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Sophorolipids from *Candida bombicola* using mixed hydrophilic substrates: Production, purification and characterization

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ABSTRACT

Sophorolipids (SLs) are glycolipids type of biosurfactants and are produced by the yeast *Candida bombicola*. Medium containing mixed hydrophilic substrate (deproteinized whey and glucose), yeast extract and oleic acid was investigated in this study for SLs production from the yeast. The produced SL was also purified and characterized in the study. At an optimum combination of the medium constituents, the yeast *C. bombicola* produced maximum 23.29 ± 0.54 g/l, 25.54 ± 1.01 g/l and 33.32 ± 0.83 g/l of the biosurfactant when fermentation was carried out in batch shake flasks, in bioreactor without pH control and in bioreactor with pH control, respectively. Produced SL was purified by silica gel column chromatography and was characterized using FTIR, ^{1}H NMR and LC–MS whose results revealed it to be (17-hydroxyoctadecenoic)-1′4″-lactone-6′6″-diacetate SL. Further, its critical micelle concentration and minimum surface tension against water were found to be 27.17 mg/l and 34.18 mN/m, respectively. Results of interfacial tension obtained using the SLs between water and either n-hexane, sunflower oil or olive oil proved its ability to solubilize non-aqueous phase liquids in water. Further, the biosurfactant was found to be stable at wide range of pHs, temperature and salt concentrations. The results of emulsifying activity and stability of the product against the tested organic solvents and oils together with its ability to solubilize fat and oil confirmed the potential of the biosurfactant in environmental applications.

1. Introduction

Sophorolipids (SLs), a glycolipids type of biosurfactant are attracting considerable attention as promising natural surfactants because of its several advantages over chemical surfactants, such as low toxicity, inherently good biodegradability and ecological acceptability. Also, SLs have found various applications in different fields such as pharmaceutical, medical, cosmetics, environmental and detergent industries [1–5]. SLs are extracellular products and produced by few types of yeast mainly *Candida* species such as *Candida bombicola*, *C. apicola*, *C. bogariensis*, and *C. batistae* [6–9]. Among these several yeasts species, *C. bombicola* is most widely studied for SLs production, due to its ability to produce large amount of the biosurfactant by utilizing low cost industrial wastes [10–12]. Since SLs or any other biosurfactants are perceived to involve higher production cost compared to chemical surfactants, efforts should be made at the level of finding suitable low cost substrates, strain development and optimization of process parameters with an overall aim to increase its volumetric productivity.

Starmerella bombicola, is a teleomorph of *C. bombicola* based on the high 18S rDNA identity (more than 98%) between the two strains and their ability to mate with each other to form ascospores [13]. *S. bombicola* NRRL Y-17069 has been studied in the authors laboratory to produce di-acetylated SLs when grown on low cost fermentative medium based on sugarcane molasses [4].

The objective of the present work was to investigate the production of SLs from *S. bombicola* using deproteinized whey and glucose as a mixed hydrophilic carbon source. Also, properties of the produced SLs, namely critical micelle concentration (CMC), minimum surface tension, interfacial tension, emulsification activity and stability towards different non-aqueous solvents, as well as effect of pH, heating time and salt concentration on the surface tension reduction by the SLs were evaluated in the study.

2. Materials and methods

2.1. Microorganism and its maintenance

The yeast *S. bombicola* NRRL Y-17069 was procured from Agricultural Research Service (ARS-Culture collection), USDA, Peoria, USA. The strain was grown, according to the supplier's instructions, for 48 h at 30 °C incubation on agar slants containing (g/l): glucose, 10; yeast extract, 3; peptone, 5; and agar, 20 (GYP-agar). The

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microorganism was sub-cultured in every 4 weeks and maintained at 4 °C in a refrigerator.

2.2. Chemicals and reagents

All chemicals and solvents used in the study were of analytical grade and supplied by either Hi-Media Pvt. Ltd., India or Merck India Ltd. Whey was a kind gift from Dynamix Dairy Industries Ltd. India. Sunflower oil, olive oil and kerosene oil, used in the study, were purchased from local market in Guwahati, India.

2.3. Seed culture preparation

The medium used for developing the seed culture contained (g/l): glucose, 100; yeast extract, 10; urea, 1, pH 6.0 [4,5,14]. 250 ml Erlenmeyer flasks containing 50 ml of the seed culture media were autoclaved at 121 °C for 20 min, and inoculated with a loop full of the microorganism freshly grown on GYP agar slant. The culture was then incubated for 48 h at 30 °C and 180 rpm in a rotating orbital incubator shaker.

2.4. Production and estimation of SLs

2.4.1. Effect of medium constituents

To study the effect of various media constituents on SLs production by *C. bombicola*, the media containing deproteinized whey, glucose, yeast extract and oleic acid at various initial concentrations, as mentioned below, was used. Deproteinization of whey protein concentrate was carried out according to the method reported by Roukas and Kotzekidou [15]. In brief, powdered cheese whey was dissolved in distilled water (approximately 200 g/l) and protein precipitation was induced by heating the whey at 90–100 °C for 20 min. Precipitated proteins were then removed by centrifugation at 4000 × *g* for 15 min, and the clear supernatant was subsequently used in the experiments. Initial experiments were carried out to study the effect of deproteinized whey concentration on SLs production and yeast growth, in which concentration of deproteinized whey was varied from 25 to 125 g/l. The other medium constituents yeast extract, urea and soybean oil were kept constant at 10, 1 and 100 g/l, respectively. To observe the effect of total hydrophilic carbon source (glucose and deproteinized whey), while glucose concentration was varied from 10 to 50 g/l, deproteinized whey concentration was varied in the range 50–90 g/l in such a way that the total hydrophilic carbon concentration in the medium was maintained at 100 g/l; concentration of the other medium constituents (yeast extract and oleic acid) were kept constant at 10 and 100 g/l, respectively. The effect of yeast extract concentration was investigated by varying its concentration from 2 to 10 g/l by keeping glucose, deproteinized whey and oleic acid at 10, 90 and 100 g/l, respectively. Similar protocol was followed for oleic acid by varying its concentration from 25 to 200 g/l in the media by keeping the respective concentrations of glucose, deproteinized whey and yeast extract constant at 10, 90 and 2 g/l.

