropped on to the oil surface.	If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is developed.
Emulsification index (%EI ₂₄)	Add 2 ml of oil to the same amount of supernatant in a glass tube, then mixing it with a vortex for 2 minutes and leaving it to stand for 24 hours.	Biosurfactants float on the upper part of the tube. %EI ₂₄ is calculated by height of the biosurfactant divided the total liquid in the tube multiplied by 100
Blue agar plate test	Prepare Bushnell Hass agar medium containing glucose (2%), CTAB (0.5 mg/ml) and methylene blue (0.2 mg/ml). Create equidistant wells using cork borer (4 mm) Add 30 μ l of supernatant into the labeled wells and incubate at 37 °C for 48-72 hours	If test is positive dark blue complex will be formed indicating the presence of anionic biosurfactants
Surface tension measurement	Pre-cultures of strains were prepared in a nutrient broth. A volume of 1 ml of inoculum was added to 100 ml mineral salt solution and 1% of filtered oil as hydrocarbon source. The mixtures with control samples (100 ml MSS and 1% filtered oil without bacterial strains) were incubated at 30 °C on shaker at 150 rpm for 3 days. Measure surface tension with a tensiometer	If surface tension in the test sample is significantly lower than the surface tension in the control then test is positive
Penetration assay	The cavities of a 96 microplate wells are filed with 150 μ l of hydrophobic paste consisting of silica gel and oil. The paste is covered with 10 μ l of oil followed by placing of coloured supernatant consisting of 90 μ l of supernatant and 10 μ l of a red staining solution	Colour will change from red to cloudy white if biosurfactants are present within 15 minutes.

224 Adapted from Mahalingam and Sampath [14]

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226 Table 4. Steps and common procedures involved in molecular characterisation of biosurfactants
227 producing microbes

Stages	Bacteria	Fungi	Yeast
Isolation and purification	Enrichment with Bushnell Hass (BH) both supplemented with 1% diesel and culturing in BH medium with 1% diesel [75]	About 1 ml of soil suspension is plated out on potato dextrose agar [33]	Enrichment and culturing of yeast was carried out on yeast extract peptone dextrose broth and agar plate respectively [80]
DNA extraction	Conventional (CTAB method) and rapid approaches	Conventional (CTAB method) and rapid approaches	Conventional (CTAB method) and rapid approaches
PCR amplification	The 27F and 1492R primer pairs used for amplification [16]	The ITSF and ITS4R primer used for amplification [78]	The ITSF and ITS4R primer used for amplification [81]
Sequencing	The amplicons were sequenced using a model ABI 3700 capillary sequencer [76]	The PCR products were sequenced by mean of the mentioned primers in an Applied Biosystem 3130 sequencer [78]	The PCR amplicons sequenced using an automatic sequencer (ABI 3730) [81]
Phylogenetic analysis	BLAST software is used in comparing sequence in GenBank. Sequences are aligned using the software CLUSTALX. Phylograms were constructed using MEGA software, with a 1,000-repetition bootstrap [77]	IT Sequences are downloaded from Gene Bank. Alignment done using the clustal W. in MEGA 7. Maximum parsimony analysis is done and branches is supported by the bootstrap (1000 replicates) method [79]	Sequence comparisons were performed using the BLAST program. Alignment by CLUSTALW and phylogenetic tree constructed with MEGA 7 software with bootstrap of 1000 repetition [82]

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230 5.2 Estimation of biosurfactant activity

231 Biosurfactant activity can be estimated by measuring its ability to change ST and hydrophilic-lipophilic
232 balance (HLB). When a significant amount of (bio)surfactant is introduced into a liquid system, a critical value (CV)
233 is reached where the ST decreases no further. Above this CV, biosurfactant monomers aggregate to form bilayers,
234 vesicles and micelles. This CV represents CMC which can be measured. Reduction of ST, IFT and CMC values can
235 be measured. A new surfactant is usually compared with a surfactant of known HLB value to predict its property.
236 The HLB value is between 0 and 20 [83]. The HLB can be calculated as follows:

$$237 \text{ HLB} = 20(\text{MWHP}/\text{MWSA})$$

238 Where MWHP stands for the molecular weight of the hydrophilic moiety, and MWSA stands for the molecular
239 weight of the whole surfactant. The HLB value provides grounds for prediction of a surfactant or biosurfactant
240 property as depicted in Table 5.

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Table 5. Corresponding predicted properties of (bio)surfactants to HLB value.

HLB value	Predicted function	Application
0-3	Anti-foaming agent	Used in fermentation process
4-6	Water/oil emulsifiers	Improving diesel fuel
7-9	Wetting agent	Aid nutrient uptake in plants
8-18	Oil/water emulsifier	Bioremediation of pollutants
13-15	Typical detergent	Industrial laundry detergents
10-18	Solubilizer	In enhanced oil recovery

243 Adapted from De *et al.* [83]

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247 6 Crude extraction of biosurfactants

248 A good number of methods exist for extracting biosurfactants among which are centrifugation, acetone
249 precipitation, ethanol precipitation, acid precipitation, ion-exchange chromatography, adsorption-desorption,
250 filtration and precipitation, foam fractionation, isoelectric focusing, ultrafiltration, dialysis and lyophilization and
251 solvent extraction [20] Solvent extraction will be explained here while others are summarized in Table 6. The
252 hydrophilic moieties of biosurfactants are soluble in non-polar solvents which make the extraction easy. Organic
253 solvents such as chloroform, methanol, butanol, hexane, acetic acid and isopropanol are commonly used for
254 biosurfactants extraction. To execute solvent extraction, the microorganisms is cultured in MSM broth for an
255 optimum incubation period on a shaker at 120 rpm at 37 °C, centrifuged at 15×10^3 rpm for 15 minutes at 4 °C. The
256 supernatant is then treated with concentrated HCl until the pH is two; and left for 24 hours at 4 °C. After 24 h
257 centrifuge the acidified supernatant at 15×10^3 rpm for 15 minutes at 4 °C and collect grey white precipitate that will
258 be formed for further extraction of the biosurfactants. Chloroform and methanol in the ratio (2:1 v/v) should be
259 added to precipitate the pellet and incubate at 30 °C for 15 minutes. Then centrifuged for 20 minutes under cooling
260 conditions and allow supernatant to evaporate by air drying. Dispense the product in sodium phosphate buffer (pH
261 7) and stored at 4 °C [42].

262

263 7 Purification of biosurfactants

264 There are good numbers of biosurfactants purification techniques, but the common ones are discussed here.

265 **7.1 Thin-layer chromatography** is a method used for the exploratory characterization of BSs. A part of the crude
266 BS is separated on a silica-gel-plate using chloroform: methanol: water (10: 10: 0.5 v/v/v) mixture. The type of
267 biosurfactant is characterized by utilising a developing solvent system with different colour developing reagent
268 like ninhydrin. This reagent is applied to detect lipopeptide as a red spot, produced by biosurfactant [84]
269 Sumaiya *et al.* [85] carried out TLC analysis and spotted sediments recovered from extracted biosurfactants on
270 a TLC plate and sprayed with phenol sulphuric acid reagent. Brown spots were developed with an Rf value of
271 0.65 which indicates lipopeptide. Rhamnolipid was the standard biosurfactant they used.

