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Biosurfactants from Fungi: A Review

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Abstract

Biosurfactants are the surfactants of microbial origin. They offer so many advantages over their synthetic counterparts due to their biodegradable and environmental friendly nature, that's why gaining much more attention in creating the era of green technology. Their applications range from cosmetic, pharmaceutical and food processes as emulsifiers, humectants, preservatives, detergents etc. The present review deals with the production, purification, characterization and applications of various fungal biosurfactants.

Keywords: Biosurfactants; Sophorolipids; Fungi; Purification; Characterization

Introduction

Biosurfactants are structurally and functionally diverse amphiphilic, surface active compounds which lower the surface and interfacial tension between individual molecules at respective surfaces and interfaces. Thus, these are very important in the living systems and can be regarded as the backbone of the biological membranes which promise the transport and exchange of the various important materials [1,2]. Biosurfactants are ecologically safe and can be applied in bioremediation processes. The microorganisms which produce biosurfactants can also be used in the various bioremediation technologies like solubilisation and removal of oil from contaminated soil, sludge in oil storage tank etc. [3]. The most important example of this is in Microbial Enhanced Oil Recovery (MEOR) which is an eco-friendly petroleum recovery process [4].

Variety of bacteria and few fungi are reported to produce biosurfactants using renewable sources [5,6]. Higher yields of 120 and 40 g/L of fungal surfactants has been reported using carbon sources like tallow fatty acid residues, animal fat, glycerol and oleic acid [7-9]. Furthermore fungi yield a good amount of biosurfactant when compared to bacteria. The reason may be presence of rigid cell wall in them [10]. The higher yield seems to make their use possible at industrial level and replacement of surfactants by biosurfactants.

Fungi Producing Biosurfactants

Where the field of production of biosurfactants by bacterial species is well explored, relatively fewer fungi are known to produce biosurfactants. Among fungi, *Candida bombicola* [11-14,7,8], *Candida lipolytica* [15,16], *Candida ishiwadae* [17], *Candida batistae* [18], *Aspergillus ustus* [19], *Ustilago maydis* [20] and *Trichosporon ashii* [21] are the explored ones. Many of these are known to produce biosurfactant on low cost raw materials. The major type of biosurfactants produced by these strains is sophorolipids (glycolipids). The structure of sophorolipids produced by *Candida batistae* [18], *Candida bombicola* [22] and *Candida sp.* SY16 [10] are given in Figure 1.

Patents on fungal biosurfactants

Due to wide industrial applications many authors have claimed for patents on biosurfactants. Some of the important patents in the last few years are listed in Table 1.

Physicochemical Parameters Affecting the Biosurfactant Production

The production of biosurfactants by the use of various culture conditions is an important aspect because a small alteration in the composition of important nutrients leads to the modification of the resulting biosurfactant. The various physicochemical factors are discussed as follows:

Carbon sources

Carbon source plays an important role in the growth as well as production of biosurfactants by the various microorganisms and it varies from species to species. When only one from glucose and vegetable oil was used for the production of biosurfactant by *Torulopsis bombicola,* a very low yield of biosurfactant was obtained but when both carbon sources were supplemented together yield increased to 70 g/L [23]. While at the concentration of 80 and 40 g/L of glucose and soybean oil, the maximum yield of sophorose lipids was obtained by *Torulopsis bombicola* [24], even higher yields of 120 g/L sophorolipids was produced with *Candida bombicola* in 8 days, when sugar and oil were used as carbon sources [13]. When canola oil and glucose were used as the carbon sources by *Candida lipolytica,* in the concentrations of 10% each, maximum yield of sophorolipids (8 g/L) was obtained [15]. Also, when the industrial residue were used for the production of biosurfactant by *Candida lipolytica,* yielded 4.5 g/L of protein-lipid-carbohydrate complex with the reduction in surface tension of distilled water from 71 to 32 mN/m [16]. Although with *Candida lipolytica* higher production of bioemulsifier was obtained, when supplemented with 1.5% glucose (w/v) [25]. *Candida antarctica* and *Candida apicola* yielded 13.4 and 7.3 g/L of sophorolipids respectively, when soap stock was supplemented in 5% v/v concentration [26]. The resting cells of