Finally the effects of vegetable oils (other than the hydrophobic carbon source, soybean oil) namely sunflower and olive oil in place of oleic acid, were studied on SLs production and yeast growth by fixing the medium composition at glucose (10 g/l), deproteinized whey (90 g/l), yeast extract (2 g/l) and vegetable oil (either sunflower or olive oil) (100 g/l). Using an initial pH of 6.0 in all the production media, experiments were carried out in triplicates in 250 ml Erlenmeyer flasks containing 50 ml of the production media and incubated at 30 °C and 200 rpm in a rotating orbital incubator shaker following inoculation with 5% (v/v) of the seed culture mentioned earlier.

2.4.2. SLs production in bioreactor

SLs production in bioreactor was carried out in a 3 l fermentor (Biotron, Spectrochem, India) with 1 l working volume. The production medium contained (g/l): deproteinized whey, 90; glucose, 10; yeast extract, 2; oleic acid, 100; initial pH 6.0. The medium was inoculated with 5% (v/v) seed culture, and the fermentation carried out for 8 days under batch operated condition at a constant controlled temperature of 30 °C; however, the pH was not controlled during the fermentation. Agitation and aeration were set at 350 rpm and 2 l/min, respectively. Samples were taken periodically for analyzing the concentrations of the yeast biomass and SLs. A second set of batch fermentation was carried out using the same fermentor, but under controlled pH condition, as reported by Hu and Ju [16]. The control strategy consisted in controlling pH at 3.5 after the initial drop from pH 6.0–3.5. All analyses were carried out in triplicate and the results from each triplicate experiment were within ±2% standard deviation.

2.4.3. Estimation of the yeast biomass and SLs production

For the yeast biomass measurement, samples taken during the experiments were extracted twice with an equal volume of ethyl acetate and isopropanol (8:2) to remove unutilized oil and SLs in the fermentation broth. Following separation of the two layers, the aqueous layer was centrifuged at 12,000 × *g* for 15 min at 25 °C and the cell pellets were washed twice with distilled water and dried to constant weight at 80 °C for determining the yeast biomass concentration.

For SLs analysis, the previously obtained ethyl acetate and isopropanol extract were vacuum-dried at 40 °C to remove the solvent. The residue was twice washed with hexane to remove any remaining oleic acid and any hydrophobic substances, viz. fatty acids and alcohols, formed during the fermentation [16]. Partially purified SLs were thus obtained after vaporizing the residual hexane at 40 °C under vacuum and its yield calculated from gravimetric analysis of the compound.

2.5. Purification of SLs produced by C. bombicola using medium based on deproteinized whey and oleic acid

SLs from the yeast fermented broth were first extracted with ethyl acetate and isopropanol (8:2) and then the solvent layer containing the SLs was separated from the broth. Following evaporation of the solvent, SLs residue was washed with hexane to obtain partially purified SLs. Further purification was carried out by silica gel column chromatography using a glass column packed with slurry containing silica gel (200–300 mesh size, Merck India Ltd.) in chloroform (HPLC grade, Merck). Chromatography was carried out by loading the column with the partially purified SLs dissolved in ethyl acetate (1–2 ml), and its elution was carried out with $CHCl_3$: methanol using gradient system (0–20% methanol). Fractions obtained from the column were analyzed by thin layer chromatography (TLC) (silica gel 60 F254/Merck Co., 20 cm × 20 cm aluminum sheets) with $CHCl_3$:methanol (9:1) as the mobile solvent. Fractions containing identical SL were pooled and the solvent removed by evaporation. Further structural characterization of the purified product was carried out by Fourier-transform infrared spectroscopy (FTIR), ^{1}H nuclear magnetic resonance (^{1}H NMR) and mass spectroscopy (MS).

2.6. Characterization of the purified SL product

2.6.1. Structural characterization

The purified SL product using silica gel column chromatography was first identified and characterized by FTIR (PerkinElmer Spectrum-One spectrophotometer, USA). While further characterization of the biosurfactant was carried out using ^{1}H NMR using

$CDCl_3$ (Mercury Plus 400 NMR Spectrometer, Varian, USA), final characterization was performed by MS (Q-TOF Premier™, Waters, USA). For MS analysis, ESI probe connected to a Micromass ZMD served as the negative ion mode.

2.6.2. Minimum surface tension, CMC and interfacial tension determination

Minimum surface tension, critical micelle concentration (CMC) and interfacial tension of SLs mixture in the study were estimated using a surface tensiometer (DCAT 11, Dataphysics Instruments, Germany) equipped with a liquid-dosing unit (LDU, Dataphysics Instruments, Germany) by Wilhelmy plate method. Wilhelmy plate used was of length 10 mm, width 19.9 mm and thickness 0.2 mm. Stock of partially purified SLs (200 mg/l) was prepared in MilliQ water and its surface tension measured at 25 °C. CMC value and minimum surface tension were calculated from the relationship between SLs concentration and corresponding surface tension. Interfacial tension measurements were carried out against n-hexane, sunflower oil and olive oil. All measurements of surface/interfacial tensions were performed in triplicate and results reported were the average of triplicate analysis.