272 **7.2 Dialysis and lyophilization** method is easy and cost-effective and widely exploited to enhance the purity of
273 biosurfactant by using seamless cellulose dialysis bags. The collected precipitate containing the biosurfactant is
274 dissolved in 5 -10 ml of sterile distilled water and dialyzed against double distilled water for 48 hours at 10 °C.
275 The dialysate is stored at 4 °C in an airtight container for further use [20]

276 **7.3 Isoelectric focusing (IEF)** is one of the new approaches used for purification of biosurfactants. Its unit
277 comprises of a single column, filled with density gradient solutions, electrolyte and non-ion conducting
278 polymers. In the presence of electric influence, pH, density gradient, the ampholyte moves in the column until it
279 reaches a neutral pH. The columns help to segregate fractions based on changes in pH. Once total separation
280 occurs, electro-focusing is discontinued and the activity of purified BE is compared with the crude form [20]
281 This procedure requires 10-12 hours at 400 V and a current of 1.5 A [86].

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285 **Table 6. Selected techniques for biosurfactants extraction**

Method	Description	Reference
Acetone precipitation	Culture is grown in a mineral salt medium supplemented with required constituents. Cell-free supernatant is mixed with ice-cold acetone to precipitate biosurfactants which is further suspended in phosphate buffer. Then mixture is incubated at 4 °C for 15 –20 h to get the precipitated biosurfactants.	Patil and Chopade [87]
Ethanol precipitation	Culture broth is centrifuged at 11,000 x g for 20 minutes at 4 °C and biosurfactant is precipitated from the supernatant by using cold ethanol.	Phetrong <i>et al.</i> [88]
Acid precipitation	Acid hydrolysis is carried out by adding concentrated HCl to the supernatant to bring down the pH to 2 for the precipitation the biosurfactants at 4 °C. Centrifugation is followed and the pellet is further extracted by using appropriate solvent. Extracted material is filtered for removal of residues and evaporated to dryness using rotary evaporator.	Nitschke and Pastore [21]
Centrifugation	Following acid precipitation, biosurfactants-containing broth can be centrifuged at 12,000 rpm for 15 min at 4 °C to be easily collected as crude product. Once the pellet is obtained, it can be dried under N ₂ and extracted with solvents.	Nitschke and Pastore [21]
Ammonium sulphate precipitation	This method is used to precipitate high molecular weight biosurfactants such as emulsan, biodispersan. In this case the biosurfactant is precipitated by salting out process and the product is purified by dialysis procedure and lyophilized	Vandana and Singh [19]
Ion exchange chromatography	This method is carved out for anionic biosurfactants. Ion exchange resin is used to attract the biosurfactants at higher pH. The biosurfactant is eluted with a buffer	Satpute <i>et al.</i> [20]

	containing 10% (v/v) ethanol. Addition of a minimum of 0.6 NaCl to the buffer releases the biosurfactant from the resin	
Adsorption-desorption	Cell-free supernatant is added directly to the adsorbent column and 0.1 M phosphate buffer (pH 6.1) is used to equilibrate it. Exhaustion of the adsorbent resin is observed by ultra violet (U.V.) absorption. A wash of distilled water is given to the resin for removal of pigments and free fatty acids. Further, elution is carried out with methanol, which can be evaporated to obtain crude biosurfactants.	Abalos <i>et al.</i> [89]
Foam fractionation	Foam is collected through fractionation column and acidified with HCl down to pH 1.0–2.0 to precipitate biosurfactants, which can be extracted with solvents. High yield of biosurfactants can be achieved by increasing the residence time of foam in the fractionation columns	Satpute <i>et al.</i> [20]
Filtration and precipitation	Precipitation was carried out with ethanol, acetone, ethanol acetic acid (1%)/5 N HCl in an equal volume of culture liquid. Extraction was performed twice to enhance the yield of biosurfactants	Turkovskaya <i>et al.</i> [22]

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8 Characterisation of biosurfactants

There are many chromatography and spectroscopic methods used to characterize biosurfactants common among them are thin chromatography (TLC), Nuclear magnetic resonance (NMR), liquid chromatography-mass spectroscopy (LC-MS), Fourier transform infrared spectroscopy (FT-IR), high-performance liquid chromatography (HPLC). Each technique has its own strength and drawbacks as indicated in Table 7. Liquid chromatography-mass spectroscopy is the most commonly used instrument [23]

8.1 Spectroscopy methods

FT-IR can elucidate some components of an unknown mixture based on functional groups. In the process, 1 mg of purified biosurfactant (dried in freeze dryer) is ground with potassium bromide (100 mg), pressed for 30 s to achieve translucent pellets. Then analyze in an FT-IR device with the spectrum ranging from 450 – 4000 cm^{-1} at a resolution of 4 cm^{-1} [90,16]

NMR provides information regarding the functional groups about the position of linkages within the lipid and carbohydrate molecules. This is based on transitions in atoms with a magnetic moment when an external magnetic field is applied. Smyth *et al.* [91] characterized glycolipid biosurfactant using NMR.

Fast atom bombardment-mass spectrometry uses a high energy beam of xenon atom and caesium ions to stammer the sample and matrix (m-nitrobenzyl alcohol) from the probe's surface. Usually, the biosurfactants are dissolved in methanol, mixed with matrix [20].

Electrospray ionization-mass spectrometry is a soft ionization method used for the production of gas-phase ions for biological molecules with high molecular weight. It is so flexible that it can be used with MS (ESI-MS/MS), LC (LE/ESI-MS), HPLC/ESI-MS) for a detailed insight of structural properties of molecules.92 Sabturani *et al.*93 used ES-MS to characterize BS derived from *P. aeruginosa* UKMP14T.

316 8.2 Chromatography methods

317

318 **Liquid chromatography-mass spectroscopy (LC-MS)** analysis of biosurfactants requires an initial purification by
319 removing the worst interferences and also to concentrate the sample to a significant quantity [23]. The LC-MS
320 utilizes differences in hydrophobicity to achieve partitioning between a non-polar stationary phase and a polar
321 mobile phase. The LC-MS technique is highly efficient in purifying and separating lipopolysaccharides (LP)
322 congeners. Liquid chromatography-MS is best suited for a characterizing an unknown lipopolysaccharide.

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324 **Gas chromatography-mass spectroscopy (GC-MS)** is used in characterizing biosurfactants where the mass
325 spectroscopy measures the MW of the compound. For this device, the sample needs hydrolytic cleavage between the
326 peptide/protein or carbohydrate/lipid portions present in the biosurfactant. The GC-MS results are analysed by fatty
327 acid derivatization to fatty acid methyl esters (FAME) and further conversion to trimethylsilyl (TMS) derivatives
328 [19].

329

330 **High-performance liquid chromatography (HPLC)** is a special kind of column chromatography used in the
331 chemical and biochemical analysis in that it can separate a mixture of surface-active compounds, identify, quantify
332 and purify separate components of biosurfactant mixture [23]. The use of HPLC has been reported in the
333 characterization, quantification and purification of BSs [94] For example, purification of LP by HPLC was carried
334 out by reversed-phase (RP)-HPLC using a semi-preparative C18 column and 0.1% trifluoroacetic
335 acid/methanol/H₂O as a mobile phase [95]

336

337 **Table 7. Chromatography and spectroscopic methods used to characterize biological molecules**

Method	Advantages	Disadvantages
LC-MS	Large commercial and public libraries No derivatization required Many modes of separation available Large sample capacity	Slow Limited commercial libraries
GC-MS	Sensitive Robust Large linear range	Slow Often requires derivatization Many analytes thermally unstable or too large for analysis
NMR	Rapid analysis High resolution No derivatization needed Non-destructive	Low sensitivity Convolutated spectra More than one peak per component Libraries of limited use due to complex matrix
HPLC	Amenable to diverse sample types Accurate Sensitive Speed Can analyze neutral, anions and cations on a single run	Lack of ideal universal detector Less separation efficiency Arduous for regulatory testing Costly

338 Adapted from Satpute *et al* [20]

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341 9 Classification of the five groups of biosurfactant

342 Biosurfactants are classified based on their biochemical constituents or the species producing them.
343 Rosenberg and Ron [96] grouped biosurfactants into LMW molecules and HMW polymers. The former efficiently
344 lower ST and IFT while the latter are expert emulsion-stabilizing agents. The main classes of LMW-BSs
345 are lipopeptides, glycolipids and phospholipids, while the HMW-BSs are particulate and polymeric surfactants (Fig.
346 2). The hydrophobic moiety of BSs is long-chain fatty acids while the hydrophilic moiety either be alcohol, amino
347 acid, carbohydrate, cyclic peptide, or phosphate carboxyl acid [13].