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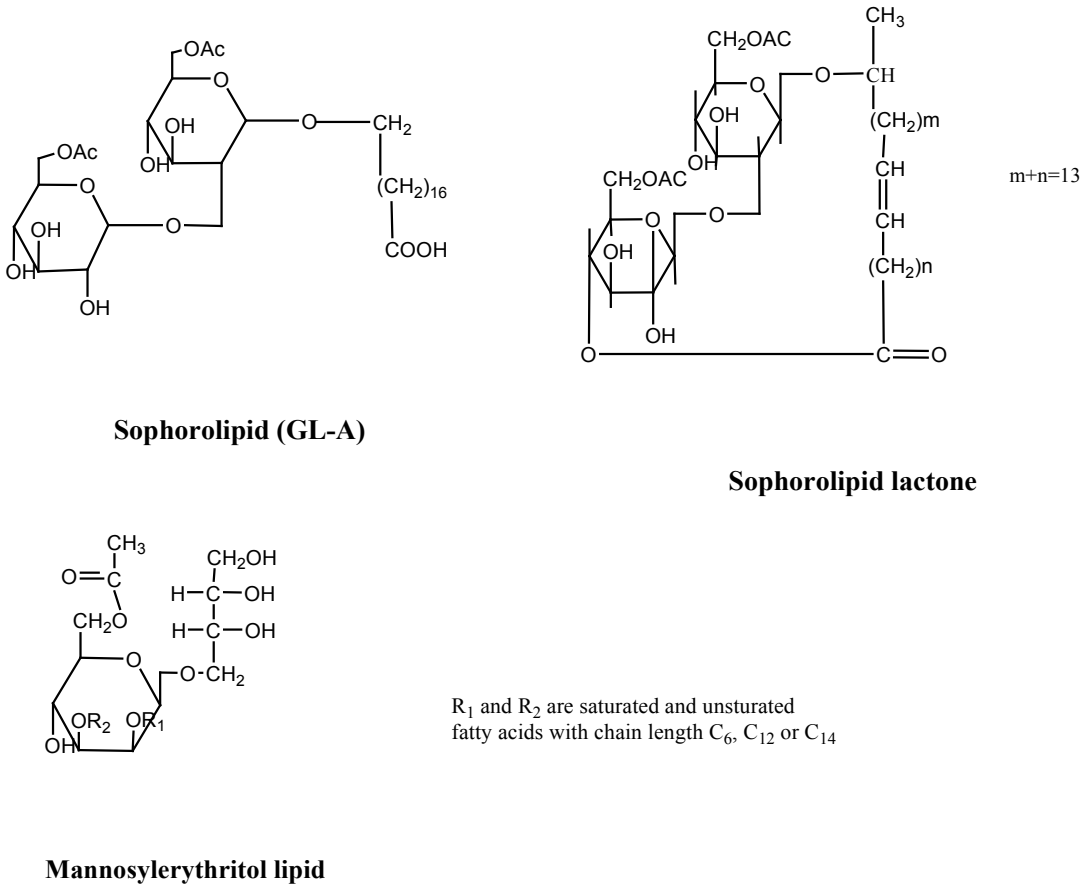

Sophorolipid (GL-A)

Sophorolipid lactone

Mannosylerythritol lipid

Figure 1: Structure of sophorolipids.

S. No.	Fungi/ type of biosurfactant	Inventor	Title of the patent	Publication No.	Publication Date/ Year
1.	Sophorolipid producer	Borzeix F	Sophorolipids as stimulating agent of dermal fibroblast metabolism	US 6057302 A	2 May 2000
2.	Sophorolipid producer	Borzeix F, Concaix	Use of sophorolipids comprising diacetyl lactones as agent for stimulating skin fibroblast metabolism	US 6596265 B1	22 July 2003
3.	*C. albicans*, *C. rugosa*, *C. tropicalis*, *C. lipolytica*, *C. torulopsis*	Awada S, spendlove R, Awada M	Microbial biosurfactants as agents for controlling pests	US 20050266036 A1	1 Dec 2005
4.	Sophorolipid producer	Gross RA, Shah V, Doncel GF	Spermicidal and virucidal properties of various forms of sophorolipids	WO 2005089522 A2	29 Sept. 2005
5.	Sophorolipid producer	Cox TF, Crawford RJ, Gregory LG, Hosking SL, Kotsakis P	Mild to the skin, foaming detergent composition	WO2011120776 A1	6 Oct 2011
6.	*Candida bombicola* ATCC 22214.	Soetaert W, De MS, Saerens K, Roelants S, Van BI	Yeast strains modified in their sophorolipid production and uses thereof	EP 2580321 A1	17 Apr 2013

Table 1: Patents on biosurfactants produced by fungi.