2.6.3. Effect of environmental parameters on surface tension reduction

The effect of environmental parameters such as salt concentration, pH and heat on surface activity of the SLs produced by *C. bombicola* was determined by varying the levels of the individual parameters one-at-a-time by keeping the other parameters at a fixed level. To study the effect of salt concentration, NaCl at different concentrations varying from 0 to 20% (w/v) were mixed with aqueous solutions of SLs (200 mg/l of SLs in MilliQ water) and its surface tension measured. Similarly, to study the effect of pH, aqueous solution containing the SLs was adjusted to desired values of pH using either 1 N NaOH or 1 N HCl and its surface tension measured. For observing the heat stability of the biosurfactant, aqueous solutions of the SLs (200 mg/l of SLs in MilliQ water) were heated to 100 °C in boiling water bath for different time intervals ranging from 0 to 120 min and then allowed to cool to room temperature before measurement of its surface tension.

2.6.4. Emulsification activity and stability

Emulsification activity and stability of the produced SLs was tested using different organic solvents, namely 1-hexadecene, benzene, paraffin light, isopropyl myristate and kerosene oil using a modified method of Cirigliano and Carman [17]. Briefly, 1 ml sample containing the SLs (0.5 mg/ml) was mixed with 1 ml of any of the oil substrate, and thereafter the mixture was shaken vigorously in a vortex mixer for 2 min and allowed to sit for 10 min before measuring its final absorbance at 600 nm. Emulsification activity was thus expressed as the absorbance of the mixture at 600 nm (A_{600}) [17]. Stability of the resulting emulsion was expressed as the decay constant (k_d) estimated from a linear relationship between absorbance (A_{600}) and time (days) as represented in the following equation:

$$\log A_{600} = -k_d \times t \quad (1)$$

2.6.5. Solubilization of fat and oil

Solubilization of fat and oil using the produced SLs were carried out by the method reported by Urum and Pekdemir [18]. Saturated fat and oil solutions were prepared by mixing 20 ml of fat (ghee) and oil (soybean oil) in 100 ml of biosurfactant solutions (27–100 mg/l) taken in 250 ml Erlenmeyer flasks. The contents in the flasks were then shaken gently (to avoid formation of emulsions) at 80 rpm in an orbital shaker set at room temperature 25 °C. The contents of the flasks were then taken in a 250 ml separating funnel and allowed to settle for 48 h, which resulted in two distinct phases: upper oil or fat rich phase and lower biosurfactant rich phase. 40 ml of the surfactant rich phase was collected and its oil or fat extracted using n-hexane [4,19]. The concentration of fat or oil following hexane extraction was obtained gravimetrically, and its solubilization ratio (SR) determined by using the following relationship:

$$SR = \frac{S_C - S_{C,CMC}}{C_S - C_{S,CMC}}$$

where S_C is the crude oil solubility at any surfactant solution concentration (C_S) and $S_{C,CMC}$ is the crude oil solubility in surfactant solution at its CMC ($C_{S,CMC}$). The experiment was carried out in triplicate and results reported were the average of triplicate analysis.

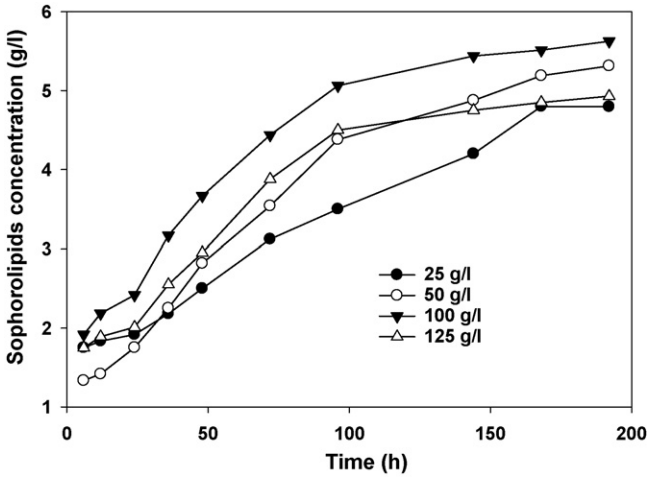

Fig. 1. Effect of deproteinized whey concentration on SLs production in the study.

3. Result and discussions

3.1. Effect of medium constituents

Medium components are known to play a vital role in the production of SLs by the yeast *C. bombicola*, and both hydrophilic and hydrophobic carbon sources are preferred for high yield of the product [20]. Compared to hydrophilic carbon source, hydrophobic carbon source in the medium highly influences SLs production by the yeast [20,21]. Nitrogen source in medium also plays an important role in SLs production as it is well known that the yeast produces biosurfactant under nitrogen limiting conditions. Therefore, optimizing the levels of these medium constituents is highly essential for maximum production of SLs. To determine the optimum concentration of deproteinized whey in the study, its concentration was varied from 25 to 125 g/l by keeping the concentrations of yeast extract, urea and soybean oil constant. Fig. 1 shows the effect of whey concentration on SLs production after 192 h of fermentation, which reveals that maximum SLs yield of 5.62 g/l was found at 100 g/l of whey. Zhou and Kosaric obtained 6.23 g/l of SLs by *C. bombicola* using cheese whey and olive oil as a carbon source in 1 l fermentor [22]. Compared to this literature report, although the yield of the product is slightly less, the result obtained in the present study is in agreement with those of Casas and Garcia-Ochoa [21], Cooper and Paddock [23] and Zhou et al. [24]. Inability of the yeast to utilize lactose present in the whey may be noted as a probable reason for the reduced SLs production in the present study [10]. Therefore, in order to enhance the SLs production by the yeast, whey along with glucose was tested in the medium keeping the final carbohydrate concentration at 100 g/l. Also, urea was omitted from the medium and in place of soybean oil oleic acid was used

