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Review

Biosurfactants Produced by Yeasts: Fermentation, Screening, Recovery, Purification, Characterization, and Applications

Natalia de Andrade Teixeira Fernandes [1], Luara Aparecida Simões [2] and Disney Ribeiro Dias [3,*]

[1] Chemistry Department, University of California, Davis, CA 95616, USA
[2] Centre of Molecular and Environmental Biology, University of Minho, 4710-057 Braga, Portugal
[3] Food Science Department, Federal University of Lavras, Lavras 37200-900, Brazil
* Correspondence: diasdr@ufla.br; Tel.: +55-35-3829-5256

Abstract: The demand for biosurfactants (BS) produced by yeast for use in industrial processes and products is increasing. Therefore, there has been an increase in the number of publications related to characterization of surfactant compounds produced by yeasts generally recognized as safe (GRAS), which has enabled their application in several industries, including the pharmaceutical and food industries. However, some of these studies use techniques that are not accurate or are no longer essential because of advancements in new technologies. Given the industrial importance of yeasts and their potential to produce BS, this study reviews the production of BS by this microorganism and the most recent industrial applications of BS. It also critically reviews a wide range of techniques used in screening of BS-producing strains, as well as those used in recovery, purification, and characterization of these surfactant compounds produced by yeasts. This review introduces diverse methodologies that are indispensable for the study of BS produced by yeast in an effort to advance BS design, synthesis, and application and introduces new perspectives in the research of these compounds to overcome the obstacles present in this field.

Keywords: bioemulsifiers; secondary metabolites; environmental; agricultural; health; medicine

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1. Introduction

Surfactants are chemical compounds found between aqueous phases with different degrees of polarity and hydrogen bonds [1,2]. These surfactants, depending on their origin, can be synthetic (surfactants) or natural (biosurfactants). Biosurfactants (BS) are composed of amphiphilic molecules with hydrophilic and hydrophobic fractions [3,4]. Their polar portion can be ionic (cationic or anionic), non-ionic, or amphoteric (possess both positive and negative charges depending on the environment in which they are present), and their non-polar portion commonly comprises a hydrocarbon chain (Figure 1). These characteristics give BS the ability to reduce surface- and interfacial stress and form emulsions, which are hydrocarbons solubilized in water or vice versa [5,6].

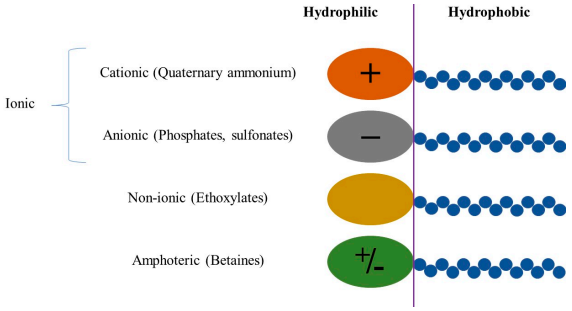

Figure 1. Types of biosurfactants based on the polarity of the head group. Source: the author.

BS have aroused considerable interest in recent decades due to properties that make them useful in different industrial activities that involve emulsification, detergency, lubrication, foaming, dispersion, or solubilization in different phases. In addition, BS have advantages over synthetic surfactants because they are complex molecules with specific functional groups and have high biodegradability (i.e., they are easily degraded by microorganisms in water and soil), stability, and efficacy in a variety of environmental conditions (temperature, pH, and salinity), as well as low toxicity and biocompatibility [7–10].

BS can be used in several industrial sectors, partially or totally replacing chemical surfactants in widely consumed products [11], such as laundry detergents [12], household cleaning products, personal hygiene products [13,14], and cosmetics [15]. In the medical field, BS can serve as antimicrobials, antitumor agents, and anti-inflammatory agents because of their bioactivity [16,17]. In agriculture, BS can improve soil quality by removing heavy metals and inhibiting activity of several pests—such as fungi, weeds, insect larvae, and nematodes—that cause drastic reductions in profits [18]. BS also play a significant role in other fields, acting as larvicidal agents against larvae of the *Aedes aegypt* mosquito—which causes diseases such as Dengue, Zika, and Chikungunya [16,19]—and used to stabilize silver and gold nanoparticles, even in the absence of conventional chemical agents [20,21].

Different microorganisms can produce BS, but use of yeast offers the great advantage of not presenting a risk of toxicity or pathogenicity; therefore, biotechnological processes based on the yeast species are granted the generally recognized as safe (GRAS) status given by the Food and Drug Administration (FDA) [22]. These microorganisms can produce BS from oleaginous substrates, including agro-industrial residues—such as glycerol, corn steeping liquor, and residual frying oil—which makes industrial application of BS produced by yeasts viable and reduces production costs [23,24]. For example, green technologies may emerge as important tools for lignocellulosic biorefineries as these are alternative routes for short-term BS production [25].

Considering reports of increased use of BS in diverse industrial sectors and growing interest in BS production and characterization, this review discusses the methods of production, detection, classification, recovery, purification, and characterization of BS produced by yeasts. This article also presents an overview of use of these natural compounds given their desirable properties.

2. Advantages and Disadvantages of Biosurfactants in Relation to Synthetic Surfactants

Surfactants are extensively used in homes and industries on a daily basis, generating a market value of USD 39,901 million worldwide in 2019 that is expected to grow to USD 52,417 million by 2025 [26]. However, despite the universality of these components, synthetic surfactants cause environmental impacts that are generally neglected since most products that incorporate them are sold as disposable and are released into the environment after disposal. Synthetic surfactants that reach aquatic environments have been reported as organic pollutants and have been detected in surface waters [27].

Synthetic surfactants in industrial, domestic, and medical wastewater become difficult to remove in water treatment plants because of the molecular properties of surfactants [28], which cause residual surfactant content to remain even after treatment. Furthermore, surfactants can also increase spread of different pollutants, such as heavy metals, causing extra problems for the ecosystem [26].

In this context, biosurfactants have emerged as an alternative to synthetic surfactants (Figure 2). In contrast to synthetic surfactants, biosurfactants are composed of natural molecules, such as lipids, sugars, and proteins, and are produced by microorganisms. This unique composition gives them preferable properties, such as better biodegradability and lower toxicity. They are able to maintain their surface properties as well as synthetic surfactants [29,30], increasing their acceptability; they generally do not pose an ecological threat.

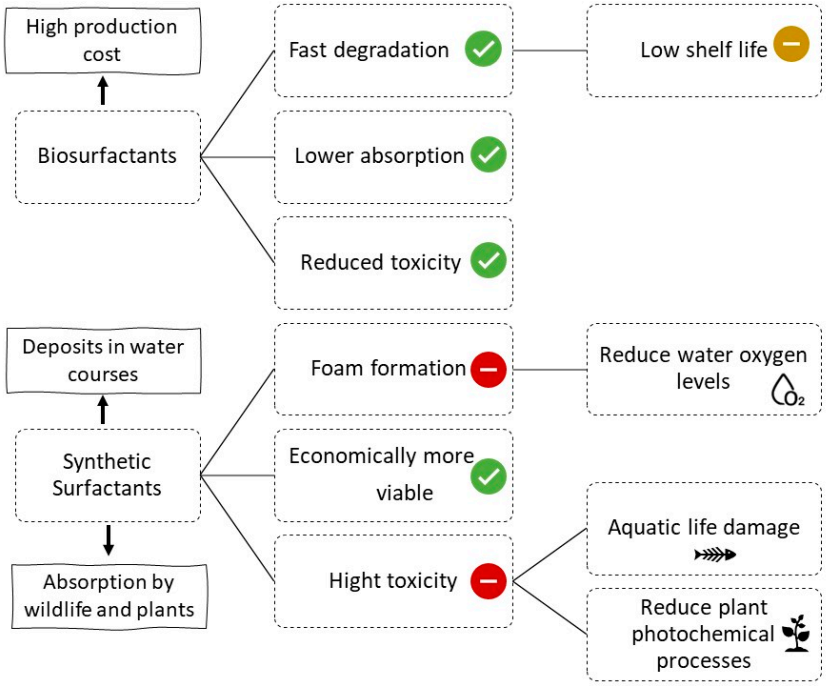

Figure 2. Synthetic surfactants and biosurfactants: comparative advantages and disadvantages.

