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 had 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

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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.

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

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

2 Biosurfactants

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

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

3 Factors affecting biosurfactant production

3.1 Biosurfactant production

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

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3.2 Factors affecting biosurfactant production

3.2.1 Nutrient factors and salt concentration

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

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

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

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

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

3.2.2 Environmental factors

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

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

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

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

Aeration and agitation are very important factors that influence BS production since they both facilitate oxygen transfer into the culture medium [26]. Adamczak and Bednarski [46] demonstrated that improved yield value of BS (45.5 g/l) was achieved when the air-flow-rate was 1 vvm and the dissolved O_2 concentration was sustained at 50% saturation. Agitation of between 120 rpm to 200 rpm is most common in microbial growth studies [56]. It is safe to conclude that incubation period remains the most unpredictable.

4 Properties of biosurfactants

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

4.1 Surface and interface activity

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

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

4.2 Tolerance to pH, ionic strength, temperature

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

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

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

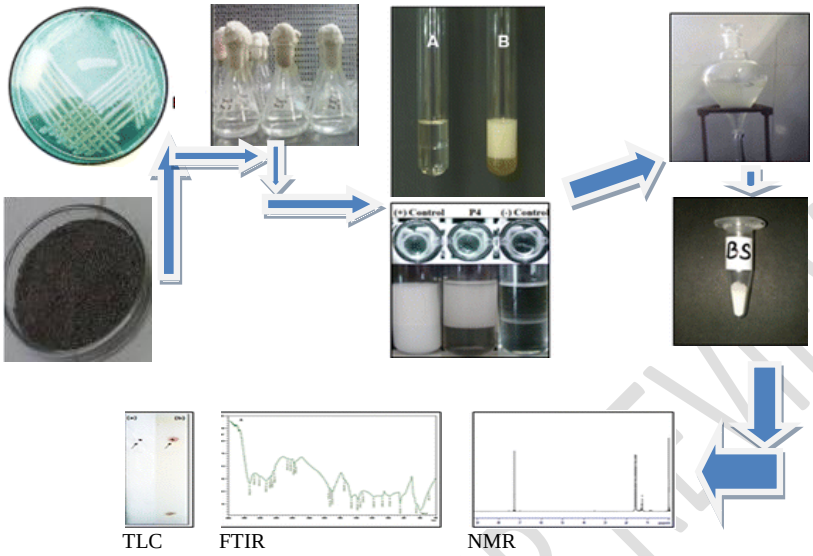

Fig. 1. Schematic presentation of a laboratory-limited biosurfactant production procedure: sampling to isolation to growth in MSM to screening of extraction and purification of biosurfactants to characterization of extracted biosurfactant. Adapted from Singh and Tiwary51

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

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

4.3 Biodegradability and low toxicity

Microbial surfactants, like other microbiologically derived compounds, can be easily degraded unlike the synthetic surfactants so they can be applied in bioremediation, biosorption and waste management [68,69]. Though very little data are available that give credence to BS toxicity, they are generally accepted as non-toxic, proving they can be used in health-related industry [26] The latter author disclosed that Corexit (a synthetic anionic surfactant) is ten times more lethal than rhamnolipids through the use of LC_{50} test against *Photobacterium phosphoreum*. This signifies that Corexit is far more toxic than rhamnolipids and possibly the comparison will be so between synthetic surfactants and microbial surfactants. A comparative study between biosurfactant from *P. aeruginosa* and a popular industrial synthetic surfactant revealed that the synthetic surfactants are toxigenic and mutagenic unlike the biosurfactants. [26]

4.4 Physiological properties

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

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

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

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

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

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

5 Basic analysis in biosurfactant study

5.1 Biosurfactant screening methods

Isolates (or supernatants) that exhibit good growth are subjected to various biosurfactants activity tests (Table 3) which include hemolytic activity test, surface tension measurements, drop collapse test; [17,72] oil spreading assay; [18] emulsification activity; [73] emulsification index (%EI24) [74] CTAB/methylene blue agar plate test [14], Penetration assay for high throughput screening [25], and molecular characterization [16] Biosurfactants producing microorganisms are characterized through this order of steps: cultural isolation, purification of isolates, DNA extraction, a polymerase chain reaction (PCR) of DNA, sequencing and phylogenetic 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 0C 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 µl 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 µl of distilled water into a Petri dish followed by the addition of 20 µl of diesel oil to the surface of water then 10 µl 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 (%EI24)	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. %EI24 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 µl of supernatant into the labeled wells and incubate at 37 0C 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 µl of hydrophobic paste consisting of silica gel and oil. The paste is covered with 10 µl of oil followed by placing of coloured supernatant consisting of 90 µl of supernatant and 10 µl 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]
225
226 Table 4. Steps and common procedures involved in molecular charaterisation 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]

5.2 Estimation of biosurfactant activity

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

HLB = 20(MWHP/MWSA)

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

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

Adapted from De *et al.* [83]

6 Crude extraction of biosurfactants

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

7 Purification of biosurfactants

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

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

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

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

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 0C 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 *et al.* [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 0C to be easily collected as crude product. Once the pellet is obtained, it can be dried under N2 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 *et al.* [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 *et al*. [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 *et al*. [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 *et al.* [22]

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.