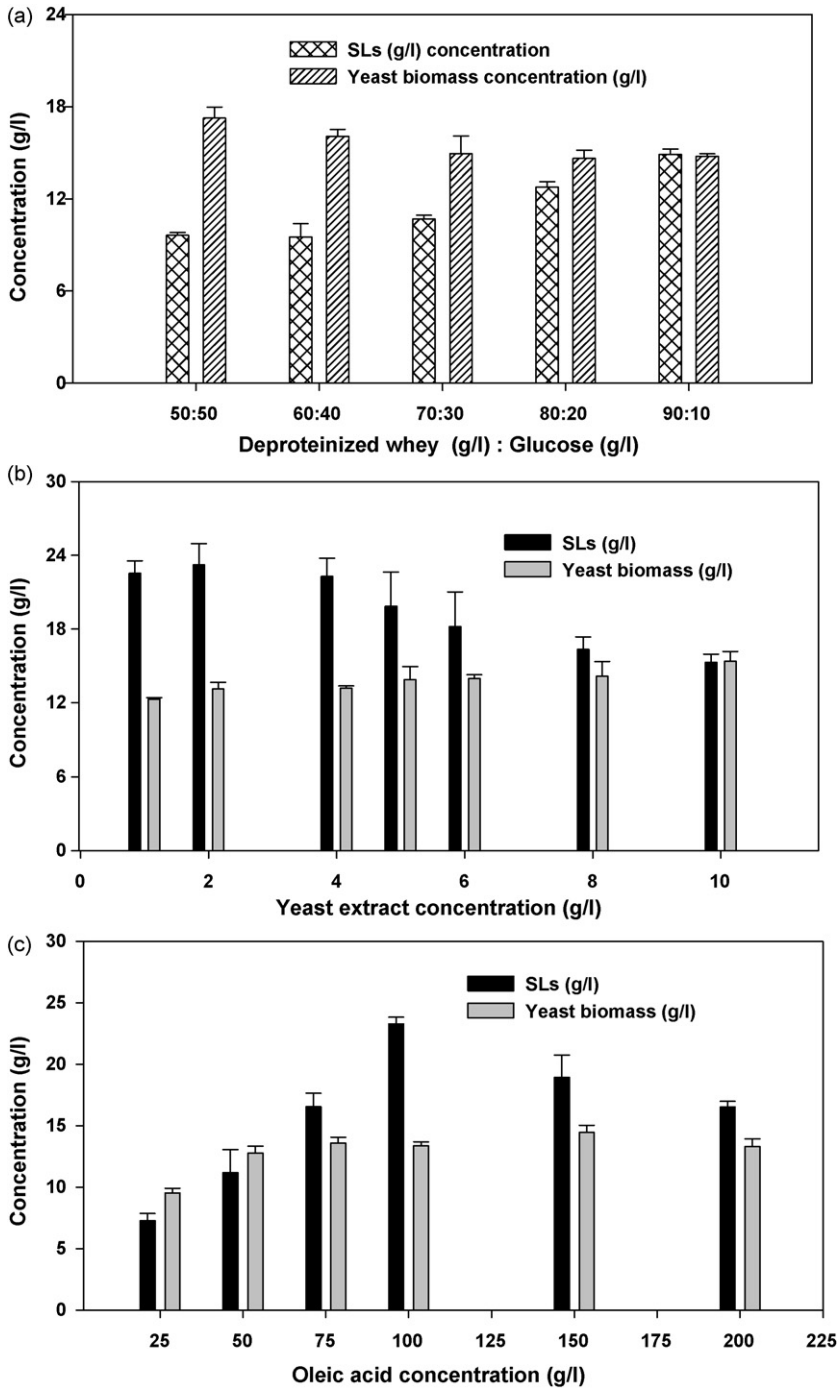

Fig. 2. Effect of glucose (a), yeast extract (b) and oleic acid (c) on SLs production and yeast biomass in media based on deproteinized whey.

as it was expected to give better yield of the product [20]. Based on this modification in the production medium, results obtained showed that addition of glucose (10 g/l) enhanced SLs production up to 14.88 g/l with 14.75 g/l of yeast biomass (Fig. 2a). The figure also reveals that while the yeast biomass was positively affected, the SLs yield decreased due to raise in glucose concentration in the medium (Fig. 2a).