348

349 **9.1 Classification based on molecular weight**

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351 **The LMW biosurfactants** are biosurfactant compounds that lower the ST and IFT at the air/water interface. They
352 are generally glycolipids (rhamnolipids, sophorolipids, trehalolipids, mannosylerythritol lipids) or lipopeptides [97]
353 and are better reducers of ST and IFT [45]

354 **The HMW biosurfactants** are known as bioemulsifiers. They show effective stabilization property with respect to
355 oil-in-water emulsions [26]. Besides, they can work at low concentrations and show considerable substrate
356 specificity [98]. Examples include emulsans, alansans, biodispersans etc. Each of the specific class is discussed
357 below.

358

359 **9.2 Classification based on chemical composition**

360

361 **9.2.1 Glycolipids**

362 Glycolipids constitute a hydrophilic carbohydrate component and a hydrophobic fatty acid chain.
363 According to Marchant and Banat [97], the hydrophilic end is made up of different sugars: rhamnose (in
364 rhamnolipids), sophorose (in sophorolipids), and mannose and erythritol (in mannosylerythritol lipids). Trehalose
365 and cellobiose lipids are other examples of glycolipids. However, most studied glycolipids are rhamnolipids.

366 a) **Rhamnolipids** are amphiphilic compounds ideally comprised of 3-hydroxy fatty acids (hydroxydecanoic
367 acid) linked through a β -glycosidic bond to mono- or di-rhamnose [26,99,100]

368 b) **Sophorolipids** are made up of disaccharide-sophorose β -linked to a long fatty acid with a chain length of
369 16 - 18 carbon atoms with the presence of unsaturation [101] They can exist in a lactonic form [97] or in an
370 acidic form [102]

371 c) **Mannosylerythritol lipids (MELs)** have 4 major structural groups having 4-O-b-D-mannopyranosyl-D-
372 erythritol linked to 2 medium-length chains of fatty acyl esters [29,103]. Though MELs exist as MEL-A,
373 MEL-B and MEL-C, the MEL-A is the most dominant [32]

374 d) **Trehalolipids** biosurfactants exist in various structural types. In some microorganisms, the disaccharide-
375 trehalose linked at C-6 and C-6 to mycolic acid which is long-chain α -branched and β -hydroxy fatty acids
376 [104]

377

378 **9.2.2 Lipopeptides and lipoproteins**

379 This class of BSs, in general, comprises of cyclic peptides connected to a fatty acid. *Bacillus* cyclic-
380 lipopeptides are formed of three different categories: fengycin, iturin and surfactin [2] Surfactin is the most studied
381 among them. Structure of surfactin is made up of 7 amino acid cyclic peptide connected to a C13–C16 fatty acid,
382 whereas iturin consists of 7 amino acids linked to C14–C17 and fengycin is composed of 10 amino acids with a fatty
383 acid chain length of C14–C18 [105]. Other examples of lipoprotein include viscosin, lichenysin, serrawettin,
384 gramicidin, polymyxin [106].

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391 **9.2.3 Fatty acids and lipids (phospholipids and neutral lipids)**

392 Many bacteria and yeasts yield appreciable amounts of these molecules during their growth on n-alkanes.
393 The HLB these molecules relate to the hydrocarbon chain length in direct proportion [45]. In *Acinetobacter* sp.,
394 phosphatidylethanolamine rich vesicles are synthesized and form optically clear micro-emulsions of oil-in-water.
395 Phosphatidylethanolamine synthesized by *R. erythropolis* while growing on n-alkane, lowers the IFT between
396 hexadecane and water to less than 1 mN/m and a CMC less than 30 [9].

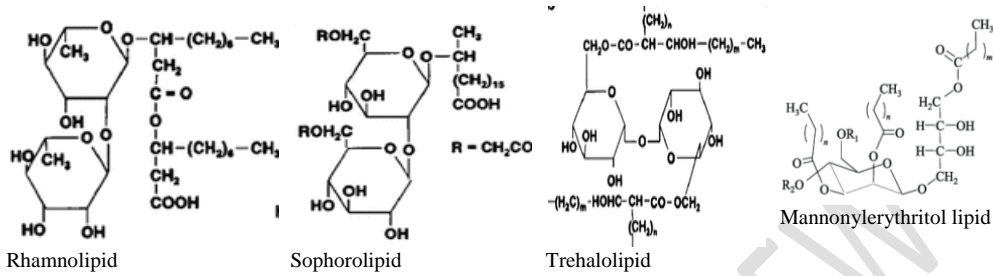
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398 **9.2.4 Polymeric and particulate biosurfactants**

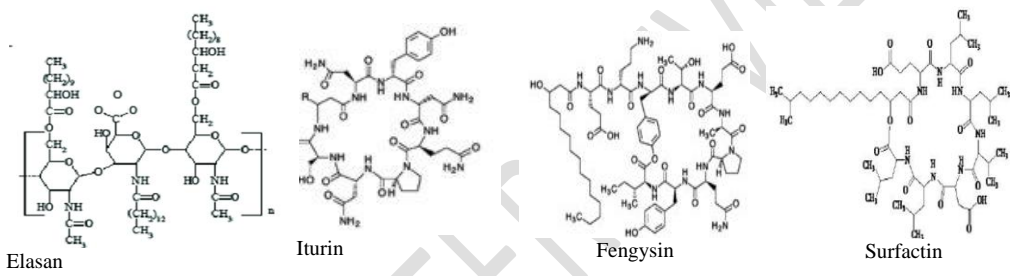
399 The best-studied polymeric BSs are emulsan, alasan, liposan, lipomanan and some other lipopolysaccharide
400 and polysaccharide-lipid (or protein) complexes. The lipopolysaccharides consist of lipid component, a core
401 polysaccharide and O-specific side chain polysaccharide bond together by covalently. Emulsan is an effective
402 emulsifying agent for oi-in-water, even at a very low concentration. Liposan is an extracellular water-soluble
403 emulsifier from *Candida lipolytica* and has 83% of carbohydrate and 17% of protein [26]. Extracellular membrane
404 vesicles (particulate BSs) can form microemulsions by partitioning hydrocarbons. These microemulsions aid alkane
405 metabolism by microbial cells [9] Vesicles of *Acinetobacter* spp. having a diameter of 20 –50 nm and a buoyant
406 density of 1.158 g/cm, were screened to possess protein, phospholipids and lipopolysaccharides. Table 8 summarises
407 the major groups of BSs produced by microorganisms.