Pseudozyma (Candida antarctica) was able to covert C_{12} to C_{18} n-alkanes into Mannosylerythritol Lipids (MEL), the yield of MEL was 140 g/L after 4 weeks and the produced biosurfactant was able to emulsify soybean oil [27]. The modification in the fatty acid constitution of final biosurfactant was observed when the fatty acid composition was changed in the fermentation media of *Candida ingens* [28].

Nitrogen sources

This is the second most important supplement for the production of biosurfactants by microorganisms. Various organic and inorganic nitrogen sources were used in the production of biosurfactants. The higher yields of sophorose lipids, biosurfactant by the fungi *Torulopsis bombicola* and *Candida bombicola* were observed using yeast extract and urea as the nitrogen source [13,8,24]. Although, the higher yields of mannosylerythritol lipid by *Candida sp.* SY16, *Candida lipolytica* and *Candida glabrata* UCP 1002 were observed with ammonium nitrate and yeast extract [10,29,15,16].

pH

pH plays an important role in the production of biosurfactants. *Candida* species produced maximum yields of biosurfactants in a wide pH range, as seen in *Candida glabrata* UCP 1002 which produced maximum biosurfactant at pH 5.7, *Candida sp.* SY16 at pH 7.8, *Candida lipolytica* at pH 5.0, *Candida batistae* at pH 6.0 [29,10,30,18]. While *Aspergillus ustus* and *Pichia anamola* produced maximum yield of biosurfactant at the pH 7.0 and 5.5 respectively [19,31].

Temperature

Various microbial processes are temperature dependent and get affected by a little change. The most favorable temperature for the production of biosurfactants by various fungi is 30°C as observed in various *Candida species* viz. *Candida sp.* SY16, *Candida bombicola, Candida batistae* and *Torulopsis bombicola* [10,8,18,23]. While, in case of *Candida lipolytica,* 27 °C was found to be the best temperature.

Incubation time

Incubation time also have a significant effect on the production of biosurfactant. Different microorganisms are able to produce biosurfactants at different time intervals. The maximum biosurfactant production by *Aspergillus ustus* MSF3 was observed after 5 days of incubation while in case of *Candida bombicola,* the incubation periods were 7, 8 and 11 days [7,13,12]. Also, the maximum biosurfactant production by *Candida bombicola* using animal fat was observed after the 68 h of incubation [8].

Purification and Analytical Methods for the Characterization of Biosurfactants

After the production of biosurfactants the most important step is their purification from the fermentation media so as to make them available for various industrial applications. This section deals with the purification and characterization methods employed for the extraction of biosurfactants from various fungal species. The purification and