The effect of yeast extract as a source of nitrogen on both yeast cell growth and SLs production was investigated. Fig. 2b shows that the concentration of yeast extract had a negative effect on the SLs production, and therefore 2 g/l was found to be the optimum concentration of yeast extract in the study. This result is in good agreement with those of Van Bogaert et al. [20] and Casas and Garcia-Ochoa [21]. This may be easily understood since nitrogen limitation leads to increased activity of the enzymes involved in SLs production, which are not fully active until the yeast reaches its stationary growth phase. However, the yeast biomass was not affected, but rather improved due to an increase in the yeast extract concentration (Fig. 2b). Zhou and Kosaric studied the effect of yeast extract on the production of SLs during cultivation of *C. bombicola* on glucose (100 g/l) and canola oil (105 g/l) and found optimum yeast extract concentration to be 4 g/l [25]. Use of a different hydrophobic carbon source than that used in the present study may be the reason for a slightly different optimum concentration of yeast extract observed by these authors.

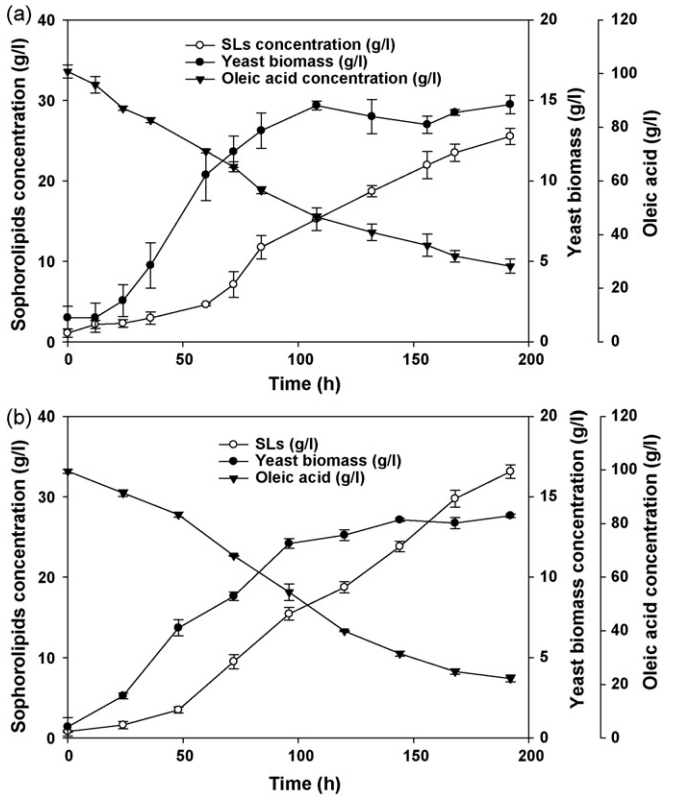

Fig. 3. SLs production under batch mode of operation in bioreactor (a) without pH control and (b) with pH control.

As the SLs production is highly influenced by the hydrophobic carbon source provided in the medium [20,21], concentration of oleic acid as well as other vegetable oils, viz. sunflower oil and olive oil, were tested in the study. Fig. 2c illustrates the influence of oleic acid on SLs production and yeast growth, which shows that oleic acid at 100 g/l is optimum for both yeast biomass and SLs production. While at optimum concentration of oleic acid, 23.29 g/l of SLs and 13.38 g/l of yeast biomass were produced, below the optimum level, the SLs concentration drastically reduced. On the other hand, when oleic acid was replaced with either sunflower oil or olive oil, SLs production considerably reduced to 2.6 and 6.2 g/l, respectively. These SLs production levels were also very much similar to those obtained with soybean oil.

Thus, the medium constituents and their optimum levels for maximum SLs production were found to be deproteinized whey (90 g/l), glucose (10 g/l), yeast extract (2 g/l) and oleic acid (100 g/l).

3.2. SLs production in bioreactor operated under batch mode of operation

At the previously found optimum concentration of the medium components, SLs production was investigated in a batch operated 3 l fermentor (with or without pH control). Fig. 3(a) and (b) shows the profiles of SLs production, yeast biomass growth and oleic acid utilization by the yeast in the fermentor operated without and with pH control, respectively. It could be seen that in the fermentor operated without pH control, the yeast was able to produce 26 g/l of SLs, which was slightly higher than the value observed from the shake flask experiment, probably due to better control of operating conditions such as aeration, temperature and agitation in the fermentor. The value was, however, found to be significantly improved at 33 g/l when the reactor was operated under controlled pH condition. Daniel et al. developed a two-step batch cultivation process

Table 1
Comparison of SLs production yield obtained in the present study using whey in batch bioreactor with the literature-reported values.

Sl. no.	Hydrophilic carbon	Hydrophobic carbon	SLs yield (g/l)	Reference
1.	DCW	Soybean oil	5.62	Present study
2.	DCW and glucose	Oleic acid	33	Present study
3.	DCW	Olive oil	6.23	[22]
4.	DCW	–	12	[26]

for SLs production using deproteinized whey in a 100 l fermentor, in which the first step consisted of growing the yeast *Cryptococcus curvatus* ATCC 20509 on deproteinized whey concentrates; in the second step the cultivation broth was disrupted with a glass bead mill and it served as medium for growth and SLs production by the yeast *C. bombicola* [26]. However, the authors reported only 12 g/l of SLs by this method which is almost three times less compared to that obtained in the present study. Table 1 summarizes the literature-reported values of SLs production yield in batch bioreactor by *C. bombicola* using whey as the hydrophilic carbon source. It is clear from the table that the SLs yield obtained in the present study is superior compared to those found in the literature, which could be attributed to the fact that the medium constituents used in the batch bioreactor experiment were all present at their optimum levels.