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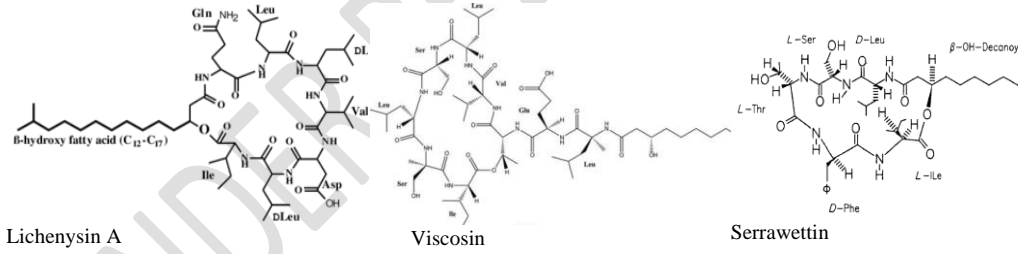
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Fig. 2. Structures of well-known biosurfactants produced by microorganisms [7, 26]

424 Table 8. Classification of biosurfactants based on chemical structure and the key microorganisms that produces the specific type of
 425 biosurfactants

Group	Type	Microbial identity	Reference
Glycolipids	Rhamnolipids	<i>Pseudomonas aeruginosa</i> , <i>Serratia rubidaea</i> SNAU02	Whang <i>et al.</i> [107], Nalini and Parthasarathi [108]
	Sophorolipids	<i>Torulopsis bombicola</i> , <i>Trichosporon asahii</i> , <i>Mucor mucedo</i> , <i>Aspergillus flavus</i> , <i>Trichoderma viridis</i> , <i>Fusarium</i> sp. S33, <i>Rhizopus oryzae</i>	Adekunle <i>et al.</i> [109], Balaji <i>et al.</i> [35], Lima <i>et al.</i> [34]
	Mannosylerythritol lipid	<i>Candida antarctica</i>	Yu <i>et al.</i> [110]
	Annosylerythritol lipids	<i>Pseudozyma rugulosa</i>	Morita <i>et al.</i> [29]
	Trehalolipids	<i>Arthrobacter paraffineus</i> , <i>Rhodococcus erythropolis</i> , <i>Gordonia amarae</i> , <i>Nocardia</i> sp	Vigneshwaran <i>et al.</i> [62]
	Cellobiolipids	<i>Ustilago maydis</i>	Shekhar <i>et al.</i> [9]
Lipopeptides	Surfactin, iturin, fengycin	<i>Bacillus subtilis</i> , <i>Bacillus mojavensis</i>	Hmidet <i>et al.</i> [111]
	Lichenysin	<i>Bacillus licheniformis</i>	Madslien <i>et al.</i> [112]
	Viscosin	<i>Pseudomonas fluorescens</i>	Alsohim <i>et al.</i> [113]
	Serrawettin	<i>Serratia marcescens</i>	Hage-Hulsmann <i>et al.</i> [114]
	Phomafungy	<i>Phoma</i> sp. S31	Lima <i>et al.</i> [34]
Fatty acids, phospholipids and neural lipids	Spiculisporic acid	<i>Penicillium spiculisporum</i>	Ishigami <i>et al.</i> [115]
	Diglycosyl diglycerides	<i>Lactobacillus fermentum</i>	Saharan and <i>et al.</i> [18]
	Glycerol-liamocin	<i>Aureobasidium pullulans</i>	Kim <i>et al.</i> [116]
	Phosphatidylethanolamine	<i>Rhodococcus erythropolis</i>	Stancu [10]
Polymeric biosurfactants	Emulsan	<i>Acinetobacter calcoaceticus</i>	Shekhar <i>et al.</i> [9]
	Alasan	<i>Acinetobacter radioresistens</i>	Toren <i>et al.</i> [117]
	Yasan	<i>Yarrowia lipolytica</i>	Yalcin <i>et al.</i> [118]
	Biodispersan	<i>Acinetobacter calcoaceticus</i> RAG-1	Rahman and Gakpe [45]
	Liposan	<i>Acinetobacter radioresistens</i> KA-53,	
	Mannoprotein	<i>Saccharomyces cerevisiae</i> , <i>Kluyveromyces marxianus</i>	Dikit <i>et al.</i> [118]
	EPS	<i>Galactomyces</i> sp. Z3, <i>Apiotrichum loubieri</i> sp. TEMOS16, <i>Geotrichum</i> spp. <i>Curvularia lunata</i> IM 2901	Yalcin <i>et al.</i> [119]
Particulate biosurfactants	Vesicles	<i>Acinetobacter calcoaceticus</i>	Muthusamy <i>et al.</i> [12]
	Whole cell		

426 **10 Advantages and disadvantages of biosurfactants**

427 Biosurfactants has its merits and draw backs as reflected in Table 9.

428

429 Table 9. Advantages and disadvantages of biosurfactants

Advantages	Disadvantages
Biosurfactants are easily degraded in the environment	Hemolytic activity of certain biosurfactants can rupture erythrocytes at 37 OC
Biosurfactants exhibits lower toxicity than the synthetic ones	Biosurfactants is characterized with very low productivity. This is because over producing strains and recombinant stains are very rare
Surfactants of biological origin have feature of compatibility thus being used in pharmaceuticals, cosmetics, food industries etc.	To get pure biosurfactants require multiple steps with attendant cost
Biosurfactants can be produced from a variety of relatively cheap raw materials	Strong foam formation hampers the improvement in production yield
Biosurfactants are effective surface and interfacial tensions reducers	Production of biosurfactants in large scale is capital intensive
Biosurfactants can be produced from industrial waste and by-products thus key into acceptable production economics	
Many biosurfactants are stable at extreme pH, salinities and temperature	
Biosurfactants are specific in their action, hence play specific functions	

430 Adapted from De *et al.* [83]

431

432 **11 Conclusion**

433 Biosurfactants are tensio-active molecules from microorganisms as metabolic products or the actual cells of
434 their surface chemistry. Besides, the known biosurfactant producers: *Pseudomonas*, *Bacillus*, *Acinetobacter*,
435 *Candida* other genera such as, *Apiotrichum*, *Aureobasidium*, *Galactomyces*, *Geotrichum* *Gordonia*, *Kluveromyces*,
436 *Phoma*, and *Yarrowia*, and host of others are now included in the list. Biosurfactants have a unique property of
437 reducing ST and IFT of adjoining liquids. Biosurfactants which are not efficient in reducing surface tensions but are
438 efficient in stabilizing emulsions are known as bioemulsifiers. Gold standard techniques employed to determine
439 biosurfactant properties are surface tension measurements, emulsification activity and emulsification index (%EI₂₄).
440 Crude extraction of biosurfactants can be achieved through a number of methods including: centrifugation, acid
441 precipitation, ion-exchange chromatography, adsorption-desorption, foam fractionation. The most common
442 technique used in purifying crude biosurfactants are thin-layer chromatography, dialysis and lyophilization, and
443 isoelectric focusing. Characterisation of biosurfactants can be achieved by using chromatography and spectroscopy
444 methods such as TLC, LC-MS, HPLC, FT-IR, NMR. Biosurfactants help microorganisms to metabolise
445 hydrocarbons, solubilize hydrophobic compounds and exhibit antimicrobial activities, thus have multifunctional
446 properties that can be relevant in industrial applications.

447

448 **Conflicts of Interest:** The authors declare no conflict of interest.