3. Biosurfactants Produced by Yeast

Yeasts have gained greater visibility in BS studies over bacteria as bacterial BS may have restricted use in the pharmaceutical and food industries due to their pathogenic and/or opportunistic characteristics. In addition, some yeasts have a higher rate of substrate conversion, producing higher amounts of BS than bacteria [31,32]. Most yeast species also offer the advantage of having GRAS status as they do not present a risk of toxicity or pathogenicity, allowing their products to be applied in a wide range of industrial sectors [33,34].

Regarding the growing industrial demand for yeasts for several applications (Figure 3), the genus *Candida* stands out for its diversity of species producing biosurfactants reported in the literature [1,35]. However, several other yeast genera have been reported recently for their potential as producers of BS—such as *Rhodotorula* [36], *Saccharomyces* [37], and *Wickerhamomyces* [16,38]—that exhibit flocculent, environmental, antimicrobial, antioxidant, and larvicidal properties. In addition, their recovery percentages are higher compared to the yields of BS originating from other microorganisms. Several studies have described increasing yields of production of BS in yeast fermentation, varying between 10 and 120 g/L [39–42]. For example, high yields of BS (120 g/L) produced by *Candida bombicola* using a residue of the meat processing industry have been reported [41]. On the other hand, a study showed that bacterium *Klebseilla* sp. produced a BS yield in the range of 0.1–6.9 g/L [43].

Yeasts are able to grow both on water-immiscible substrates, such as vegetable oils and hydrocarbons, and on water-soluble compounds, such as carbohydrates and glycerol [33,44]. The conditions of cultivation and composition of the medium determine the production and composition of the BS since hydrophobic substrates have high moisture and protein content that cause their rapid degradation and influence survival of producing microorganisms, which use the set of carbon and energy sources for growth [45,46]. This combination of carbon sources with insoluble substrates facilitates intracellular diffusion because it increases solubility of water-insoluble compounds and facilitates their transport to the cell [47]. Table 1 shows BS production by different yeasts from different substrates, highlighting the vast diversity of substrate possibilities.

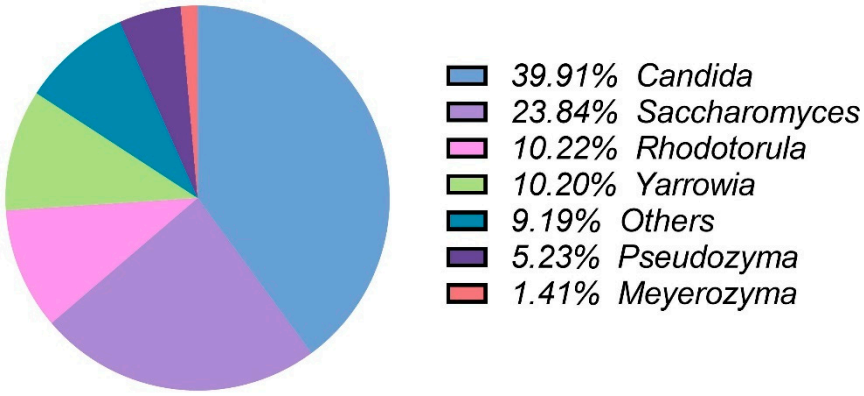

Figure 3. Publications on the main genera of BS-producing yeasts in the last 5 years. Data collected from Google Scholar searches on the number of articles published from 2018 to 2022 (n = 9020), excluding reviews.

Table 1. Yeast-producing BS using different substrates.

Biosurfactant-Producing Microorganism	Biosurfactant Type	Substrate	Properties	References
Debaryomyces hansenii CBS767	Glycolipid	Soybean oil	Significant emulsification activity and lowering of surface tension	[48]
Candida utilis UFPEDA1009	Glycolipid	Canola waste frying oil and glucose	Replace animal fat in formulation of cookies	[37]
Rhodotorula sp.CC01	Glycolipid	Landfill leachate and Olive oil	Potential in remediating petroleum hydrocarbons	[49]
Candida sphaerica UCP 0995	Glycolipid	Groundnut oil refinery and corn steep liquor	Recovered motor oil adsorbed in a sand sample	[50]
Saccharomyces cerevisiae URM 6670	Rhamnolipid	Soybean waste frying oil and 1% corn steep liquor	Emulsifiers in a salad dressing formulation	[51]
Candida lipolytica UCP0988	Glycolipid	Animal fat and corn steep liquor	Recovering residual oil from oil-saturated sand	[52]
Meyerozyma guilliermondii	Glycolipid	Used soybean oil	Solubilize cadmium from the sewage sludge	[53]
Candida tropicalis UCP0996	-	Sugarcane molasses, corn steep liquor, waste frying oil	Motor oil spreading efficiency	[54]
Cutaneotrichosporon mucoides UFMG-CM-Y6148	Sophorolipid	Sugarcane bagasse hydrolysate	Highlighted emulsifying properties in kerosene	[25]
Starmerella riodocensis	Sophorolipid	Glucose and palm oil	Emulsification activity against kerosene and antifungal activity against *Candida albicans*	[55]

One of the first and most relevant studies of production of yeast BS was by Pareilleus (1979) [56], in which yeast *Candida lipolytica* was shown to produce a complex extracellular polymer with a protein, a lipid, and a carbohydrate portion and exhibited emulsifying properties when grown in n-tetradecane or a mixture of linear hydrocarbons. Some years later, Cooper and Paddock (1984) [57] used two types of carbon sources—carbohydrate and vegetable oil—to obtain large yields of BS from *Candida bombicola* (formerly called *Torulopsis bombicola*), producing a mixture of glycolipids that were found by thin-layer chromatography, showing six components for α-naphthol-positive.

Further research continued to explore the ability of yeasts to produce BS. Ilori et al. (2008) [58] evaluated the potential for hydrocarbon degradation and the emulsifying activities of BS produced by two yeast strains—*Saccharomyces cerevisiae* and *Candida albicans*—obtained from a polluted pond. Both strains grew effectively using crude oil and diesel as carbon sources and the BS exhibited antimicrobial activities against *Escherichia coli* and *Staphy-*

lococcus aureus. This work demonstrated that these strains represented a new class of BS producers with the potential for use in a variety of biotechnological and industrial processes, particularly in the pharmaceutical industry. The following year, Hirata et al. (2009) [59] showed production of a glycolipid BS by yeast *C. bombicula* using soybean oil as a carbon source; this BS exhibited low foaming with high detergency and also presented low cytotoxicity and readily biodegradable properties.

More recently, a BS produced by *Rhodotorula* sp. using wastewater from olive mills as a substrate has been investigated. The isolated yeast strain was considered a potent producer of a BS that was partially characterized as a complex of glycolipoprotein groups exhibiting interesting properties, such as low critical micellar concentration, a significant reduction in surface tension, strong emulsifying activity, and great potential for application in remobilization of polluted soil hydrocarbons, with a removal rate greater than 95% [36].

Another study evaluated the toxicological profile of polymeric nanoparticles encapsulated in a polylactic acid–polyethylene glycol (PLA–PEG) BS in mice. The BS was isolated from *C. parapsilosis* and partially characterized by FTIR and GC MS, revealing the presence of a phenol or alcohol group with the possible presence of an amide in the structure. A PLA–PEG copolymer was synthesized using 72 k Da PLA and 6 k Da PEG, and the histopathological results from the selected vital organs revealed that BS and/or encapsulated polymeric nanoparticles can be considered safe since no toxicological characteristics were observed in the tissue histopathology. Therefore, it can be deduced that nanoparticle-encapsulated biosurfactants are non-toxic and can provide a safe and suitable platform for biomedical applications in the future [60].