3.3. Purification and characterization of the produced SLs

Typical structure of SLs consists of a sophorose (dimeric glucose) linked by a glycosidic bond through a hydroxyl group located at the penultimate position of an 18-carbon fatty acid [4,7,14]. Since the biosurfactant occurs as a mixture of compounds containing macrolactone and open-chain or free acid forms, purification of the major SL followed by its structural characterization was carried out in the present study. By silica gel column chromatography, four different types of SLs were purified and the major SL (denoted as SL-1) was found to be white crystalline in nature, which was further characterized by FTIR, ^{1}H NMR and MS.

3.4. Structural characterization

The SL-1 obtained by column chromatography of the SLs mixture was initially identified and characterized by FTIR. Fig. 4 shows the FTIR spectra of the SL-1 which reveals a broad band at 3403 cm^{-1} corresponding to the O–H stretch in its structure. The spectra also reveals that asymmetrical stretching (ν_{as} CH_2) and symmetrical stretching (ν_s CH_2) of methylene groups occurred at 2926 and 2854 cm^{-1}, respectively; further band at 1624 cm^{-1} was

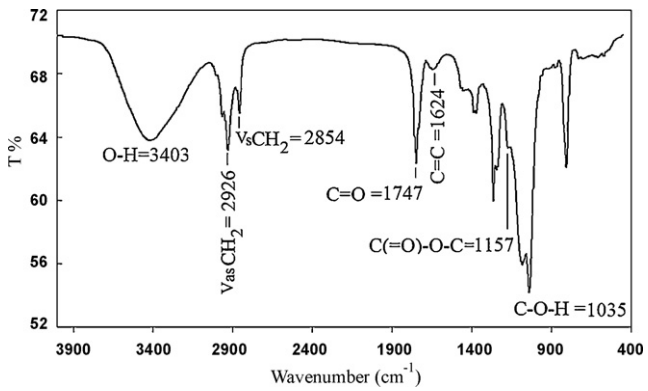

Fig. 4. FTIR spectra of the purified SL-1.

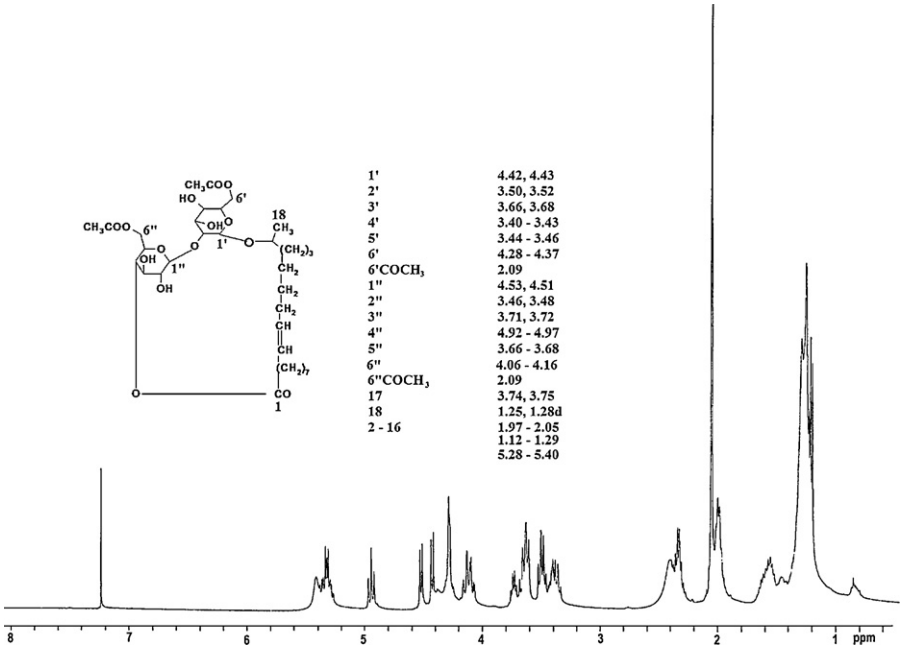

Fig. 5. ^{1}H NMR spectra of the purified SL-1.

observed due to stretching of the unsaturated C=C bonds in the SL-1 molecule [4,27].

While the absorption band at 1747 cm^{-1} indicated the presence of lactone (C=O) group in SL-1, a missing band at 1445 cm^{-1} corresponding to C–O–H in-plane bending of carboxylic acid (–COOH) confirmed the product to be a lactonic type of SL. Further, the stretch of C–O band of C(=O)–O–C in lactones was represented by a band at 1157 cm^{-1}. Moreover, sugar C–O stretch of C–O–H groups was found to be at 1035 cm^{-1}. All these structural details were similar to those reported in the literature for lactonic SLs, which, therefore, confirmed the fermentation product to be lactonic SL [27].

An ^{1}H NMR spectrum of the purified SL-1 was taken in $CDCl_3$ and it was assigned to a typical glycolipid-type structure (Fig. 5). The protons of Glucose-H-1′ and Glucose-H-1″ (Fig. 5) were resonated at 4.42 and 4.43, and at 4.51 and 4.53 ppm, respectively. The other protons of two glucoses were resonated at 3.40–4.50 ppm. Multiple signals of protons at 1.12–1.29 ppm revealed the existence of a fatty acid chain moiety, and signals at 5.28–5.40 ppm revealed –CH=CH–group in the fatty acid chain of SL-1. Also, a signal at 2.09 ppm revealed the presence of (–$COCH_3$) group in SL-1 [4,28].