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452 References

- 453 1. Udoh T, Vinogradov J. Experimental investigations of behaviour of biosurfactants in brine solutions relevant to
454 hydrocarbon reservoirs. *Colloids and Interfaces*. 2019;3(1):24.
- 455 2. Liu JF, Mbadanga SM, Yang SZ, Gu JD, Mu BZ. Chemical structure, property and potential applications of
456 biosurfactants produced by *Bacillus subtilis* in petroleum recovery and spill mitigation. *International Journal of*
457 *Molecular Sciences*. 2015;16(3): 4814-4837.
- 458 3. Pradhan A, Bhattacharyya A. An Alternative Approach for Determining Critical Micelle Concentration:
459 Dispersion of Ink in Foam. *Journal Surfactants Detergent*. 2018;21(5):745-50.
- 460 4. Sobrinho HB, Luna JM, Rufino RD, Porto ALF, Sarubbo, LA. Biosurfactants: classification, properties and
461 environmental applications. *Recent Developments in Biotechnology*. 2013;11: 1-29.
- 462 5. Karanth NGK, Deo PG, Veenanadig NK. Microbial production of biosurfactants and their importance. *Current*
463 *Science*. 1999;77: 116-126.
- 464 6. e Silva NMPR, Rufino RD, Luna JM, Santos VA, Sarubbo LA. Screening of *Pseudomonas* species for
465 biosurfactant production using low-cost substrates. *Biocatalysis and Agricultural Biotechnology*. 2014;3(2): 132-
466 139.
- 467 7. Md F. Biosurfactant: production and application. *Journal of Petroleum and Environmental*
468 *Biotechnology*. 2012;3(4): 1-5.
- 469 8. Ron EZ, Rosenberg E. Natural roles of biosurfactants: Minireview. *Environmental Microbiology*. 2001;3(4): 229-
470 236.
- 471 9. Shekhar S, Sundaramanickam A, Balasubramanian T. Biosurfactant producing microbes and their potential
472 applications: a review. *Critical Reviews in Environmental Science and Technology*. 2015; 45(14): 1522-1554
- 473 10. Stancu MM. Response of *Rhodococcus erythropolis* strain IBBPo1 to toxic organic solvents. *Brazilian Journal*
474 *of Microbiology*. 2015;46(4): 1009-1018.
- 475 11. Pacwa-Plociniczak M, Plaza GA, Piotrowska-Seget Z, Cameotra, S. S. Environmental applications of
476 biosurfactants: recent advances. *International Journal of Molecular Sciences*. 2011;12(1): 633-654.
- 477 12. Muthusamy K, Gopalakrishnan S, Ravi TK, Sivachidambaram P. Biosurfactants: properties, commercial
478 production and application. *Current Science*. 2008;94(6): 736-747.
- 479 13. Santos DKF, Rufino RD, Luna JM, Santos VA, Sarubbo LA. Biosurfactants: multifunctional biomolecules of
480 the 21st century. *International Journal of Molecular Sciences*. 2016;17(3): 401.
- 481 14. Mahalingam PU, Sampath N. Isolation, characterization and identification of bacterial biosurfactant. *European*
482 *Journal of Experimental Biology*. 2014;4(6): 59-64.
- 483 15. Budsabun T. Isolation of biosurfactant producing bacteria from petroleum contaminated terrestrial samples that
484 collected in Bangkok, Thailand. *Procedia-Social and Behavioral Sciences*. 2015;197: 1363-1366.
- 485 16. Elazzazy AM, Abdelmoneim TS, Almaghrabi, OA. Isolation and characterization of biosurfactant production
486 under extreme environmental conditions by alkali-halo-thermophilic bacteria from Saudi Arabia. *Saudi Journal of*
487 *Biological Sciences*. 2015;22(4): 466-475.
- 488 17. Dhail S. Isolation of potent biosurfactant producing bacteria from oil spilled marine water and marine
489 sediments. *African Journal of Biotechnology*. 2012;11(103): 16751-16757.

- 490 18. Saharan BS, Sahu RK, Sharma D. (2011). A review on biosurfactants: fermentation, current developments and
491 perspectives. Genetic Engineering and Biotechnology Journal. 2011;1: 1-14.
- 492 19. Vandana P, Singh D. Review on biosurfactant production and its application. International Journal of Current
493 Microbiology and Applied Sciences. 2018;7(8): 4228-4241.
- 494 20. Satpute SK., Banat IM, Dhakephalkar PK, Banpurkar G, Chopade BA. Biosurfactants, bioemulsifiers and
495 exopolysaccharides from marine microorganisms Biotechnology Advances. 2010; 28(4), 436-450.
- 496 21. Nitschke M, Pastore GM. Production and properties of a surfactant obtained from *Bacillus subtilis* grown on
497 cassava wastewater. Bioresource Technology. 2006; 97(2): 336-341.
- 498 22. Turkovskaya OV, Dmitrieva TV, Muratova AYA. biosurfactant-producing *Pseudomonas aeruginosa*
499 strain. Applied Biochemistry and Microbiology. 2001;37(1): 71-75.
- 500 23. Biniarz P, Łukaszewicz M, Janek T. Screening concepts, characterization and structural analysis of microbial-
501 derived bioactive lipopeptides: a review. Critical Reviews in Biotechnology. 2017;37(3): 393-410.
- 502 24. Gordillo A, Maldonado MC. Purification of peptides from *Bacillus* strains with biological
503 activity. Chromatography and its Applications. 2012;11: 201-225.
- 504 25. Maczek J, Junne S, Gotz P. Examining biosurfactant producing bacteria-an example for an automated search for
505 natural compounds. Application Note CyBio AG. 2007.
- 506 26. Roy AA. review on the biosurfactants: properties, types and its application. Journal of Fundamentals of
507 Renewable Energy and Applications. 2017;8: 1-14.
- 508 27. Mukherjee S, Das P, Sen R. Towards commercial production of microbial surfactants. Trends in Biotechnology.
509 2006;24(11): 509-515.
- 510 28. Geetha SJ, Banat I. M., & Joshi, S. J. Biosurfactants: Production and potential applications in microbial
511 enhanced oil recovery (MEOR). Biocatalysis and Agricultural Biotechnology. 2018;14: 23-32.
- 512 29. Morita T, Fukuoka T, Imura T, Kitamoto D. Mannosylerythritol lipids: production and applications. Journal of
513 Oleo Science. 2015;64(2): 133-141.
- 514 30. Amaral, PFF, da Silva JM, Lehoccky BM, Barros-Timmons AMV, Coelho MAZ, Marrucho IM, Coutinho JAP.
515 Production and characterization of a bioemulsifier from *Yarrowia lipolytica*. Process Biochemistry. 2006; 41(8):
516 1894-1898.
- 517 31. Zinjarde SS, Pant A. (2002). Emulsifier from a tropical marine yeast, *Yarrowia lipolytica* NCIM 3589. Journal
518 of Basic Microbiology. 2002;42(1): 67-73.
- 519 32. Niu Y, Fan L, Gu D, Wu J, Chen Q. Characterization, enhancement and modelling of mannosylerythritol lipid
520 production by fungal endophyte *Ceriporia lacerate* CHZJU. Food chemistry. 2017;228: 610-617.
- 521 33. Raja M, Praveena G, William SJ. Isolation and identification of fungi from soil in Loyola college campus,
522 Chennai, India. International Journal of Current Microbiology and Applied Science. 2017;6(2): 1789-1795.
- 523 34. Lima JMS, Pereira JO., Batista IH, Neto, PDQC, dos Santos JC, de Araújo SP, de Azevedo JL. Potential
524 biosurfactant producing endophytic and epiphytic fungi, isolated from macrophytes in the Negro River in Manaus,
525 Amazonas, Brazil. African Journal of Biotechnology. 2016;15(24): 1217-1223.
- 526 35. Balaji V, Arulazhagan P. Ebenezer, P. Enzymatic bioremediation of polyaromatic hydrocarbons by fungal
527 consortia enriched from petroleum contaminated soil and oil seeds. Journal of Environmental Biology. 2014;35: 3-9.
- 528 36. Bhardwaj G, Cameotra SS. Chopra, H. K. Biosurfactants from fungi: a review. Journal of Petroleum and
529 Environmental Biotechnology. 2013;4(06): 1-6.
- 530
531