In addition to yeasts demonstrating high conversions of substrates and their BS products being suitable for applications in the food and pharmaceutical industries—as most do not present risks and have GRAS status—it is clear that BS produced by yeasts have been successful in diverse applications—from antimicrobials to bioremediations and even in the medical field. Therefore, studies of BS produced by yeasts have increased as there is a competitive market between BS and chemical surfactants.

4. Applications of Biosurfactants Produced by Yeast

Diversity of chemical structures of BS leads to a variety of functions (Figure 4), which include their interaction with hydrophobic chemicals. Because of this capacity, BS influence absorption, transport, and biodegradation of hydrocarbons and xenobiotics, which enables bioremediation applications [61]; their ability to form stable micelles enables their application in nanoemulsion formulations and other drug delivery systems used against major diseases, such as thrombosis, Alzheimer's disease, cancer, etc. [62]. The amphipathic nature of BS allows them to interact with polar and non-polar surfaces, as well as charged surfaces forming the first layer and acting as wetting agents. This amphipathic ability also assists in microbial adhesion and anti-adhesion at interfaces, enabling applications in the pharmaceutical, agricultural, food, and medical industries [63].

4.1. Medicine and Health

BS have emerged as promising molecules due to their structural versatility and diverse properties that can be widely used in the pharmaceutical, medical, and cosmetic industries, mainly due to their surface activity. Therefore, BS can be used as antibacterial, antifungal, and antiviral operators; particles safe modulators; antibodies; quality treatments; cancer therapies; constituents of drug delivery systems; or emulsifiers in cosmetics.

Strains *Candida albicans* and *Candida glabrata* were used to produce BS, which were then evaluated for their antibacterial properties; the BS of both strains showed antibacterial activity against pathogenic Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) bacteria at a concentration of 60 mg/L [64]. The antimicrobial and anti-adhesive activity of BS isolated from *Candida lipolytica* were determined by measuring the percentages of growth inhibition obtained for various microorganisms; it was shown that this BS inhibited growth of different

strains of *Streptococcus* and *Staphylococcus aureus* by more than 90% at one concentration of 12 mg/L. Recently, the BS produced by yeast *Wickerhamomyces anomalus* exhibited antibacterial properties against *Bacillus cereus*, inhibiting and killing the bacterium at a concentration of 60 mg/L [16].

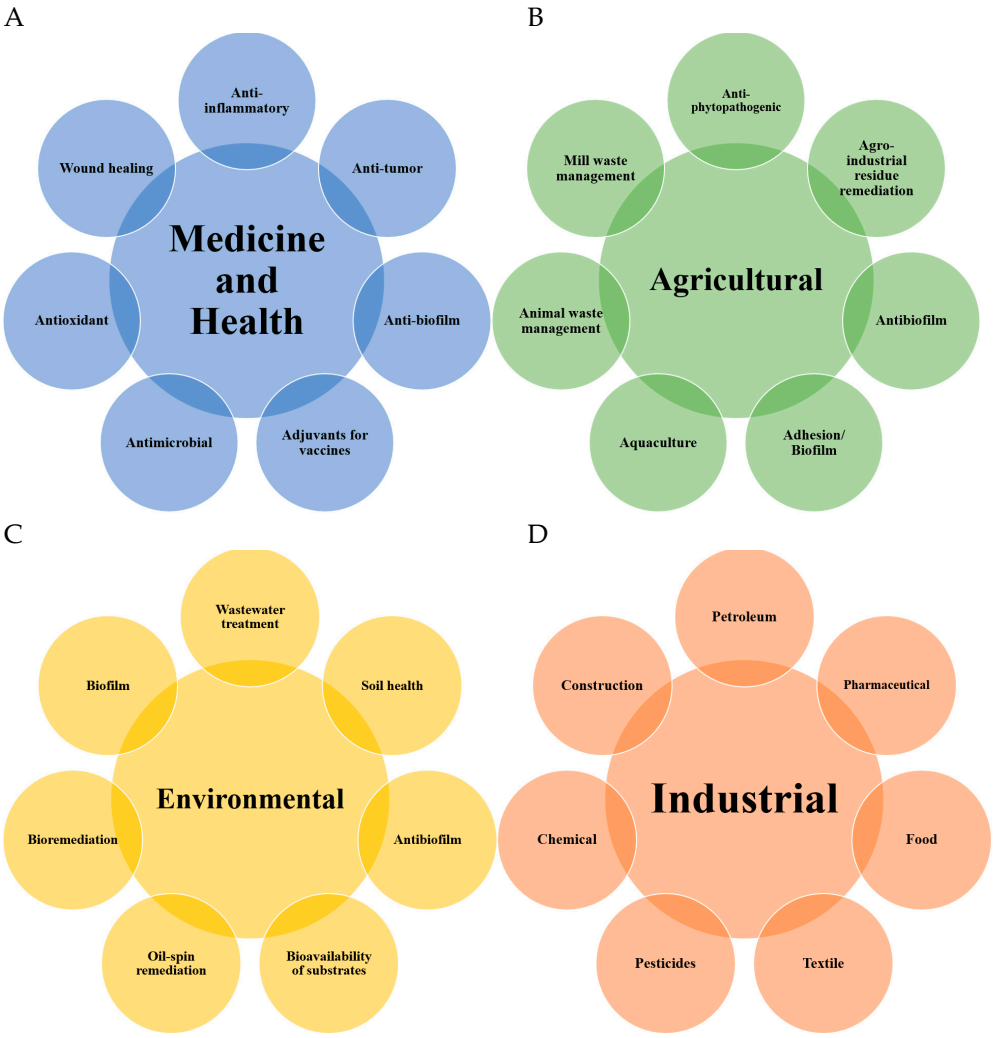

Figure 4. Properties and applications of biosurfactants in the (**A**) medical/health sector; (**B**) agricultural sector; (**C**) environmental sector; and (**D**) industrial sector. Source: modified from [63].

Mannosilitritol lipid (MEL), a glycolipid BS produced by the yeast of the *Candida* species, has been reported to have neurological and immunological properties, along with antimicrobial potential, while the succinyl trehalose lipid produced by *Rhodococcus* species could inhibit certain viruses [65]. Yeast *Candida bombicola* has produced a sophorolipidic BS that exhibited spermicidal activities, was considered hostile to the human immunodeficiency virus (HIV), and was cytotoxic. The diacetate ethyl ester subsidiary of this sophorolipid was the strongest spermicide and virucide in the arrangement of sophorolipids examined [66].

Other works can be found in the literature for these applications, such as the one published by Akiyode et al. (2016) [67] that concluded that BS were effective in retarding the growth of the tested cancer cell lines and, therefore, may be potential candidates for use in human cancer therapy. The physicochemical characteristics of BS suggest that their mechanism of action may be due to activity in the cell membrane.

BS produced by yeast also showed larvicidal activity against larvae of the *Aedes aegypt* mosquito, which causes diseases such as Dengue, Zika, and Chikungunya. Marcelino et al.

(2017) [19] showed that the BS produced by yeast *Scheffersomyces stipitis* killed 100% of the larvae 12 h after application of BS in concentrations of 800 and 1000 mg/L. Another study showed the larvicidal activity of BS produced by *W. anomalus*, where 100% of deaths were obtained at a low concentration of 63 mg/L in 24 h [16].

