

Biosurfactants from Yeasts

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Abstract

Biosurfactants are surface active compounds derived from living organisms, mainly microorganisms. Almost all surfactants currently in use are chemically derived from petroleum. However, in recent years, the interest in chemical surfactants has been substituted by an increase in the use of microbial surfactants. Compared with chemical surfactants, biosurfactants have more advantages, for example, their lower toxicity, higher biodegradability and better environmental compatibility. Most known biosurfactants are of bacterial origin, and only a few biosurfactants come from yeasts and molds. Biosurfactants have not yet been employed extensively in industry because of the relatively high production and recovery costs involved. To reduce the costs of biosurfactant production, it is necessary to select microorganisms capable of high-yield biosurfactant production. This review describes screening of biosurfactant producing-yeasts, the estimation of biosurfactant activity as well as medium components which affect biosurfactant production. The characterization of biosurfactants from yeasts is also discussed.

Keywords: Biosurfactants, biosurfactant activity, screening, yeasts

Introduction

Biosurfactants are produced by various microorganisms. Their properties of interest are: in changing surface active phenomena, such as lowering of surface and interfacial tension, wetting and penetrating actions, spreading, hydrophilicity and hydrophobicity actions [1]. They are categorized mainly by their chemical compositions and their microbial origins. The major classes of biosurfactants include glycolipids, lipopeptides and lipoproteins, phospholipids, fatty acids, polymeric biosurfactants and particulate biosurfactants [2]. These molecules reduce surface and interfacial tension in both aqueous solutions and hydrocarbon mixtures [3]. Biosurfactants are important biotechnological products with a wide range of applications in many industries such as food, cosmetic and pharmaceutical industries [4]. Furthermore, biodegradability, ecological safety,

production from renewable resources, and functionality under extreme conditions are significant advantages of biosurfactants over chemical surfactants [5]. Minimal studies have reported biosurfactants synthesized by yeasts [6] and most of them are related to bacteria [7] and marine microorganisms [8]. This article aims to provide an overview of the production of biosurfactants from yeasts and their characterization. **Table 1** summarizes recent studies on the type and microbial origin of biosurfactants, especially yeasts and fungi. In general, their structure includes a hydrophilic moiety consisting of amino acids or peptides for anions or cations; mono-, di-, or polysaccharides; and a hydrophobic moiety consisting of unsaturated and saturated fatty acids.

Table 1 Types of biosurfactants produced by yeasts.

Biosurfactant	Microorganism	Reference (s)
Glycolipids	<i>Absidia corymbifera</i> F-295	Batrakov <i>et al.</i> [9]
	<i>Symptodiomyces paphiopedili</i>	Kulakovskaya <i>et al.</i> [10]
	<i>Candida sphaerica</i> UCP0995	Sobrinho <i>et al.</i> [11]
	<i>Candida bombicola</i>	Solaiman <i>et al.</i> [12]
	<i>Candida bogoriensis</i>	Kitamoto <i>et al.</i> [13]
	<i>Pseudozyma aphidis</i>	Rau <i>et al.</i> [14]
	<i>Wickerhamiella domercqiae</i>	Jing <i>et al.</i> [15]
Sophorose lipids	<i>Candida ishiwadae</i>	Thanomsub <i>et al.</i> [16]
Monoacylglycerols		
Mannosylerythritol lipid		
Phospholipids and Fatty acids	<i>Candida sp.</i> SY16	Kim <i>et al.</i> [17]
	<i>Candida antarctica</i>	Kim <i>et al.</i> [18]
	<i>Pseudozyma sp.</i>	Fukuoka <i>et al.</i> [19]
Glycerophospholipids	<i>Absidia corymbifera</i>	Batrakov <i>et al.</i> [20]
	<i>Issatchenkia orientalis</i>	Katamai <i>et al.</i> [21]
Oleic acid		
Polymeric biosurfactants	<i>Candida lipolytica</i>	Sarubbo <i>et al.</i> [22]
Proteins-carbohydrates and lipids complex	<i>Candida glabrata</i> UCP 1002	Sarubbo <i>et al.</i> [23]

Screening of potential biosurfactant-producing yeasts

Hemolytic activity was carried out as described by Carrillo *et al.* [24]. Isolated strains were screened on blood agar plates containing 5 % (v/v) blood and incubated at 45 °C for 24 - 48 h. Hemolytic activity was detected as the presence of a clear zone around a colony. The large number of false negatives and positives obtained with the blood agar lysis method and its poor correlation to surface tension demonstrated that it is not a reliable method to detect biosurfactant production.