- 532
533 37 Shah S, Prabhune A. Purification by silica gel chromatography using dialysis tubing and characterization of
534 sophorolipids produced from *Candida bombicola* grown on glucose and arachidonic acid. *Biotechnology letters*.
535 2007;29(2): 267-272.
- 536 38. Zhang J, Xue Q, Gao H, Lai H, Wang, P. Production of lipopeptide biosurfactants by *Bacillus atrophaeus* 5-2a
537 and their potential use in microbial enhanced oil recovery. *Microbial Cell Factories*. 2016;15(1): 168.
- 538 39. Tabatabaee A, Mazaheri-Assadi M, Noohi AA, Sajadian V, Isolation of biosurfactant producing bacteria from
539 oil reservoirs. *Iranian Journal of Environmental Health Science and Engineering*. 2005;2(1): 6-12.
- 540 40. Prasad N, Dasgupta S, Chakraborty M, Gupta S. Isolation and characterization of biosurfactant producing
541 bacteria for the application in enhanced oil recovery. In *IOP Conference Series: Earth and Environmental*
542 *Science* (Vol. 78, No. 1, p. 012017). IOP Publishing. 2017.
- 543 41. Maneerat S, Phetrong K. Isolation of biosurfactant-producing marine bacteria and characteristics of selected
544 biosurfactant. *Songklanakarin Journal of Science and Technology*. 2007;29(3): 781-791.
- 545 42. Batool R, Ayub S, Akbar I. Isolation of biosurfactant producing bacteria from petroleum contaminated sites and
546 their characterization. *Soil and Environment*, 2017;36(1): 35-44.
- 547
548 43. Qiao N, Shao Z. Isolation and characterization of a novel biosurfactant produced by hydrocarbon-degrading
549 bacterium *Alcanivorax dieselolei* B-5. *Journal of Applied Microbiology*. 2010;108(4): 1207-1216.
- 550
551 44. Nwachi AC, Onochie CC, Iroha IR, Agah VM, Agumah BN, Moses IB, Ogebeja DO. Extraction of biosurfactants
552 produced from bacteria isolated from waste-oil contaminated soil in Abakaliki Metropolis, Ebonyi State. *Journal of*
553 *Biotechnology Research*. 2016;2(4): 24-30.
- 554 45. Rahman PK, Gakpe E. Production, characterisation and applications of biosurfactants-Review. *Biotechnology*.
555 2008;7: 360-370.
- 556 46. Adamczak M, Bednarski W. Influence of medium composition and aeration on the synthesis of biosurfactants
557 produced by *Candida antarctica*. *Biotechnology Letters*. 2000;22(4): 313-316.
- 558 47. Maqsood MI, Jamal A. Factors affecting the rhamnolipid biosurfactant production. *Pakistan Journal of*
559 *Biotechnology*. 2011;8(1): 1-5.
- 560 48. Tahzibi A, Kamal F, Mazaheri-Assadi M. Improved production of rhamnolipids by a *Pseudomonas aeruginosa*
561 mutant. *Iranian Biomedical Journal*. 2004;8(1): 25-31.
- 562 49. Ikhwan AZN, Nurlaila, HS, Ferdinand FDK, Fachria R, Hasan AEZ, Yani M, Setyawati, I. Preliminary study:
563 optimization of pH and salinity for biosurfactant production from *Pseudomonas aeruginosa* in diesel fuel and crude
564 oil medium. In *IOP Conference Series: Earth and Environmental Science* (Vol. 58, No. 1, p. 012056). IOP
565 Publishing. 2017.
- 566 50. Chen J, Huang PT, Zhang KY, Ding FR. Isolation of biosurfactant producers, optimization and properties of
567 biosurfactant produced by *Acinetobacter* sp. from petroleum-contaminated soil. *Journal of Applied Microbiology*.
568 2012;112(4): 660-671.
- 569 51. Auhim HS, Mohamed AI. Effect of different environmental and nutritional factors on biosurfactant production
570 from *Azotobacter chroococcum*. *International Journal of Advances in Pharmacy Biology and Chemistry*. 2013;2:
571 477-481.
- 572 52. Jagtap S, Yavankar S, Pardesi K, Chopade, B. Production of bioemulsifier by *Acinetobacter* species isolated
573 from healthy human skin. *Indian Journal of Experimental Biology*. 2010;48: 70-76.
- 574 53. Fontes GC, Amaral F, Filomena P, Nele M, Coelho Z, Alice M. Factorial design to optimize biosurfactant
575 production by *Yarrowia lipolytica*. *BioMed Research International*. 2010;821306: 1-8.

576
577 54. Morais IM, Cordeiro AL, Teixeira GS, Domingues VS, Nardi RM, Monteiro AS, Alves RJ, Siqueira EP, Santos
578 VL Biological and physicochemical properties of biosurfactants produced by *Lactobacillus jensenii* P 6A and
579 *Lactobacillus gasseri* P 65. Microbial Cell Factories. 2017;16(1):155.

580 55. Yeh MS, Wei YH, Chang JS. Enhanced Production of Surfactin from *Bacillus subtilis* by addition of solid
581 carriers. Biotechnology Progress. 2005;21(4): 1329-1334.

582 56. Singh P, Tiwary BN. Isolation and characterization of glycolipid biosurfactant produced by a *Pseudomonas*
583 *otitidis* strain isolated from Chirimiri coal mines, India. Bioresources and Bioprocessing. 2016;3(1): 42-57.

584 57. Deleu M, Paquot M. From renewable vegetables resources to microorganisms: new trends in
585 surfactants. Comptes Rendus Chimie. 2004;7(6-7): 641-646.

586 58. Mukherjee AK (2007). Potential application of cyclic lipopeptide biosurfactants produced by *Bacillus subtilis*
587 strains in laundry detergent formulations. Lett Appl Microbiol 45(3): 330-335.

588 59. Mnif I, Chaabouni-Ellouze S, Ghribi, D. (2012). Optimization of the nutritional parameters for enhanced
589 production of *B. subtilis* SPB1 biosurfactant in submerged culture using response surface
590 methodology. Biotechnology research international. 2012;2012: 1-9.

591 60. Asad A, Ikramullah M, Asif S, Ahmad A, Jamal A, Jaffri A. Evaluation of lipopeptide (surfactin) production by
592 *Bacillus subtilis*. Biomedical. 2010; 26: 34-3.
593

594 61. Ohadi M., Dehghannoudeh G, Shakibaie M, Banat IM, Pournamdari M, Foroontanfar H. Isolation,
595 characterization, and optimization of biosurfactant production by an oil-degrading *Acinetobacter junii* B6 isolated
596 from an Iranian oil excavation site. Biocatalysis and Agricultural Biotechnology. 2017;12: 1-9.

597 62. Vigneshwaran C, Vasantharaj K, Jerold M, Sivasubramanian V. Production of biosurfactants and its application
598 in bioremediation. In Environmental Sustainability Using Green Technologies (pp. 279-298). CRC Press. 2016.