BS can also be used in cosmetics—in the same way as chemically synthesized surfactants—and in detergency, emulsification, demulsification, humidification, foaming, dispersion, solubilization of hydrophobic substances, or modification of surfaces [68]. Takahashi et al. (2012) [69] investigated the antioxidant properties of different MEL derivatives (A, B, and C). All MEL derivatives tested showed antioxidant activity in vitro, but MEL-C, which was produced from soybean oil by *Pseudozyma hubeiensis*, showed the highest rates of elimination of the DPPH radical (50.3% at 10 mg/mL) and superoxide anion (50% at 1 mg/mL), had greater protective activity against oxidative stress, and showed the highest antioxidant activity (50.3% at 10 g/L). Based on their results, the authors suggested that MELs have potential as anti-aging ingredients for skin care.

4.2. Agriculture

BS can be used to enhance agribusiness as they play an important role in helping microorganism products used for biocontrol, such as parasitism, antimicrobial, rivalry, and protection measures. BS can help organisms to adsorb soil particles surrounded by toxins, thus reducing the dispersion path between the assimilation site and the bio-uptake site by microorganisms. In addition, BS do not have any unfavorable impact on humans or plants and, therefore, can be used in biological control of plant diseases [66]. Use of BS can provide protection to plants directly because of antimicrobial properties against phytopathogenic pathogens and indirectly through the process of stimulating plant defense systems, called the "induced systemic resistance" mechanism; by inducing this mechanism, BS make plants more resistant and less susceptible to attack by pathogens [70].

A sophorolipidic BS produced by yeast *Rhodotorula babjevae* that was isolated from an agricultural field was evaluated for antifungal activity against phytopathogenic fungi and exhibited promising activity against *Colletotrichum gloeosporioides*, *Fusarium verticilliodes*, *Fusarium oxysporum*, and *Trichophyton rubrum*. The in vitro antifungal activities of the purified BS were estimated based on the minimum inhibitory concentration (MIC) values and were obtained in the BS concentration range of 62–1000 μg/mL [71].

Another recent work also showed the antifungal activity of a BS produced by yeast against species of phytopathogenic fungi *Aspergillus flavus, Aspergillus niger, Cercospora sorghi, Colletotrichum truncatum, Fusarium verticillioides,* and *Fusarium solani*. This BS was produced by yeast *W. anomalus* and inhibited mycelial growth in up to 95% of all phytopathogenic fungi evaluated in concentrations of 30–50 μg/mL, showing that BS can be applied in agriculture to control diseases and aid in biological control [16].

A study investigated application of modified BS (Figure 5) produced by yeast in plant diseases and produced more evidence of the plant-disease control properties of microbial BS. The investigators reported that BS derivatives exhibited significant antifungal activity against 18 phytopathogens (*Alternaria tomatophilia*, *Alternaria solani*, *Alternaria alternata*, *Aspergillus niger*, *Aureobasidium pullulans*, *Botrytis cinerea*, *Chaetomium globosum*, *Fusarium asiaticum*, *Fusarium globaminum*, *Fusarium cereals*, *Fusarium austroamericana*, *Fusarium oxysporum*, *Penicillium chrysogenum*, *Penicillium digitatum*, *Penicillium funiculosum*, *Phytophthora infestans*, *Phytopthora capsici*, and *Ustilago maydis*) and seven bacterial plant pathogens (*Acidovorax carotovorum*, *Erwinia amylovora*, *Pseudomonas syringae*, *Pectobacterium carotovorum*, *Ralstonia solanacearum*, *Pseudomonas cichorii*, and *Xanthomonas campestris*). The minimum inhibitory concentrations ranged from 0.009 to 10 mg/mL, and the BS was also effective against zoospores of pathogen *Plasmopara viticola*, which showed loss of viability and lysis occurring at a concentration of 50–500 μg/mL of BS [72].

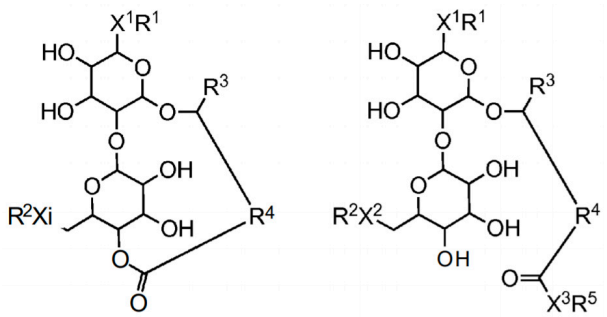

Figure 5. Modified sophorolipids for inhibition of plant pathogens. Source: [72].

4.3. Bioremediation and Oil Recovery

Bioremediation is a process that uses microorganisms to accelerate degradation of environmental contaminants. Biosurfactants increase the surface area, solubility, and bioavailability of hydrophobic water-insoluble substrates, stimulating growth of oil-degrading microorganisms and improving their ability to utilize hydrocarbons [73].

The crude BS produced by yeast *Candida lipolytica* (UCP 0988) was evaluated for removal of heavy metals and petroleum derivatives. The crude BS removed about 96% of Zn and Cu and reduced the concentrations of Pb, Cd, and Fe from the specimen. The BS removed 20% of the residual oil using a permeability apparatus. The results show that BS can be applied in technologies where removal of heavy metals and petroleum derivatives is desirable. These results demonstrate the versatility of biomolecules of an amphipathic nature [74].

In another study, the BS of *Candida sphaerica* was tested for demulsification of engine oil emulsions, with values around 40%. The crude BS was able to disperse approximately 90% of the oil droplets in seawater and proved to be non-toxic to the native marine microbiota. These results indicate the potential for applying the BS produced by *C. sphaerica* in the oil industry as a complement to the remediation processes involving contaminated water [75]. The same group showed the following year that the BS of *Candida bombicola* also demonstrated the ability to remove oil, with 70% of the engine oil adsorbed to the porous surface. The results obtained with this BS showed this biomolecule's promising properties for use in bioremediation of hydrophobic compounds [76].

Another study, also involving yeast of the genus *Candida*, analyzed the BS of *Candida lipolytica*. The crude BS did not present toxicity for bivalve *Anomalocardia brasiliana*, for microcrustacean *Artemia salina,* or for three species of vegetable seeds. It stimulated degradation of motor oil by microorganisms native to seawater, and the cell-free crude extract removed about 30–40% of Pb and Cu from the sand. These results indicate that the BS produced has great potential to be applied as a bioremediation agent in the oil industry for cleaning up oil spills, with additional potential to be used in other industries [77].

Recently, other studies have also shown the potential for BS produced by yeasts in removing engine oil. Derguine-Mecheri et al. (2021) [36] evaluated the BS produced by *Rhodotorula* sp.YBR and obtained a recovery rate of 98% ± 0.28%. Demonstrating efficient results in the improved removal of hydrophobic contaminants from polluted soils makes this BS a promising potential candidate for environmental applications. In addition, Santos et al. (2021) [78] demonstrated a new formulation of low-cost, biodegradable, and non-toxic BS by *Candida sphaerica* and investigated the dispersion capacity of engine oil. This biomolecule did not show a cytotoxic effect when placed in contact with the L929 cell line, proving to be harmless to the environment. It has also proven effective in removing oil adsorbed on soil, with a dispersion capacity of 90%, presenting potential in bioremediation applications aimed at recovering environments polluted by oily residues.

In addition to applications in bioremediation and removal of heavy metals from soil, another application evaluated using BS produced by yeast was in bioleaching assays. *Meyerozyma guilliermondii* showed, in the tests performed, the ability to remove metal in anaerobic sewage sludge and solubilize 15.9% of the sewage sludge's cadmium [53].

5. Screening Methods for Detection of Biosurfactants

Although several yeast strains are already known to produce BS, many species have been described as new producers of BS; for this reason, there are main methods used to screen, detect, or evaluate potential BS-producing microorganisms, with each having its own advantages and disadvantages (Table 2).

Table 2. Screening methods to evaluate potential BS-producing microorganisms and their advantages and disadvantages.