Kim *et al.* [25] reported 20 colonies of yeast-forming halos on agar plates covered with crude oil which were isolated from soil samples. Isolated colonies were cultured in the isolation liquid medium containing *n*-hexadecane, soybean oil, or glucose as a carbon source. Three strains showing a large halo (more than 2 cm in diameter) were selected when the drop collapse technique was used to check activity. The yeast strain, *Candida sp.* SY16 was capable of effectively emulsifying crude oil, vegetable oil and hydrocarbons. In

addition, it can reduce the surface tension of the culture broth to 30 dyne/cm.

More than 200 strains were isolated from oil and wastewater samples. These yeast strains were cultured in fermentation media, 11 strains showing large halos (more than 3 cm in diameter) were isolated by measuring the oil film-collapsing activity of the culture broth by the drop collapse technique. A yeast strain Y2A producing a large amount of biosurfactant was isolated from oil-containing wastewater and identified as *Wickerhamiella domercqiae* [15].

Among 81 yeast strains showing growth in the basal salt medium supplemented with 1 g/L weathered crude oil as a sole carbon source, 5 strains showed high biosurfactant activity which was tested for by an oil displacement area (ODA) test [26]. The biosurfactant property was also confirmed by emulsification activity (modified from Cooper and Goldenberg, [27]). As a result, the highest activity was exhibited by the strain SR4 grown in the optimized medium supplemented with 1 g/L of a mixture of xylene isomers. Sequence analysis of 18S rDNA indicated

that the SR4 strain belongs to *Issatchenkia orientalis* (99 % identity) [21].

The estimation of biosurfactant activity

The fields of microbial surfactants are largely attributed to the development of quick, reliable methods for screening biosurfactant-producing microbes and assessing their potential. The measurement of surface tension has traditionally been used to detect biosurfactant production and most other methods that measure the surface properties of biosurfactants use surface tension reduction as the standard [28]. To date 4 methods have been used to detect the biosurfactant activity namely (1) oil displacement area (ODA), (2) surface tension reduction, (3) drop collapse and (4) emulsification activity (EA).

The oil spread technique was carried out according to Morikowa *et al.* [29]. Fifty ml of distilled water was added to Petri dishes followed by addition of 100 μ l of crude oil to the surface of the water. Then, 10 μ l of the culture filtrates was put on the crude oil surface. The diameter of the clear zone on the oil surface was recorded.

Surface tension is a property of the surface of a liquid that causes it to behave as an elastic sheet. Thus it is a measure of the maximum force needed to detach the ring from the liquid surface. Surface tension can be measured with a ring-tensiometer (Kruss Digital-Tensiometer 10, Hamburg, Germany) at room temperature [30]. The ring hanging from the balance hook is immersed into the liquid. Then, the ring is slowly pulled up, by lowering the sample cup. Finally, the force applied on the ring while pulling through the surface is recorded. The surface tension of distilled water is 72.0 mN/m.

The drop-collapse technique involves using a polystyrene lid of 96-microtiter plates as described by Bodour and Miller-Maier [31]. A one-hundred μ l culture supernatant is added to wells of a 96-well microtiter plate lid, then 5 μ l of crude oil was added to the surface of the culture supernatant. Biosurfactant-producing culture gave flat drops. Aliquots from a culture of each strain were analyzed on 2 separate microtiter plates.

Emulsification is the process by which emulsions are prepared. An emulsion is a mixture of 2 immiscible substances. One substance (the dispersed phase) is dispersed in the other (the continuous phase). The emulsifying activity

(%EA) is determined using a modification of the method described by Cooper and Goldenberg [27]. To measure emulsification activity, 1 ml of *n*-hexadecane is added to 1 ml of the supernatant in a test tube. The mixture is mixed with a vortex mixer at high speed for 2 min and allowed to stand for 10 min and 24 h (emulsification index). The emulsion stability is determined after 10 min (%EA) and 24 h {the emulsification index (EI-24)} and calculated by dividing the measured height of the emulsion layer by the height of the oil or hydrocarbons phase and multiplying by 100.

Furthermore, the emulsifying activity was determined using a modified turbidometric method [32] and expressed as the turbidity at 620 nm. The surfactants (1 mg) were introduced into a 50 ml flask with distilled water to make 10 ml, then 0.1 ml of each hydrophobic liquid was added. The mixtures were incubated at 30 °C with reciprocal shaking (160 strokes per min) for 1 h and allowed to stand for 10 min. The lower phase was taken and its turbidity measured at 620 nm. Soybean oil, *n*-hexadecane/2-methylnaphthalene (1:1, v/v), and crude oil (Arabian light, 1 % sulfur content) were used as the hydrophobic liquids. A non-surfactant reaction was used as the control.

The oil spreading technique better predicted biosurfactant production than the drop collapse method. The use of the drop collapse method as a primary method to detect biosurfactant producers, followed by the determination of the biosurfactant concentration using the oil spreading technique, constitutes a quick and easy protocol to screen and quantify biosurfactant production [26].