599 63. Satpute SK, Kulkarni GR, Banpurkar AG, Banat IM, Mone, NS, Patil RH, Cameotra SS. Biosurfactant/s from
600 Lactobacilli species: Properties, challenges and potential biomedical applications. Journal of Basic Microbiology.
601 2016;56(11): 1140-1158.

602 64. Gudina EJ, Teixeira JA, Rodrigues LR. (2010). Isolation and functional characterization of a biosurfactant
603 produced by *Lactobacillus paracasei*. Colloids and Surfaces B: Biointerfaces. 2010;76(1): 298-304.

604 65. Salari R, Salari R. Investigation of the best *Saccharomyces cerevisiae* growth condition. Electronic Physician.
605 2017;9(1): 3592–3597.

606 66. Sena HH, Sanches MA, Rocha DF, Segundo-Filho WO, de Souza ÉS, de Souza JV. Production of biosurfactants
607 by soil fungi isolated from the Amazon forest. International Journal of Microbiology. 2018;5684261: 1-8.

608 67. Qazi MA, Subhan M, Fatima N, Ali MI, Ahmed S. Role of biosurfactant produced by *Fusarium* sp. BS-8 in
609 enhanced oil recovery (EOR) through sand Pack column. International Journal of Bioscience, Biochemistry and
610 Bioinformatics. 2013;3(6): 598-604.

611 68. Gharaei-Fathabad, E. Biosurfactants in pharmaceutical industry: a mini-review. America Journal of Drug
612 Discovery and Development. 2011;1(1): 58-69.

613 69. Sepahy AA, Assadi MM, Saggadian V, Noohi A. Production of biosurfactant from Iranian oil fields by isolated
614 Bacilli. International Journal of Environmental Science and Technology. 2005;1(4): 287-293.

615 70. Kaczorek E. Effect of external addition of rhamnolipids biosurfactant on the modification of gram positive and
616 Gram negative bacteria cell surfaces during biodegradation of hydrocarbon fuel contamination. Polish Journal of
617 Environmental Studies. 2012;21(4).

- 618 71. Yuliani H, Perdani MS, Savitri I, Manurung M, Sahlan M, Wijanarko A, Hermansyah H. Antimicrobial activity
619 of biosurfactant derived from *Bacillus subtilis* C19. *Energy Procedia*. 2018;153: 274-8.
- 620 72. Jamal P, Mir S, Alam MZ, Nawawi WMFW. Isolation and selection of new biosurfactant producing bacteria
621 from degraded palm kernel cake under liquid state fermentation. *Journal of Oleo Science*. 2014;63(8): 795-804.
- 622 73. Raval MS, Gund SS, Shah NR, Desai RP. (2017). Isolation and characterization of biosurfactant producing
623 bacteria and their application as an antibacterial agent. *International Journal of Pharma and Bio Science*, 8(3):
624 302-310.
- 625 74. Hashemi SZ, Fooladi J, Ebrahimipour G, Khodayari S. Isolation and identification of crude oil degrading and
626 biosurfactant producing bacteria from the oil-contaminated soils of Gachsaran. *Applied Food Biotechnology*.
627 2016;3(2): 83-89.
- 628 75. Patel D, Nataraj M. Study of biosurfactant producing bacteria and preliminary characterization of biosurfactant
629 produced by *Bacillus* species isolated from petroleum contaminated soil. *The Journal of Microbiology*,
630 *Biotechnology and Food Sciences*. 2016;6(2): 823.
- 631 76. Pereira RM., Silveira ÉLD, Scaquitto DC, Pedrinho EAN, Val-Moraes SP, Wickert E, Lemos, EGDM.
632 Molecular characterization of bacterial populations of different soils. *Brazilian Journal of Microbiology*. 2006;37(4):
633 439-447.
634
- 635 77. Rodrigues L, Banat IM, Teixeira J, Oliveira, R. (2006). Biosurfactants: potential applications in medicine.
636 *Journal of Antimicrobial Chemotherapy*. 2006;57(4): 609-618.
- 637 78. Toledo FL, Gonzalez-Lopez J, Calvo C. Production of bioemulsifier by *Bacillus subtilis*, *Alcaligenes faecalis*
638 and *Enterobacter* species in liquid culture. *Bioresource Technology*. 2008;99(17): 8470-8475.
- 639 79. Adnan M, Alshammari E, Ashraf SA, Patel K, Lad K, Patel M. Physiological and molecular characterization of
640 biosurfactant producing endophytic fungi *Xylaria regalis* from the cones of *Thuja plicata* as a potent plant growth
641 promoter with its potential application. *BioMed Research International*. 2018.
642
- 643 80. Thapa S, Shrestha R, Tirewal A, Sharma A, Yuvraj KC. Isolation of yeast from soil and different food samples
644 and its characterization based on fermentation. *Nepal Journal of Biotechnology*. 2015;3(1): 29-34.
- 645 [81. Rodrigues AMD, Pinheiro REE, Costa JA, Santos JTO, Poli JS, Rosa CA, Nóbrega, MMGP. Comparison
646 between morphophysiological and molecular methods for the identification of yeasts isolated from
647 honey. *International Food Research Journal*. 2018;25(1): 418-422.
- 648 82. Maji J, Mukhopadhyay BC, Mitra S, Biswas SR. Molecular characterization of Yeasts and Bacteria isolated
649 from Handia, an Indian traditional rice fermented alcoholic beverage. *American Journal of Current Microbiology*.
650 2018;6(1): 1-12.
- 651 83. De S, Malik S, Ghosh A, Saha R, Saha B (2015) A review on natural surfactants. *RSC Advances*. 2015;5(81):
652 65757-65767.
653
- 654 84. Maheswari NU, Parveen LF. Comparative study of biosurfactant by using *Bacillus licheniformis* and
655 *Trichoderma viride* from paper waste contaminated soil. *International Journal of Chemical Sciences*. 2012;10(3):
656 1687-1697.
657
- 658 85. Sumaiya M, Anchana-Devi C, Leela K. (2017). A Study on Biosurfactant Production from Marine Bacteria.
659 *International Journal of Scientific and Research*. 2017;7(12): 139-145.
- 660 86. Garfin DE (2000) Isoelectric focusing: In *Handbook of Bioseparation* (Ed. Ahuja, S). Pp 263-298, Academic
661 Press San Diego

662 87. Patil JR., Chopade, BA. Bioemulsifier production by *Acinetobacter* strains isolated from healthy human skin. *US*
663 *Patent No. US 2004/0138429 A1*. 2003.
664

665 88. Phetrong K., Aran H, Maneerat, S. (2008). Production and characterization of bioemulsifier from ass marine
666 bacterium, *Acinetobacter calcoaceticus* subsp. anitratus SM7. *Songklanakarin Journal of Science and Technology*.
667 2008;30(3): 297-305.
668

669 89. Abalos A, Pinazo A, Infante MR, Casals M, Garcia F, Manresa A. Physicochemical and antimicrobial properties
670 of new rhamnolipids produced by *Pseudomonas aeruginosa* AT10 from soybean oil refinery wastes. *Langmuir*.
671 2001;17(5): 1367-1371.

672 90. Antoniou E, Fodelianakis S, Korkakaki E, Kalogerakis N. (2015). Biosurfactant production from marine
673 hydrocarbon-degrading consortia and pure bacterial strains using crude oil as carbon source. *Frontiers in*
674 *Microbiology*. 2015;6: 274-287.