Methods	Advantages	Disadvantages
Hemolytic activity	Easy visualization	The method is not specific; Some biosurfactants do not show any hemolytic activity; Can give many false negative and false positive results.
Blue agar plate method	Easy visualization; Efficient method	It is specific for anionic biosurfactants; The medium can be harmful and inhibits growth of some microbes.
Agar plate overlaid with hydrocarbons	Easy visualization; Efficient method	It takes a long time (one week) for the results.
Axisymmetric drop shape analysis	Requires a very small number of cells	Equipment is required; Complex calculation; Different samples cannot be measured in parallel.
Modified drop collapse method	Fast and easy method; Requires no specialized equipment and just a small volume of samples	If the sample contains a small amount of BS, false negative results can occur.
Oil spreading method	Fast, easy, and efficient method; Requires no specialized equipment and just a small volume of samples	-
Emulsification assay	Simple screening method	Surface activity and emulsification capacity do not always correlate. Consequently, this method gives just an indication of the presence of biosurfactants.
Emulsification index	A simple and efficient method	-
Tensiometric measurement	Accuracy and ease of use	It requires specialized equipment; Measurements of different samples cannot be performed simultaneously.

(-) No disadvantage found.

5.1. Hemolytic Activity

This is a qualitative screening test for detection of BS producers, in which solid media are used—normally nutrient agar supplemented with 5% fresh whole blood, called blood agar [79]. The isolates are seeded and incubated at the required temperature according to the needs of each microorganism for 48 h. Visual inspection for hemolysis may be an indication of lysis of red blood cells due to rupture of the cell membrane caused by the presence of active surface molecules. Hemolytic activity has been considered an unreliable criterion for detection of BS activity [80]; however, it is very difficult to test the BS productivity of a culture under different conditions directly on the agar [81].

5.2. Blue Agar Plate Method

This technique was developed for detection of glycolipids using mineral salt agar (MSA) supplemented with a carbon source (2%), 0.5 mg/mL of cetyltrimethylammonium bromide (CTAB), and 0.2 mg/mL methylene blue (MB) [82]. This technique uses anionic BS to form a pair of insoluble ions with the CTAB-MB cationic; formation of a dark blue halo around the culture is considered positive for production of BS (Figure 6). It is an excellent technique that has been used widely for detection of BS glycolipids.

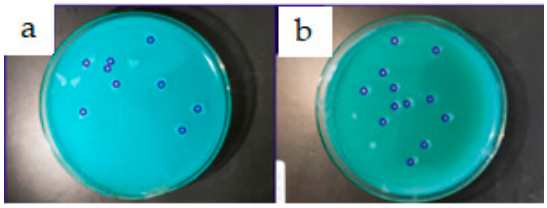

Figure 6. Blue agar plate method. BS positive (dark blue halo). (**a**,**b**). Source: [83].

5.3. Agar Plate Overlaid with Hydrocarbons

Pure isolates are plated on Luria–Bertani agar plates coated with hydrocarbons—such as kerosene, hexadecane, benzene, toluene, diesel, or crude oil—and incubated for one week at the desired temperature. Colonies surrounded by an emulsified halo are detected as producers of BS (Figure 7) [84]. This is an efficient method where the observation of an emulsified halo around the culture is a direct indication of the BS producer.

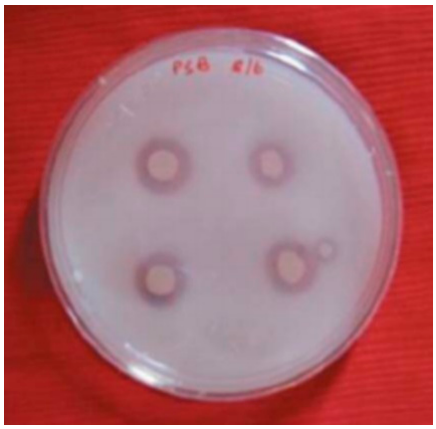

Figure 7. Agar plate overlaid with hydrocarbons. Positive emulsified halo. Source: [85].

5.4. Axisymmetric Drop Shape Analysis (ADSA)

This technique simultaneously determines the contact angle and the surface tension of the liquid from the profile of a drop on a solid surface (Figure 8). The cells are suspended in a buffer solution or in broth cultures, a drop of each suspension is placed on the surface of fluoroethylene propylene, and the profile of the drop is determined with a counter monitor depending on the time (up to 2 h). The surface stresses of the suspensions are calculated from the droplet profile with ADSA. Only suspensions producing BS show a reduction in surface tension, which depends on the concentration of the product and/or the number of microorganisms producing BS. This technique requires a very small number of cells [86].

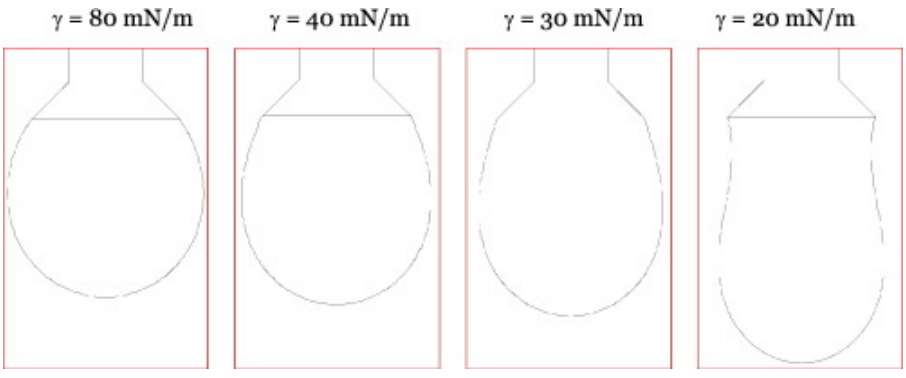

Figure 8. Axisymmetric drop shape analysis. Pendant drop shapes with different surface tensions using a 10 µL drop. Source: [87].

5.5. Modified Drop Collapse Method

Microplates are thinly coated with oil, a 5 µL sample of the culture broth is added to the center of the well, and observations are made for 1 min. If the drop of a sample collapses within the coated oil, it is an indication of the presence of BS in the culture broth [88,89]. This is a technique of rapid detection. However, if the sample contains a small amount of BS, false negative results can occur [80].

5.6. Oil Spreading Method

For this method, 20 µL of oil is added to 50 mL of distilled water in a Petri dish and 10 µL of the culture broth is added to the middle of the surface of the oil-coated water. If there is an emulsified halo around the culture broth, it is considered positive for production of BS (Figure 9) [90]. This is one of the fastest methods for detecting presence of BS producers and is considered efficient.

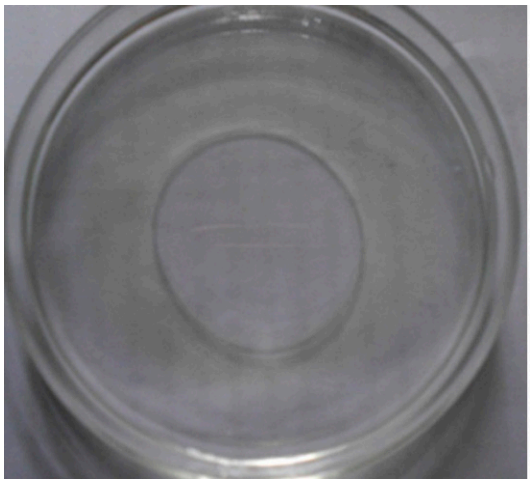

Figure 9. Oil spreading method. Positive emulsified halo. Source: [91].

5.7. Emulsification Assay (EA)

The culture broth is centrifuged at 10.000 rpm for 15 min and then 3 mL of the supernatant is mixed with 0.5 mL of oil or hydrocarbon and vortexed vigorously for 2 min. The mix is left undisturbed for 1 h to separate the aqueous and oily phases (Figure 10). The absorbance of the aqueous phase is measured using a spectrophotometer at 400 nm and the uninoculated broth is used as a blank; an absorbance of 0.01 units multiplied by the dilution factor is the unit of emulsification activity per milliliter [92].