However, the measurement of surface tension is time-consuming, which makes it inconvenient to use for screening of a large number of isolates. The drop collapse technique depends on the principle that a drop of a liquid containing a biosurfactant will collapse and spread completely over the surface of the oil [31]. This method is easy and can be used to screen a large number of samples [20], but it has not been correlated to surface tension reduction to confirm its reliability. The oil spreading technique measures the diameter of clear zones caused when a drop of a biosurfactant-containing solution is placed on an oil-water surface [29].

Emulsification activity for culture broth was also tested using xylene, toluene, petroleum and diesel oils. Although surface tension reduction is

found to be a good measure of biosurfactant production, it did not correlate well with emulsion ability. Several bacteria isolates had good emulsifying abilities with all hydrocarbons tested. The simplicity of the above techniques allows effective screening of biosurfactant-producing microorganisms. Other more conclusive tests such as surface tension measurement should be carried out for confirmation of the obtained results [34].

Medium components affecting the growth and biosurfactant production by yeasts

Candida antarctica SY 16 required a vegetable oil as the carbon source to produce a biosurfactant, mannosylerythritol lipid (MEL-SY16). Biosurfactant production was 31 g/L after 7 days in a batch culture and was not growth associated. In a two-stage culture, glycerol and oleic acid were used as an initial and a feeding carbon source, respectively and 41 g/L biosurfactant was produced after 8 days [35]. In addition, in fed-batch fermentation, glucose and soybean oil (1:1, w/w) were used in combination as the initial carbon sources for cell growth, and soybean oil was used as the carbon source during the MEL production phase [17].

Amezcu-Vega *et al.* [36] investigated the effect of culture medium composition on biosurfactant production and their total fatty acids content, as well as the surface tension of media, and biomass production by *Candida ingens*. A factorial experimental design was used to evaluate the combined effect of C/P, C/N inorganic, C/Fe, C/Mg ratios and yeast extract concentration. The highest biosurfactant production was reached when high C/Fe and high C/P ratio variables were combined; biosurfactant concentration increased 3.42 fold. The variable with the highest effect on net decrease in surface tension (DST) and fatty acids percentage of *C. ingens* biosurfactant was yeast extract. The average DST (25 mN/m) and fatty acids percentage (34.7 %) values were enhanced at high yeast extract concentration of 1 g/L. The main conclusion of this study was that the medium composition affected the biosurfactant production by *C. ingens*. It was also observed that the surface tension and total fatty acids of the biosurfactant were modified as the media composition changed.

Issatchenkia orientalis PO1.2 can produce biosurfactant when 0.1 % waste lubricating oil was used as a carbon source. The results of emulsification activity (%EA) from the supernatant of *I. orientalis* PO1.2 with soy bean oil, palm oil or rice bran oil, showed the best %EA with rice bran oil (71.67 %) [37].

The summary of biosurfactant production by yeasts with various carbon sources is shown in **Table 2**.

Recovery of biosurfactants

Biosurfactants are unlikely to be produced at low cost if extensive refining is required. Process development must therefore be focused around biosurfactants which can be recovered by simple and inexpensive techniques such as gravity separation. The most common biosurfactant recovery methods are either extraction with solvents (e.g. chloroform-methanol, dichloromethane-methanol, butanol, ethyl acetate) or acid precipitation. However, there are reports of the use of ammonium sulfate precipitation, crystallization centrifugation, adsorption, foam fractionation etc. [38].

Glycolipids produced by *Torulopsis bombicola* were extracted by chilled ethyl acetate after adsorption on charcoal [39]. The mannosylerythritol lipid produced by *Candida* spp. may be sedimented by centrifugation and then extracted with either ethanol or methanol [25]. Bryant [40] improved the method for isolating the biosurfactant glycolipids by using diafiltration and isopropanol precipitation. This method removed protein and isolated the glycolipids by using organic extraction and salicylic acid chromatography.

Nocardia sp. L-417 strain grown in *n*-hexadecane as a carbon source produced 2 types of biosurfactant that had different characteristics. These biosurfactants were purified by procedures including ammonium sulfate fractionation, chilled acetone and hexane treatments, silica-gel column chromatography and Sephadex LH-20 gel filtration consecutively. The biosurfactant type I had strong properties as an emulsifying agent and as an emulsion-stabilizing agent, whereas type II showed a strong ability to reduce surface tension [41].

Table 2 Production of biosurfactants by yeasts with various carbon sources.