8.2 Chromatography methods

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

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

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

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 Convoluted spectra More than one peak per component Libraries of limited use due to complex maxtrix
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

Adapted from Satpute *et al* [20]

9 Classification of the five groups of biosurfactant

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

9.1 Classification based on molecular weight

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

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

9.2 Classification based on chemical composition

9.2.1 Glycolipids

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

a) **Rhamnolipids** are amphiphilic compounds ideally comprised of 3-hydroxy fatty acids (hydroxydecanoic acid) linked through a β-glycosidic bond to mono- or di-rhamnose [26,99,100]
b) **Sophorolipids** are made up of disaccharide-sophorose β-linked to a long fatty acid with a chain length of 16 - 18 carbon atoms with the presence of unsaturation [101] They can exist in a lactonic form [97] or in an acidic form [102]
c) **Mannosylerythritol lipids (MELs)** have 4 major structural groups having 4-O-b-D-mannopyranosyl-D-erythritol linked to 2 medium-length chains of fatty acyl esters [29,103]. Though MELs exist as MEL-A, MEL-B and MEL-C, the MEL-A is the most dominant [32]
d) **Trehalolipids** biosurfactants exist in various structural types. In some microorganisms, the disaccharide-trehalose linked at C-6 and C-6 to mycolic acid which is long-chain α-branched and β-hydroxy fatty acids [104]

9.2.2 Lipopeptides and lipoproteins

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

9.2.3 Fatty acids and lipids (phospholipids and neutral lipids)

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

9.2.4 Polymeric and particulate biosurfactants

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

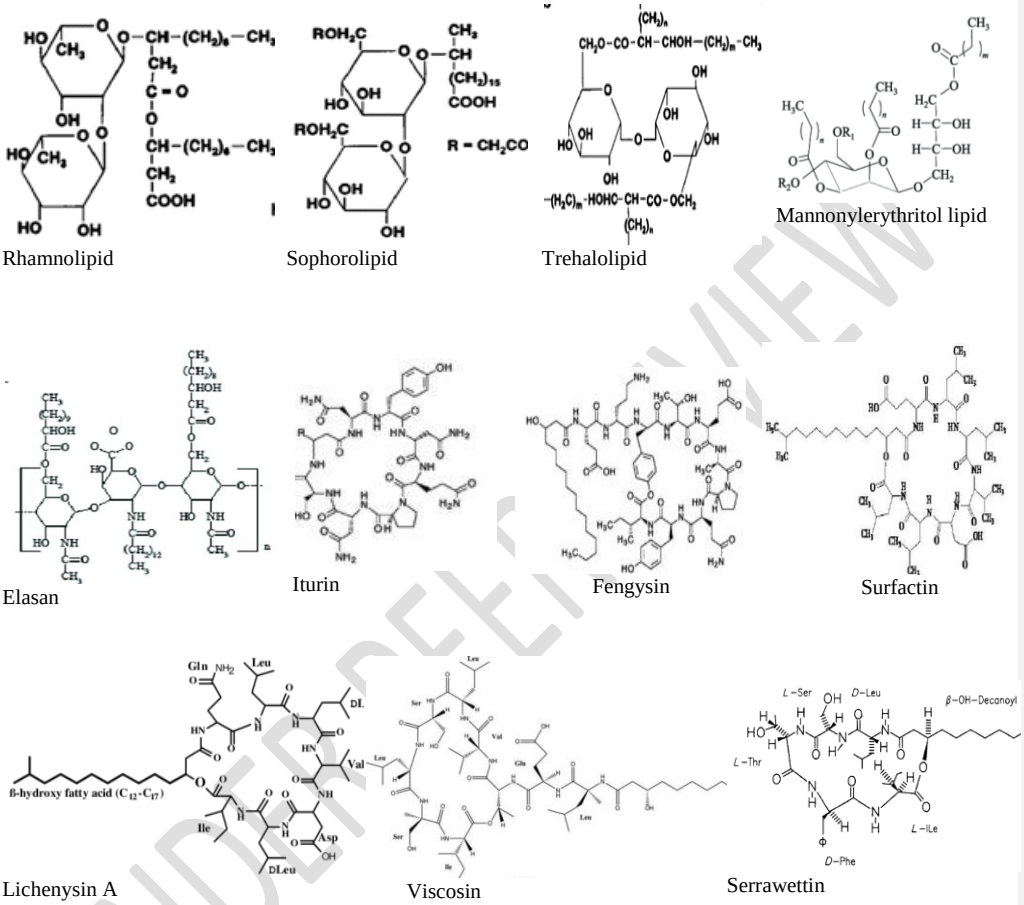

Fig. 2. Structures of well-known biosurfactants produced by microorganisms [7, 26]

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

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

10 Advantages and disadvantages of biosurfactants

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

Table 9. Advantages and disadvantages of biosurfactants

Advantages	Disadvantages
Biosurfactants are easily degraded in the environment	
Biosurfactants exhibits lower toxicity than the synthetic ones	Hemolytic activity of certain biosurfactants can rupture erythrocytes at 37 0C
Surfactants of biological origin have feature of compatibility thus being used in pharmaceuticals, cosmetics, food industries etc.	Biosurfactants is characterized with very low productivity. This is because over producing strains and recombinant stains are very rare
Biosurfactants can be produced from a variety of relatively cheap raw materials	To get pure biosurfactants require multiple steps with attendant cost
Biosurfactants are effective surface and interfacial tensions reducers	Strong foam formation hampers the improvement in production yield
Biosurfactants can be produced from industrial waste and by-products thus key into acceptable production economics	Production of biosurfactants in large scale is capital intensive
Many biosurfactants are stable at extreme pH, salinities and temperature	
Biosurfactants are specific in their action, hence play specific functions	

Adapted from De *et al.* [83]

11 Conclusion

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

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

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