Recently, Tavares et al. [93] have proposed a quick and reproducible method to determine the emulsifying activity, based on the ability of biosurfactants/bioemulsifiers (BS/BE) to form stable emulsions when mixed with n-heptane. In a 4 mL screw cap glass tube (10 × 75 mm, ND10 caps with PTFE septum) add 1 mL of n-heptane to 1 mL of an aqueous solution containing the surfactant/cell-free culture broth. Mix vigorously for 2 min, using a vortex, and leave the tube to rest for 10 min in an upright position before analyzing. Repeat the process with progressively greater volumes of surfactant/cell-free culture broth until complete emulsification of the organic phase is observed (Figure 10). With this method, 1 emulsifying unit (1 U) is the minimum volume of product (Volmin of emulsifier/surfactant, up to 1 mL) needed to form and maintain 100% emulsion in the organic phase. This can then be used to obtain the emulsifying activity (EA) in U/mL by EA = 1 U/Volmin (mL) [93]. This method allows the direct comparison between distinct substances with surfactant properties, giving a quick quantification of substances of multiple origins and chemical natures.

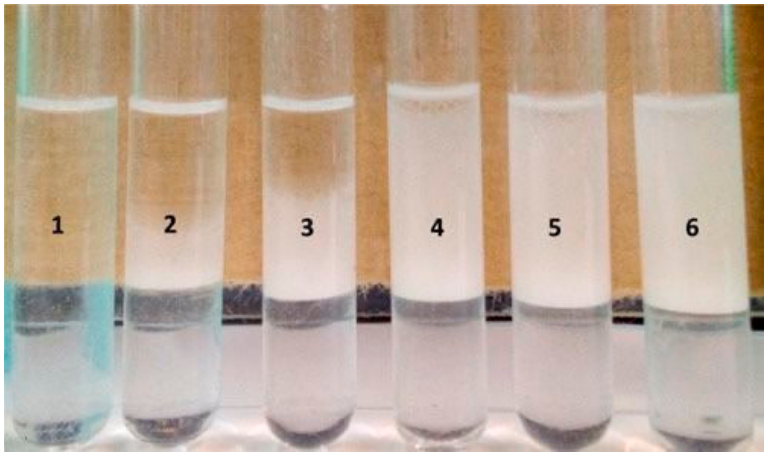

Figure 10. Emulsification assay. Emulsification tests using 1 mL of n-heptane as organic phase and 1 mL of aqueous phase containing increasing amounts of crude BS/BE (0.352 g/L solution)—1: control (0), 2: 100 µL, 3: 200 µL, 4: 325 µL, 5: 350 µL, and 6: 400 µL. Using 350 µL of this crude 100% emulsion of organic phase is attained. Source: [93].

5.8. Emulsification Index (EI)

Emulsification activity is measured by calculating the height of the emulsion for determining EI. This technique is performed by adding kerosene or oil to the culture broth (1:2 *v*/*v*); the tube with the sample and kerosene is vortexed for 2 min and left to stand for 24 h. The height of the emulsion is measured in the layers formed between the aqueous layer and the kerosene layer (Figure 11). EI stability designates the strength of a surfactant [94].

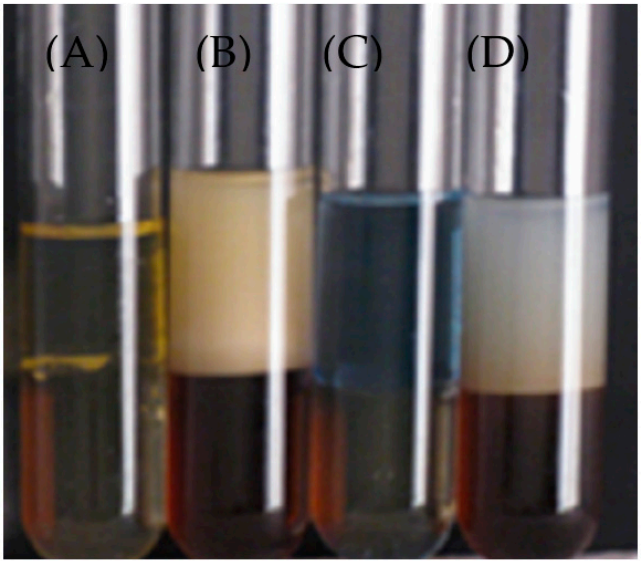

Figure 11. Emulsification index. (**A**) Kerosene—negative control; (**B**) emulsification of kerosene by culture supernatant; (**C**) diesel oil—negative control; and (**D**) emulsification of diesel oil by culture supernatant. Source: [95].

5.9. Tensiometric Measurement

Measurement of surface tension using a tensiometer is one of the most common methods in which cell-free supernatant is used. The Wilhelmy plate method, the Du Noüy ring method (Figure 12), the maximum tensile strength method, and the slope drop method are all known for measuring surface tension [96]. It is not feasible to measure surface tension of many isolates at the preliminary selection level because exact measurement

of surface tension is difficult to obtain and requires precise equipment and adequate environmental conditions.

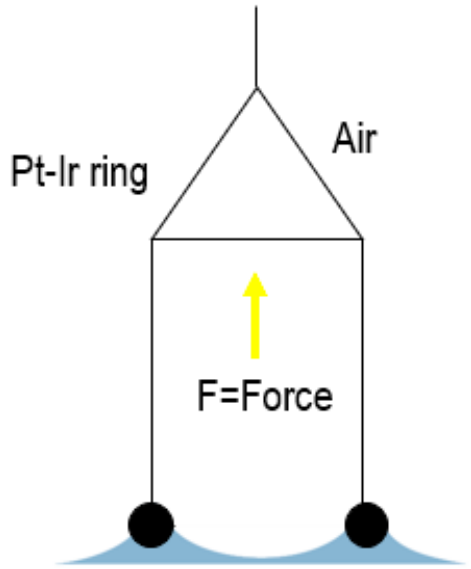

Figure 12. Du Noüy ring method. The force (yellow arrow) referring to the wetted length acting on a ring as a result of the tension of the withdrawn liquid lamella when moving the ring from one phase to another is measured via this method. Source: the author.

6. Biosurfactant Fermentation

Microorganisms can synthesize BS during growth and metabolism; this synthesis usually occurs by resting microbial cell systems, in addition to growth-dependent production. BS are biologically produced from various substrates including hydrocarbons, hydrophobic mixtures, chemicals, solvents, hydrophobic mixtures, vegetable oils, waste oil residues, and dairy products, among others [16,97–99]. Therefore, low-cost raw materials are essential for overcoming the high costs of BS production. However, proper management and use of harmful and non-harmful waste generated in the world is necessary [99].

BS synthesis can be natural or induced by presence of different compounds, varying pH values, temperatures, inoculum sizes, aeration, stresses, and agitation speeds. BS yield and production can also be affected by elements such as carbon, nitrogen, iron, sulfur, phosphorus, and manganese [97,100]. More research is essential to optimize production of BS and improve their applications on an industrial scale considering the numerous environmental complexities and factors that limit synthesis and use of BS [101].

There are currently two ways of producing BS: submerged fermentation (SmF), or liquid fermentation, and solid-state fermentation (SSF). SmF is the methodology most used by the scientific community, while SSF is still not very well studied but promising [102]. SSF is a microbial process that occurs mainly on the surface of solid materials that have the property of absorbing or containing water, with or without soluble nutrients [103,104]. Both techniques can use the same producing microorganisms, but the results can be significantly different due to the large differences in conditions between the two types of culture regimes. Furthermore, for a given bioprocess, SSF usually reduces the overall cost compared to liquid fermentation [105] due to the low volume of water used.