S. No.	Fungi	Type of biosurfactants	Solvents used in the purification processes	Determination of various parameters by analytical methods	References
1.	*Torulopsis bombicola*	Sophorose lipid	Hexane, Chloroform, Ethyl acetate	**Glucose**- Dinitrosalicylic acid [32] **Soybean oil**- Wjis method [33] **Sophorose lipid**- Phenol sulphuric acid [34] **Cell growth**- Plotting absorbance at 650 nm against dry cell weight	[24]
2.	*Candida bombicola*	Sophorolipids (SL)	Not mentioned	**Biomass**- Measured as optical density at 650 nm **Nitrogen**- With an Orion 720A free ammonia electrode. **Phosphorus and glucose**- HPLC	[11]
3.	*Candida bombicola*	Sophorolipid	Pentane and Ethyl acetate	**Hydroxyl fatty acid –** Methanolysis [35]	[12]
4.	*Candida ingens*	Not mentioned	Ethyl acetate extraction [36]	**Biomass**- [37] **Surface tension**- Fisher tensiometer	[28]
5.	*Candida bombicola*	Sophorolipid	n-Hexane and Ethyl acetate [38]	**Biomass**- O.D. at 650nm in a spectrophotometer **Oil**- Dry weight after n-hexane removal by evaporation. **Sophorolipid**- Conc. is checked by dry weight after evaporation of ethyl acetate and structure was analysed by HPLC **Glucose**- HPLC	[13]
6.	*Candida bombicola*	Sophorolipid	Not mentioned	Not mentioned	[14]
7.	*Candida bombicola*	Sophorolipid	Ethyl acetate and Hexane [39]	**Ni^{2+} estimation**- Inductively coupled plasma mass spectrometry [40]	[7]
8.	*Candida bombicola*	Sophorolipid	Hexane or Ethyl acetate	**Protein** -Lowry method [41] **Total carbohydrates**- anthrone assay [42] **Total intracellular lipids**- [43] **Amino acid-** HPLC	[8]
9.	*Candida lipolytica*	Protein-lipid-polysaccharide complex	Ethyl acetate extraction	**Protein**- Total protein test kit from Lab test Diagnostica, S.A., Brazil. **Sugars-** Phenol–sulfuric acid method [34] **Lipid-** Gravimetric estimation [44]	[15]
10.	*Candida lipolytica*	Protein-lipid-carbohydrate complex	Chloroform [30]	**Protein**- Total protein test kit from Lab test Diagnostica, S.A., Brazil. **Sugars-** Phenol–sulfuric acid method [34] **Lipid-** Gravimetric estimation [44]	[16]
11.	*Candida ishiwadae*	Glycolipid	Ethyl acetate and Hexane [45]	**Fatty acid analysis-** GC-MS	[17]
12.	*Candida batistae*	Sophorolipid	Ethyl acetate extraction	**Acid form of SLs/ Lactone form-** HPLC	[18]
13.	*Aspergillus ustus*	Glycolipoprotein	Ethyl acetate, diethyl ether and dichloromethane	**Protein**- [46] **Carbohydrate**- Phenol–sulphuric acid method [47] **Lipid**- For free fatty acid using the method of [48]	[19]
14.	*Ustilago maydis*	Glycolipid	Chloroform:Methanol extraction [49]	**Biomass**- [49] **Free fatty acids-** [50] **Lipase activity**- [51]	[20]
15.	*Trichosporon ashii*	Sophorolipid	Chloroform and methanol extraction	**Carbohydrate-** Anthrone reagent method [52] **Lipid**- [53] **Protein**- [46]	[21]

Table 2: Purification and analytical methods for the characterization of biosurfactants.

analytical methods for the characterization of fungal biosurfactants are given in Table 2.

Characterization of Biosurfactants by Various Chromatographic and Spectroscopic Techniques

For the complete structure elucidation of biosurfactants, various chromatographic and spectroscopic techniques were used. A combination of these techniques is very helpful in the characterization of the compound. These techniques are discussed as follows:

Thin layer chromatography (TLC)

This is the most important and preliminary technique for the characterization of various types of biosurfactants. The various solvent systems and developer employed in thin layer chromatography are given in Table 3.

Gas chromatography- mass spectroscopy (GC-MS)

In the structure elucidation of sophorolipids by *Candida batistae,* for the confirmation of fatty acids, the sample was investigated for GC-MS analysis. The major peak at Retention Time (RT) 51.5 min. was supposed due to be 18-hydroxyoctadecenic acid and this was confirmed by comparing the sample with the authenticated sample produced by *Starmerella bombicola*. The Fragmentation pattern of the sample were observed at m/z 312 $[M]^+$ (relative intensity, 1.3); m/z 294 $[M\text{-}H_2O]^+$ (relative intensity, 2.4), m/z 31 $[CH_2CH{=}CH_2]^+$ (relative intensity, 52). In addition to the major peaks additional peaks were also observed (e.g. m/z 31, m/z 55, m/z 67, m/z 81, m/z 95, m/z 110, m/z 123, m/z 137, m/z 151, m/z 165, m/z 213, m/z 237, m/z 262 and m/z 280). The major peak for the standard sophorolipids was observed at 36.9 min. and this reveals the presence of 17-hydroxyoctadecenic acid. The Fragmentation pattern of the standard peak were observed at m/z 312 $[M]^+$ (relative intensity 0.6), m/z 294 $[M\text{-}H_2O]^+$ (relative intensity, 18), m/z 45 $[CH_3\text{-}CH{=}OH]^+$ (relative intensity, 57). Here, also the additional peaks were observed (e.g. m/z 29, m/z 55, m/z 67, m/z 81, m/z 95, m/z 109, m/z 123, m/z 137, m/z 151, m/z 165, m/z 213, m/z 265 and m/z 279) [18]. For the structure elucidation of monoacylglycerols produced by *Candida ishiwadae*, the fatty acid moieties methyl oleate and linoleyl of the compounds (a & b) having Retention Factor (Rf) values 0.23 and 0.17 was determined by the methanolysis. From the combined data of ^{1}H-NMR and mass spectral data, the compounds (a & b) were determined to be 1-oleylglycerol and 1-linoleylglycerol. Further confirmation was done from their molecular weights, 356 and 354 [17]. In the structure elucidation of sophorolipids produced by the *Candida bombicola*, hydroxyl-acid methyl esters were liberated by the methanolysis and were confirmed by GC-MS. The 16-hydroxydecanoic acid was confirmed by comparing the fragmentation pattern with the standard 16-hydroxyhexadecanoic acid purchased from Sigma Aldrich. Also, an isomer 15-hydroxyhexadecanoic acid was confirmed because of the availability of the same fragmentation pattern in the library [12].