675 91. Smyth TJP, Perfumo A, Marchant R, Banat IM. Isolation and analysis of low molecular weight microbial
676 glycolipids. In *Handbook of Hydrocarbon and Lipid Microbiology*. Pp. 3705-3723, Springer, Berlin, Heidelberg.
677 2010.

678 92. Banerjee S, Mazumda S. Electrospray ionization mass spectrometry: a technique to access the information
679 beyond the molecular weight of the analyte. *International Journal of Analytical Chemistry*. 2012;282574: 1-40.

680 93. Sabturani N, Latif J, Radiman S, Hamzah A. Spectroscopic analysis of rhamnolipid production by *Pseudomonas*
681 *aeruginosa* UKMP14T. *Malasian Journal of Analytical Science*. 2015;20(1): 31-43.

682 94. Janek T, Lukaszewicz M, Rezanka T., Krasowska, A. (2010). Isolation and characterization of two new
683 lipopeptide biosurfactants produced by *Pseudomonas fluorescens* BD5 isolated from water from the Arctic
684 Archipelago of Svalbard. *Bioresour Technol*. 2010;101(15): 6118–6123.

685 95. Pathak KV, Keharia H. Application of extracellular lipopeptide biosurfactant produced by endophytic *Bacillus*
686 *subtilis* K1 isolated from aerial roots of banyan (*Ficus benghalensis*) in microbially enhanced oil recovery
687 (MEOR). *3 Biotech*. 2014;4(1): 41-48.

688 96. Rosenberg E, Ron, E. Z. (1999). High-and low-molecular-mass microbial surfactants. *Applied Microbiology and*
689 *Biotechnology*. 1999;52(2), 154-162.

690 97. Marchant R, Banat, I. M. Biosurfactants: a sustainable replacement for chemical surfactants?. *Biotechnology*
691 *letters*, 2012;34(9): 1597-1605.

692 98. Uzoigwe C, Burgess JG, Ennis CJ, Rahman PK. Bioemulsifiers are not biosurfactants and require different
693 screening approaches. *Frontiers in Microbiology*. 2015;6: 245.

694 99. Abdel-Mawgoud AM, Lépine F, Déziel E. Rhamnolipids: diversity of structures, microbial origins and
695 roles. *Applied Microbiology and Biotechnology*. 2010;86(5): 1323-1336.

696 100. Vatsa P, Sanchez L, Clement C, Baillieul, F, Dorey S. (2010). Rhamnolipid biosurfactants as new players in
697 animal and plant defense against microbes. *International Journal of Molecular Sciences*. 2010;11(12): 5095-5108.

698 101. de Oliveira MR, Magri A, Baldo C, Camilios-Neto D, Minucelli T, Celligoi, MAPC. (2015). Sophorolipids A
699 promising biosurfactant and it's applications. *International Journal of Advanced Biotechnology and Research*.
700 2015;6: 161-174.
701

702 102. van Bogaert IN, Saerens K, de Muynck C, Develter D, Soetaert W, Vandamme, EJ. Microbial production and
703 application of sophorolipids. *Applied Microbiology and Biotechnology*. 2007;76(1): 23-34.

704 103. Fukuoka T, Morita T, Konishi M, Imura T, Kitamoto, D. Characterization of new types of mannosylerythritol
705 lipids as biosurfactants produced from soybean oil by a basidiomycetous yeast, *Pseudozyma shanxiensis*. *Journal of*
706 *Oleo Science*. 2007;56(8): 435-442.

707
708 104. White DA, Hird LC, Ali ST. Production and characterization of a trehalolipid biosurfactant produced by the
709 novel marine bacterium *Rhodococcus* sp., strain PML026. *Journal of Applied Microbiology*. 2013;115(3): 744-755.

710 105. Lang S. (2002). Biological amphiphiles (microbial biosurfactants). *Current Opinion in Colloid and Interface*
711 *Science*. 2002;7(1-2): 12-20.

712 106. Gautam KK, Tyagi VK. (2006). Microbial surfactants: a review. *Journal of Oleo Science*. 2006;55(4): 155-166.

713 107. Whang LM, Liu PWG, Ma CC, Cheng SS. Application of biosurfactants, rhamnolipid, and surfactin, for
714 enhanced biodegradation of diesel-contaminated water and soil. *Journal of Hazardous Materials*. 2008;151(1): 155-
715 163.

716 108. Nalini S, Parthasarathi R. Optimization of rhamnolipid biosurfactant production from *Serratia rubidaea*
717 SNAU02 under solid-state fermentation and its biocontrol efficacy against *Fusarium* wilt of eggplant. *Annals of*
718 *Agrarian Science*. 2018;16(2): 108-115.

719 109. Adekunle AT, Ester BB, Olabisi A, Peter OSB, Joshua IUJ, Alfa S. (2015). Characterization of new
720 glycosophorolipid-surfactant produced by *Aspergillus Niger* and *Aspergillus flavus*. *European Journal of*
721 *Biotechnology and Bioscience*. 2015;3(4): 34-39.

722
723 110. Yu H, Xiao H, Wang D. Effects of soil properties and biosurfactant on the behavior of PAHs in soil-water
724 systems. *Environmental System Research*. 2014;3(1):6.

725 111. Hmidet N, Ben Ayed H, Jacques P, Nasri M. Enhancement of surfactin and fengycin production by *Bacillus*
726 *mojavensis* A21: application for diesel biodegradation. *BioMed research international*. 2017;2017.

727 112. Madslie EH, Rønning HT, Lindbäck T, Hasse B, Andersson MA, Granum PE. Lichenysin is produced by
728 most *Bacillus licheniformis* strains. *Journal of Applied Microbiology*. 2013;115(4): 1068-1080.

729 113. Alsohim AS, Taylor TB, Barrett GA, Gallie J, Zhang XX, Altamirano-Junqueira AE, Jackson RW. The
730 biosurfactant viscosin produced by *Pseudomonas fluorescens* SBW 25 aids spreading motility and plant growth
731 promotion. *Environmental Microbiology*. 2014;16(7): 2267-2281.

732
733 114. Hage-Hulsmann J, Grunberger A, Thies S, Santiago-Schubel B, Klein AS, Pietruszka J, Binder D, Hilgers F,
734 Domrose A, Drepper T, Kohlheyer D. Natural biocide cocktails: Combinatorial antibiotic effects of prodigiosin and
735 biosurfactants. *PLoS one*. 2018;13(7): e0200940.

736
737 115. Ishigami Y, Zhang Y, Ji F. (2000). Spiculisporic acid. Functional development of biosurfactant. *Chimica Oggi*.
738 2000;18(7-8): 32-34.

739
740 116. Kim JS, Park NH, Kim, CG. US Patent No. 8,642,793. Washington, DC: U.S. Patent and Trademark Office.
741 2014.

742 [117. Toren A, Navon-Venezia S, Ron EZ, Rosenberg E. (2001). Emulsifying activities of purified alasan proteins
743 from *Acinetobacter radioresistens* KA53. *Applied and Environmental Microbiology*. 2001;67(3): 1102-1106.

744 118. Dikit P, Maneerat S, Musikasang H, H-kittikun A. (2010). Emulsifier properties of the mannoprotein extract
745 from yeast isolated from sugar palm wine. *Science Asia*. 2010;36: 312-318.

746
747 119. Yalçın HT, Ergin-Tepebaşı G, Uyar E. Isolation and molecular characterization of biosurfactant producing
748 yeasts from the soil samples contaminated with petroleum derivatives. *Journal of Basic Microbiology*. 2018;58(9):
749 782-792.

750

751