Finally, to elucidate the molecular weight of the purified SL-1, MS analysis was performed. The mass spectrum of the compound is presented in Fig. 6. The mass to charge ratio (m/z) at 687.08 (M-1) in the spectra revealed that the molecular weight of SL-1 is 688 D, which is the molecular weight of (17-hydroxyoctadecenoic)-1′4″-lactone-6′6″-diacetate SL [29].

3.5. Surface tension, CMC and interfacial tension of the produced SLs

Fig. 7 depicts the results of CMC and minimum surface tension in water due to the produced SLs in the study. These values along with other properties of the biosurfactant are summarized in Table 2. In literature, CMC of the SLs produced from *C. bombicola* grown on medium containing sugarcane molasses, yeast extract,

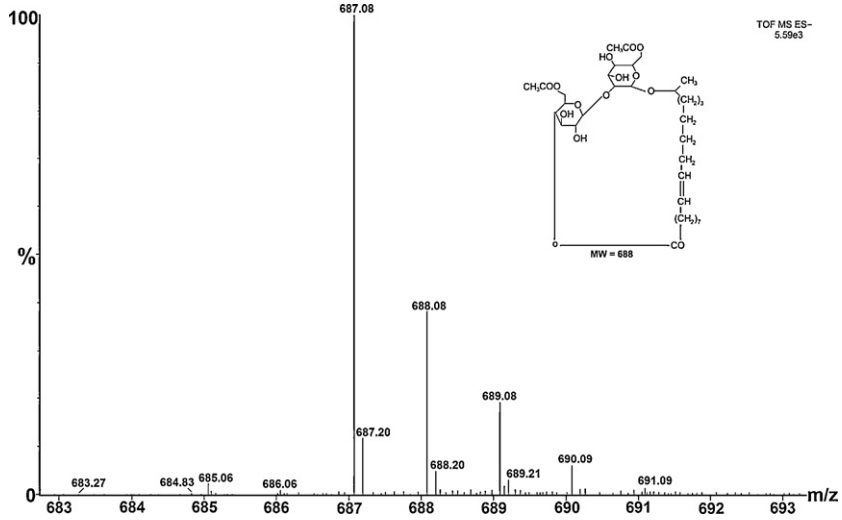

Fig. 6. MS spectra of the purified SL-1.

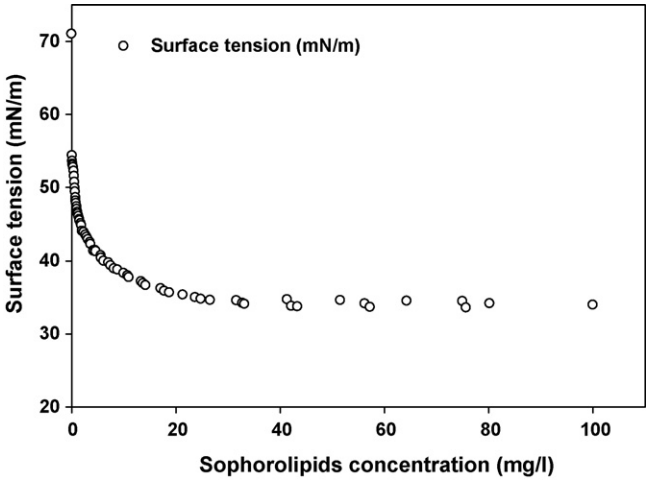

Fig. 7. CMC and minimum surface tension reduction by the SLs produced using deproteinized whey and oleic acid.

urea and soybean oil was estimated to be 59.43 mg/l [4]. Similarly, Otto et al. reported a high value of 130 mg/l for CMC and 39 mN/m for minimum surface tension due to a mixture of SLs produced in a two-stage process using *C. curvatus* and *C. bombicola* grown on deproteinized whey and rapeseed oil as the carbon sources [29]. Compared to these literature-reported CMC values for the SLs, the results obtained in the present study suggest that the partially purified biosurfactant provided superior properties in terms of surface tension reduction and its low value of CMC.

With respect to its capacity to reduce the interfacial tension between water and tested hydrophobic substances (n-hexane, sunflower oil, olive oil), Table 2 suggests that the biosurfactant is very much effective. Thus, the efficiency of the biosurfactant in reducing the interfacial tension between water and various hydrophobic substances makes it more attractive for use in microbial enhanced oil recovery (MEOR) and enhanced oil recovery.

3.6. Effect of environmental parameters on surface tension reduction

For successful industrial applications of any biosurfactant, it is important that it remains stable at extremes of temperature, pH

Table 2
Properties of the SLs produced by the yeast: (a) CMC, surface tension, interfacial tension and solubilization ratio and (b) emulsification properties.

Properties		Value
(a)		
CMC		27.17 (mg/l)
mST		34.18 (mN/m)
IFT against n-hexane		0.99 (mN/m)
IFT against sunflower oil		3.44 (mN/m)
IFT against olive oil		4.46 (mN/m)
SR for oil		26.0×10^{-2}
SR for fat		83.33×10^{-2}
Non-aqueous phase liquid	Emulsification activity ($A_{600\,nm}$)	k_d, Decay constant (day^{-1})
(b)		
Kerosene	0.880	−4.752
Isopropyl Myristate	0.816	−3.168
Benzene	0.443	−3.456
1-Hexadecene	0.828	−2.448
Paraffin	0.854	−3.6

CMC, critical micelle concentration; mST, minimum surface tension; IFT, interfacial tension; SR, solubilization ratio.