Biosurfactants	Yeasts	Carbon source	Reference
Mannosylerythritol lipid MEL-A, -B and -C	<i>Candida antarctica</i> T-34	<i>n</i> -Octadecane	Kitamoto <i>et al.</i> [13]
MEL-A, -AB and -C	<i>Candida antarctica</i> KCTC 7804 <i>Ustilago maydis</i> DSM 4500	Glycerol, oleic acid Sunflower oil	Kim <i>et al.</i> [25] Spoeckner <i>et al.</i> [42]
Sophorolipid SL mixture	<i>Candida bombicola</i> ATCC 22214	Whey, rapeseed oil	Daniel <i>et al.</i> [43]
SL (lactone)	<i>Candida apicola</i> IMET 43147	Glucose, sunflower oil	Hommel <i>et al.</i> [44]
Cellulose lipid CL-A, -B and -C	<i>Ustilago maydis</i> ATCC 14826	Coconut oil	Fautz <i>et al.</i> [45]
Fatty acid Oleic acid	<i>Issatchenkia</i> <i>orientalis</i> SR4	Glucose, weathered crude oil, xylene	Katamai <i>et al.</i> [21]

Mannoprotein in the cell wall of *Saccharomyces cerevisiae* was extracted by autoclaving in a citrate buffer (pH 7), and purified. The yield of emulsifier extracted from a commercial strain of *S. cerevisiae* was 80 - 100 g/kg. The mannoprotein consisted of approximately 380 - 410 g/kg protein and 210 g/kg carbohydrate [46].

Biosurfactant production by the *Issatchenkia orientalis* SR4 was performed in an optimized medium supplemented with 1 g/L of a mixture of xylene isomers. The culture supernatant (10 L) obtained was extracted twice with an equal volume of mixtures of chloroform/methanol (2:1) (modified from Mercade *et al.* [47]). The crude extract was separated by chromatography on a silica gel column by sequential elution with hexane, ethyl acetate and methanol. All fractions were collected, dried and tested for their biosurfactant activities [21].

Characterization of biosurfactants

Kim *et al.* [18] described the molecular weight of MEL-SY16 from *Candida* sp. SY16 was 634 Da and its hydrophobic lipophilic balance (HLB) was 8.8. It decreased the surface tension to 30 dyne/cm at a critical micelle concentration of 15.8 μ M, and the minimum interfacial tension against kerosene was 0.1 dyne/cm. MEL-SY16 was stable from pH 4 to 10, up to 90 °C for 1 h, and against NaCl up to 100 mM.

The molecular weight of the bioemulsifier mannoprotein from *Saccharomyces cerevisiae* was estimated to be 14,000 - 15,800 Da by the SDS-PAGE method. Physical and chemical stability analysis showed that emulsions of 60 % oil in water, 8 g/L bioemulsifier and 5 - 50 g/L sodium chloride were stable for 3 months at 4 °C from pH 3 - 11 [46].

Monoacylglycerols; 1-linoleylglycerol and 1-oleylglycerol produced by *Candida ishiwadae* exhibited higher surfactant activities tested by the drop collapse test than several artificial surfactants such as sodium dodecyl sulfate [16].

The fraction with the highest surfactant activity from *Issatchenkia orientalis* SR4 was purified further using preparative HPLC on Inertsil ODS-3 eluting with 95 % aqueous acetonitrile containing 0.05 % trifluoroacetic acid (TFA). The fragmentation pattern and a mass spectral library-search suggested that the compound corresponding to this peak was methyl oleate. This preliminary identification was confirmed by co-injection of authentic methyl oleate. Finally, the active compound was analyzed by ODS-HPLC and compared with authentic oleic acid. HPLC analysis was performed on an Inertsil ODS-3 HPLC column. The active compound showed the same retention time as authentic oleic acid, and the identity was confirmed by co-injection. This is the first report on a biosurfactant, oleic acid produced by yeast, *I. orientalis* SR4 when xylene was used as a carbon source. The purified compound showed almost the same surface activity as an authentic oleic acid, and the activity was higher than that of synthetic surfactants such as SDS, triton X-100 and triton X-114. Emulsification activity (%EA) of this purified compound in comparison with authentic oleic acid and synthetic surfactants. The %EA of the purified compound was equal to the %EA of authentic oleic acid. However, it was lower than those of the synthetic surfactants [21].

Conclusions

Biosurfactants synthesized by yeasts are now much studied. Screening of potential biosurfactant producing yeasts isolated from soil, oil contaminated soil and wastewater were assessed for surface tension reduction and emulsifying properties. The medium components, especially carbon sources for biosurfactant production could be divided into 3 categories, carbohydrates, hydrocarbons and vegetable oils. Biosurfactants can be recovered by simple and inexpensive techniques such as gravity separation or extraction with solvents. In addition, biosurfactants from yeasts should be characterized because it is important to develop them to broaden the spectrum of specific properties and applications. Considering the current social and technological development, utilization of biosurfactants, which are environmentally friendly and highly functional materials, should be strongly encouraged. Thus the

biosurfactant could become the flagship product for clean technology in the future.

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