Production of biosurfactants in bioreactors is a process that involves monitoring the conditions involved, being essential to choose an appropriate culture method (continuous, batch, or fed-batch), which depends on type of microorganism and type of bioreactor [106,107]. Continuous cultivation of microorganisms is one of the growing methodologies [108] and is mainly characterized by the constant growth rate of the microorganism in a constant environment (parameters such as pH, substrate concentrations, metabolic products, and oxygen are all constant) [6]. Continuous culture of microbes is carried out

in bioreactors called chemostats, a type of bioreactor to which the prepared substrate is constantly supplied, while the culture liquid comprising the remaining nutrients, the end products, and the microbial culture is continuously withdrawn simultaneously to maintain a constant culture volume [109,110].

Batch fermentation is widely used in fermentation industries to produce various microbial products. In this process, microorganisms and substrates are supplied to a batch bioreactor for product synthesis [111]. Batch fermentation is a simple way to conduct and ensures controlled environments within the bioreactor. However, during the fermentation process, competitive changes can occur in microbial biomass, acid concentration, and by-product concentration (chapter). The batch bioreactor consists of a mechanically agitated vessel, which may include a gas sprayer, an insulating jacket to regulate temperature changes, a pH meter, and air sprinklers [112,113]. Despite being an easy process, a large expense is involved, and a great deal of time is spent, including emptying, filling, and cleaning bioreactors [114].

The fed-batch process is a customized form of batch fermentation and is more popular in the bioprocessing sector [110]. Microorganisms are inoculated and cultured in the batch system for a period after introducing nutrients into the fermenter to feed them [115]. In fed-batch systems, the constant feed flow of the substrate allows the target secondary metabolites to reach very high concentrations. The benefit of this culture method is that the level of the fed substrate can be managed at the target level [116]. Furthermore, fed-batch systems can be applied when large amounts of biomass are required [117,118].

7. Recovery and Purification of Biosurfactants

Although choice of substrates used in production of BS has an influence on the general process, productivity, and cost of BS, extraction and purification of BS for a marketable product constitutes 60–80% of the total production costs [119]. Therefore, the latest advances in economical and efficient techniques to recover and purify biosurfactants from fermentation broth are reviewed in this study (Table 3).

Table 3. The latest techniques for recovering biosurfactants from the fermentation broth produced by yeasts.

Microbial Source	Recovery Method of BS	Reference
Saccharomyces cerevisiae	Acid precipitation followed by solvent extraction with 1:2 methanol (extract: methanol).	[120]
Wickerhamomyces anomalus	Adsorption–desorption chromatography (Amberlite XAD2) using methanol as eluent.	[16]
Saccharomyces cerevisiae	Solvent extraction using ethyl acetate followed by centrifugation and filtration and addition of saturated NaCl and anhydrous $MgSO_4$.	[121]
Rhodotorula sp.YBR	Acid precipitation followed by solvent extraction with thrice an equal volume of ethyl acetate and methanol (2/1, *v*/*v*).	[36]
Candida stellata	Ethanol precipitation (500 mL for 1 L of broth—1:2 *v*/*v*) followed by centrifugation.	[35]
Candida parapsilosis	Acid precipitation followed by centrifugation.	[60]
Candida sphaerica	Solvent extraction using ethyl acetate (1:1 *v*/*v*) followed by other solvent extraction using twice the amount of hexane. After extraction, the product was treated with a base and crystallized.	[78]

The most common approaches for recovery and purification of BS involve acid precipitation [122] and extraction of organic solvents, such as ethyl acetate [122], chloroform, and methanol [123]. For further purification, different chromatographic methods have been used [124,125]. However, other methods used to recover BS are also mentioned in this review.

7.1. Acetone Precipitation

After fermentation, the cell-free supernatant is mixed with ice-cold acetone to precipitate the emulsifiers, which are then suspended in a phosphate buffer. Then, the mixture is incubated at 4 °C for 15–20 h to obtain the precipitated BS [126]. This method has been used by several researchers for purification of BS [127–129]. It is a fast and relatively inexpensive method without the need for specialized equipment.

7.2. Ethanol Precipitation

As with acetone precipitation, ethanol precipitation uses cell-free fermentation broth, which, normally, is mixed with cold ethanol in a 3:1 ratio (ethanol: culture broth). Invally et al. (2018) confirmed that ethanol precipitation did not cause BS loss. It was shown that ethanol precipitation of BS produced by *Acinetobacter calcoaceticus* was the most efficient method when compared to other precipitation methods [130].

7.3. Ammonium Sulfate Precipitation

This method uses $(NH_4)_2SO_4$ for precipitation and is used widely for high-molecular-weight BS and compounds rich in proteins. This method was introduced by Rosenberg et al. (1979) for precipitation of BS from *Arthrobacter*. For this method, 30% or more of $(NH_4)_2SO_4$ is used and added directly to the fermentation broth without removing the cells; it is then left to stand overnight. Subsequently, this precipitate is suspended in 3% saturated $(NH_4)_2SO_4$ and, after centrifugation, is added to the $(NH_4)_2SO_4$ supernatant again to reach the final concentration of 40%; or, if desired, the resulting precipitate is centrifuged and extracted with ether. Currently, research uses cell-free broth at 4 °C followed by addition of saturated $(NH_4)_2SO_4$. After cooling, the pellet is resuspended in $(NH_4)_2SO_4$ and the pellet obtained after centrifugation is dissolved in water and extracted with an equal volume of hexane to remove residues. The product is subsequently purified by dialysis and lyophilized [131–133].

7.4. Acid Precipitation

This method is used widely in recovery of BS because it is an easy, inexpensive, and readily available method. The BS is purified from the cell-free supernatant, acid hydrolysis is carried out using concentrated HCl to lower the pH to 2.0, and it is left at 4 °C overnight. The BS becomes insoluble at the low pH [134], and proteins and lipids precipitate [135]. After cooling overnight, the precipitate is centrifuged and the pellet is subsequently extracted using solvents [136,137]. The extracted material is filtered to remove residues and evaporated using a rotary evaporator.

7.5. Centrifugation

After acid precipitation, the broth containing BS can also be centrifuged at 12,000 rpm for 15 min at 4 °C to be easily collected as a crude product. Once the pellet is obtained, it can be dried under N_2 and extracted with solvents [137].

7.6. Crystallization

Once the BS is precipitated/extracted, it is redissolved in an organic solvent. The reaction is associated with a reduction in temperature, which crystallizes the BS; therefore, it becomes less soluble in solvents [138].

7.7. Adsorption–Desorption

The interaction of BS molecules with polystyrene resins (XAD 2 or 16) is used for purification of BS since their molecules can be adsorbed and desorbed in these resins. The process is initiated by applying cell-free culture broth directly to the adsorbent column, followed by washing with three volumes of demineralized water to remove unabsorbed compounds. Subsequently, the BS is adsorbed and eluted with three volumes of methanol and removed by evaporation to obtain the crude BS [97]. In this recently developed

approach, the BS is adsorbed on polymeric resins and later desorbed with organic solvents. The main advantages of this technique are rapid recovery in one step and obtaining high-quality purified BS.

Dubey and Juwarkar (2005) [139] suggested that adsorption–desorption on wood-based activated carbon can be used because the same carbon can be reused for three consecutive cycles of BS adsorption; this process offers a good example of continuous BS recovery from a fermentation broth using an in situ method that reduces use of high-cost solvents, results in less degradation, and prevents product inhibition.

7.8. Foam Fractionation

This technique was first reported in 1920 and is used to enrich dissolved compounds [140]. The basic principle of this technique is separation of adsorptive bubbles so that the air bubbles generated by an aeration system move to the top of the liquid surface, leading to formation of a foam fraction [141]. A certain amount of liquid is trapped between the air bubbles and is, therefore, lost within the foam; however, the force of gravity causes the trapped liquid to drain, leading to the foam's collapse. This collapsed foam contains surface and non-surface compounds that are more concentrated than the initial liquid [142]. This technique was previously described and recently used and is considered a low-cost and ecologically acceptable method, suitable for the process of concentrating products diluted in culture media or extracts [132,143].