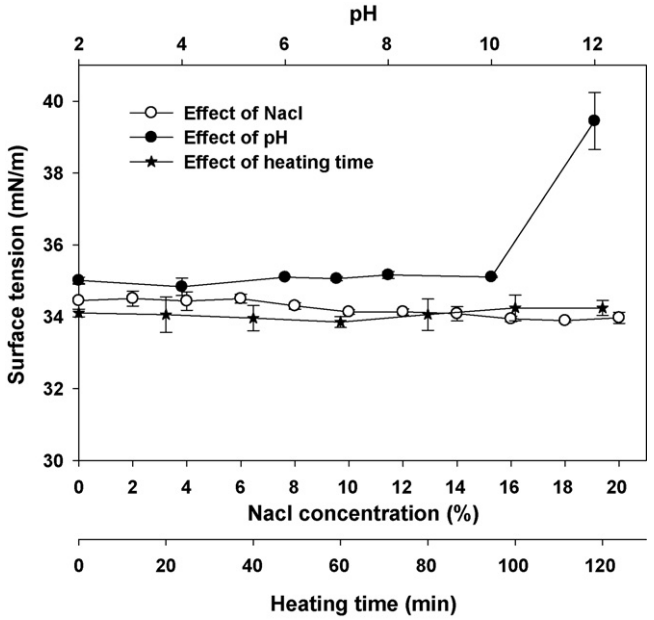

Fig. 8. Effect of salt concentration, pH and heating time on surface tension reduction by the SLs produced in the study.

and ionic strength. Hence the effect of NaCl concentration, heating time and pH on surface tension reduction by the SLs produced in the study was investigated.

Fig. 8 shows the effect of these factors on surface tension reduction by the SLs, from which it is clear that the biosurfactant is highly active at all salt concentrations tested. In literature, Ghojavand et al. reported that biosurfactant produced by a member of the *Bacillus subtilis* group (PTCC 1696) showed a maximum surface activity at salt concentrations in the range 4–8% (w/v) [30]. Further, unhindered surface activity of the SLs in the present study up to 2 h of incubation in boiling water revealed very good thermal stability of the product. Fig. 8 also shows that the SLs was highly active over a broad pH range of 2–10; however, above pH 10 its activity decreased sharply and the surface tension increased up to 39.45 ± 0.79 mN/m.

3.7. Emulsification activity and stability of the produced SLs

It is understood that emulsification property of any surfactant depends on not only the carbon source used for its production but also on the test solvent [4]. Therefore, different non-aqueous phase liquids were tested as a substrate for emulsification activity and stability due to the produced SLs, and the values are presented in Table 2. It could be seen that among the different non-aqueous phase liquids tested, all the substrates, except benzene, showed very good emulsification activity; in case of stability of the emulsions, the stability due to 1-hexadecene was the best followed by isopropyl myristate, benzene, paraffin and kerosene oil (Table 2). These emulsification activities of the SLs in the study were found to be better than the values obtained previously with the SLs produced by the same yeast but using sugarcane molasses and soybean oil as substrates [4].

3.8. Solubilization of fat and oil

Solubilization ratio, as noted before was determined from slope of a plot between SLs concentrations and oil/fat solubilization beyond its CMC. Fig. 9 shows the profiles of solubilization of oil (soybean) and fat (ghee) in the study, from which the solubilization ratios were calculated and are mentioned in Table 2. Urum and

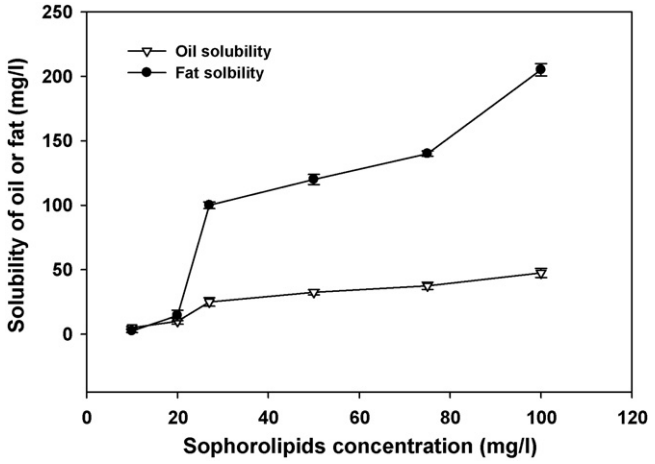

Fig. 9. Solubilization of fat and oil in aqueous phase using various concentrations of the SLs produced in the study.

Pekdemir studied the solubilization of crude oil by different surfactants and found rhamnolipid and aescin to be suitable surfactants with solubilization ratio of 57.9×10^{-4} [18]. Thus, the results of solubilization ratio obtained in the present study clearly demonstrate a very good potential of the SLs for solubilizing high fat and oil that are common in dairy industry wastewater.

4. Conclusion

Low cost medium based on deproteinized whey (9%) along with glucose (1%) was used as a hydrophilic carbon source for SLs production by the yeast *C. bombicola*. The yield and properties (CMC and minimum surface tension) of the partially purified product in this study using mixed hydrophilic substrates was better compared to those reported in the literature using only deproteinized whey in batch fermentation. The purified SL (SL-1) was confirmed to be (17-hydroxyoctadecenoic)-1′4″-lactone-6′6″-diacetate sophorolipid by FTIR, ^{1}H NMR and LC–MS. The properties of the produced SLs in terms of its CMC, minimum surface tension, interfacial tension, emulsification activity, emulsification stability, oil solubilization activity and its stability over a wide range of temperature, pH and ionic strength revealed very high potential of the biosurfactant in environmental applications.

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