High performance liquid chromatography (HPLC) and Liquid chromatography-mass spectroscopy (LC-MS)

In the structure elucidation of mannosylerythritol lipid from *Candida sp.* SY16, the acid hydrolysate of glycoside gave two spots on TLC at Rf values 0.38 and 0.46 respectively and corresponded to D-mannose and meso-erythritol. Also, in the HPLC chromatogram the peaks at RT 10.5 and 7.8 min. corresponded to D-mannose and meso-erythritol respectively. The molar ratio of D-mannose and meso-erythritol was calculated by integration of HPLC peak areas against the known concentrations of the authenticated standards and hence calculated to be 1:1 [10]. The comparative studies of sophorolipids from two carbon sources soybean and glucose by *Pichia anamola* were done. The HPLC of the biosurfactant from glucose and soybean medium showed the peaks at retention time of 9.269 (m/z 675.687, 691.880 and 708.062) and 9.779 min. (m/z 659.499, 675.627 and 691.830). These were compared with the standard sophorolipids sample having a peak at RT of 9.646 (m/z 648.760 and 650.816). The comparative study is given in Table 4 [31] .

Proton nuclear magnetic resonance spectroscopy (^{1}H-NMR)

In the ^{1}H-NMR spectrum of sophorolipids produced by *Starmellela bombicola,* the duplet at 1.20 ppm was assigned as $-CH_3$ at

Fungi	Biosurfactant type	Solvent system	Identification of functional group	Developer	References
Candida bombicola	Sophorolipid	Chloroform:Methanol:Water (65:15:2) [54]	Diacylated lactone	Not mentioned	[12]
Candida lipolytica	Protein-lipid-polysaccharide complex	(i) Hexane:Isopropyl ether: Acetic acid (15:10:1) (ii) Chloroform:Methanol:water (65:24:4)	**Protein**-40.2%, **Carbohydrate-** 24.0% **Lipid-** 19.5%	**Ninhydrin**-Free amino groups **Iodine vapors**-Lipids **α-napthol-H_2SO_4** - Sugar [55]	[15]
Torulopsis petrophilum	Glycolipid	Chloroform:Methanol:Water (65:15:2)	**Not mentioned**	**α-napthol**	[53]
Candida batistae	Sophorolipid	Chloroform:Methanol (8:2)	**Not mentioned**	**Anthrone/sulfuric acid:** [34]	[18]
Pichia anamola	Sophorolipid	Chloroform:Methanol:Acetic acid (65:25:4)	**Not mentioned**	**Iodine vapour and Molisch reagent**	[31]

Table 3: Various solvent systems and developer employed in TLC method.

Sample	RT from HPLC	$[M+H]^+$	Molecular Weights (Da)	Hydroxy carboxylic acid of sophorolipid
Sophorolipid (standard)	9.646	648.760 650.816	647 650	C18+Na C20:1
Biosurfactant from glucose medium	9.269	675.687 691.880 708.062	675 691 707	C20+Na C20+Na ^{Ox} C18:1+Ac+Ac
Biosurfactant from glucose medium	9.779	659.499 675.627 691.830	658 675 691	$[C20]_{Lactone}$ C20+Na C20+Na ^{Ox}

Ox is the oxidized form of sophorolipids

Table 4: Retention times and $[M+H]^+$ of mass spectroscopy for peaks from standard sophorolipid, glucose medium and soybean oil medium samples.