7.9. Solvent Extraction

For this technique, the BS is concentrated from the supernatant by addition of $ZnCl_2$ and extracted twice with solvents. The organic phases are evaporated to dryness and analyzed using the TLC technique [144]. Hydrophobic portions of BS are soluble in some solvents that aid in the extraction and separation of the crude product. Different solvents—such as chloroform, methanol, ethyl acetate, dichloromethane, butanol, pentane, hexane, acetic acid, diethyl ether, and isopropanol—are commonly used for extraction of BS, but these solvents are toxic and expensive; therefore, it is necessary to use cheaper and less toxic solvents for recovery of BS [96].

8. Characterization of Biosurfactants

Use of analytical chemistry techniques to prove the production of and characterize surfactant compounds is efficient and conclusive. These techniques include thin-layer chromatography (TLC), Fourier transformation infrared spectroscopy (FTIR), high-performance liquid chromatography and mass spectrometry (HPLC–MS), tandem mass spectrometry (MS/MS), and nuclear magnetic resonance (NMR). All these techniques require (partial) purification of surface-active compounds from samples of cell-free supernatants [145]. Different techniques require different levels of sample purity; therefore, it is necessary to be careful with the purity of each sample to obtain significant characterization of surfactant compounds.

8.1. Thin-Layer Chromatography (TLC)

TLC is one of the most used techniques to detect BS. The sample is dissolved in solvents appropriate for each type of analysis and applied to the TLC plate, which is then placed in a closed chamber with the mobile phase (solvent or mixture of solvents). The mobile phase migrates on the plate; thus, the components also migrate but at different rates, resulting in separation. After the race, spots can be seen in UV light or by chemical treatment [146]. To detect BS, the solvent system depends on the type of compound of interest: organic and inorganic solvents that can be dissolved and are not volatile are preferred. Sometimes, acetic acid, diethyl ether, ethyl acetate, n-hexane, and pyridine are necessary for mobility of BS functional groups [96]. A summary of solvents and treatments for visualizing the spots used for detection of different BS produced by yeasts is provided in Table 4.

Table 4. Solvents and chemical treatments for visualization of TLC spots used for characterization of BS produced by yeast.

Microbial Source	Mobile Phase	Visualization	Reference
Rhodotorula babjevae	Chloroform: methanol: water (65:15:2 *v*/*v*)	Iodine fumes and Anthrone reagent	[71]
Scheffersomyces stipitis	Chloroform: methanol: distilled water (65:15:1 *v*/*v*)	Seebach reagent	[19]
Cyberlindnera saturnus	N-hexane: ethyl acetate (5:3 *v*/*v*) and Acetonitrile: methanol: water (4:2:1 *v*/*v*)	UV light, Ninhydrin reagent, Anthrone reagent, and Rhodamine 6G reagent	[147]
Cutaneotrichosporon mucoides	Chloroform: methanol (19:1 *v*/*v*)	Seebach reagent	[25]
Rhodotorula sp.YBR	Acetone: acetic acid: water (70:20:10 *v*/*v*) for amino acids, Chloroform: methanol: water (60:30:10) for sugars, and Chloroform: methanol: water (65:25:10 *v*/*v*) for lipids	Iodine fumes, Ninhydrin reagent, and Molisch reagent	[36]

8.2. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR has been proposed as a rapid technique that enables identification of surfactant compounds and their quantification [148]. However, FTIR alone should not be used to conclusively identify the type of surfactant compound produced by a microorganism. As an FTIR principle, the chemical bonds present in the compound produce a specific spectrum that can be detected when analyzing a sample in comparison to a known pattern of a specific compound or to patterns that have analogous chemical groups [148]. However, as these chemical bonds are not exclusive to surfactant compounds and can be present in many other extracellular compounds produced by microorganisms, the sample must have a high level of purity to allow this detection to be specific [145]. This technique has been used in a series of studies that report production of BS by yeast strains [21,35,37].

8.3. High-Pressure Liquid Chromatography (HPLC)

This technique consists of a stationary phase (a solid column over which the mobile phase continuously flows the components of the sample solution), a mobile phase (the injected sample solution is carried through the injector port), and a detector (a response is emitted due to sample elution, and, subsequently, a peak in the chromatogram is signaled). In this technique, the components migrate according to the non-covalent interactions of the compound with the column; this separation is based on polarity. Separate products can be detected in individual peaks, and these fractions can be collected for analysis of the structure of each fraction [96,149]. The BS can be separated and identified successively when the HPLC is coupled to an evaporative light-scattering detector (ELSD) or when mass spectrometry (MS) is employed. HPLC, along with MS, is important to provide the molecular mass of each fraction [122].

8.4. Tandem Mass Spectrometry (MS/MS)

The MS/MS technique employs two stages of mass analysis to selectively examine fragmentation of specific ions in an ion mixture. The types of instruments that can be used to perform this experiment are based on separation of mass analysis events over time or measurements on physically separate analyzers [150]. In the MS/MS technique, a single congener can be fragmented and the resultant daughter ions analyzed [151], providing more precision for characterization of BS compounds being produced by microbial strains [145].

8.5. Nuclear Magnetic Resonance (NMR)

This technique is based on transitions of atoms with a magnetic moment when an external magnetic field is applied and absorbance of radiation by a nucleus in a strong magnetic field. Absorption of radiation causes the nuclear spin to realign or rotate in the direction of higher energy; as soon as the energy is absorbed, the nuclei will reissue radiation and return to a low energy state. The transition energy of NMR entirely depends on the intensity of the magnetic field and a factor of proportionality for each nucleus called the magnetogyric ratio. NMR provides information about the functional groups as well as the position of the bonds within the carbohydrate and lipid molecules. The exact location of each functional group can be obtained; information on structural isomers is also possible with the help of a series of NMR experiments and solvents, such as acetic acid, acetone, benzene, chloroform, dimethyl sulfoxide, methanol, pyridine, and water. BS must be dissolved and applied to a series of 1D (1H and 13C) and 2D (such as COZY, TOCSY, HMQC, and HMBC) experiments by NMR and analyzed. This technique has been used to determine chemical structures of BS since 1960 [96,152–154].

8.6. Raman Spectroscopy

In biological and chemical systems, Raman spectroscopy has been used successfully to characterize structural conformation, functionalities, and molecular composition of lipid bilayers and surfactant mono- and bilayers—including interdigitation of lipid and surfactant chains or tail groups in bilayers [155,156].

Recently, purified BS extracted from *Pseudozyma* yeast strains were characterized using the traditional Raman scattering and surface-enhanced Raman scattering methods and exhibited similar vibrations in the regions of $2700-2800\ cm^{-1}$ and $2850-3050\ cm^{-1}$, which correspond, respectively, to asymmetric and symmetric C–H stretching vibrations of terminal CH_2 and CH_3 groups. This work demonstrated that Raman spectroscopy can be a marker-free and efficient method to identify BS and differentiate the level of saturation in an acid chain [157].

9. Final Considerations

Effective screening methodologies and improved purification techniques play a vital role in obtaining better-quality biosurfactants at greater quantities. Biosurfactants produced by yeasts have been shown to be effective in applications in several industrial areas; the possibilities for these applications in the pharmaceutical and food industries are ever increasing. Additionally, yeasts are better producers than bacteria since they produce a greater amount of BS in less time. Currently, the commercial success of biosurfactants is limited by the high cost of production. Therefore, optimized conditions for growth and production, use of renewable and economically viable substrates, and use of microorganisms with higher substrate conversion rates would help to produce more profitable and economically viable biosurfactants.

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