ω-end of fatty acid (C-18) and the sextet at 3.74 ppm was assigned as hydroxylated methine group (-CHOH-) at ω-1 position (C-17). While, the sophorolipids produced by *Candida batistae* showed two multiplets at 3.58 and 3.80 ppm and were assigned as the hydroxylated methylene group (-OCH_2-) at ω-end of fatty acid (C-18) [18]. For the structure elucidation of monoacylglycerols produced by *Candida ishiwadae*, the compounds with Rf values 0.23 and 0.17 were subjected for ^{1}H-NMR. For Rf 0.17, the five protons at δ 3.5-4.2 corresponded to the carbinol protons which indicated the presence of glycerol moiety. The glycerol moiety contains a substituent at –OH group attached to Cl-atom as indicated by high values at δ 4.10 and 4.18. Also, the additional protons at δ 0.9-2.5 and two olefinic protons at δ 5.38 suggested the presence of monounsaturated fatty acid moiety. Also, for the Rf value 0.23 the compound elucidated was monoacylglycerol with an additional double bond [17]. For the structure elucidation of mannosylerythritol lipid by *Canida sp.* SY16, the protons resonating at 5.45, 4.93, 4.26 and 4.44 ppm confirms the acylation positions of sugar moiety. They had lower chemical shifts in comparison to the lipid-free sugar moiety protons which resonate at 4.00, 3.62, 3.72 and 3.88 ppm. The triplet at 0.90 ppm and singlet at 2.08 was assigned due to methyl (-CH_3), broad peak at 1.28-1.40 ppm was assigned due to –CH_2- groups in fatty acids, multiplet at 5.30-5.38 ppm was assigned =CH_2- in unsaturated fatty acids [10].

Carbon nuclear magnetic resonance spectroscopy (^{13}C-NMR)

In ^{13}C-NMR spectrum of sophorolipids produced by *Starmellela bombicola,* peak at 20.7 were assigned as the ω-end carbon (C-18) and the one at 77.4 ppm were assigned as ω-1 carbon (C-17) of fatty acid respectively. While the peak at 69.7 ppm in the ^{13}C-NMR spectrum of sophorolipids produced by *Candida batistae* was assigned as the ω-end carbon (C-18) [18]. In the structure elucidation of mannosylerythritol lipid from *Candida sp.* SY16, peaks at 23.7-33.1 ppm were assigned as –CH_2- groups in fatty acids and peaks at 128.8, 129.7, 130.2 and 131.1 ppm were assigned as four =CH- groups in unsaturated fatty acids [10].

Applications

Biosurfactants have numerous applications in the bioremediation processes, food industries, cosmetic industries and biomedical fields. The various reported applications of the fungal biosurfactants are as follows:

Microbial enhanced oil recovery and cleaning of oil tanks

The Sophorolipids from *Candida lipolytica* and *Candida bombicola* are very promising in the cleaning of oil tanks, decontamination of polluted areas, microbial enhanced oil recovery, industrial cleaning, low-end consumer products and house-hold applications [16,7]. The biosurfactants from *Torulopsis bombicola* and *Aspergillus ustus* MSF3 were used for the release of bitumen from the contaminated soil and for the degradation of hydrocarbons [23,19]. Mannosylerythritol lipids from *Candida antarctica* have potential applications in the removal and biodegradation of hydrocarbons in oil-contaminated soil and were also used to rinse oil and grease from the contaminated soil [27,55].

Food and oil industry

Biosurfactants are able to stabilise various types of emulsions, so are valuable for the food industry. The biosurfactants from the *Candida lipolytica* and *Saccharomyces cerevisiae* are good choices for the food and oil industries [15] further, the biosurfactant from *Saccharomyces cerevisiae* used as a single cell protein [56]. The bioemulsifier, liposan from *Candida lipolytica* was able to stabilize the emulsions of vegetative oils and water. It was also able to stabilize the cottonseed oil, corn oil, soybean oil and peanut oil emulsions [57-60].

Biomedical field

The biosurfactants are extensively useful in the biomedical fields. They possess significant anti-biological activities. The biosurfactant from the *Aspergillus ustus* MSF3 have significant antimicrobial activity against the *Candida albicans* and gram-negative bacterium [19].

Cosmetic industry

The biosurfactants are capable of their usage in the cosmetic industry due to their skin friendly properties. Sophorolipids from the mutant strain *Candida bomobicola* ATCC 22214 have great uses in the cosmetic industries due to their anti-radical properties, stimulation of fibroblast metabolism and hygroscopic properties to support healthy skin physiology [14].

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