Appl Biochem Biotechnol (2009) 158:663–674
DOI 10.1007/s12010-008-8449-z

Production, Characterization, and Properties of Sophorolipids from the Yeast *Candida bombicola* using a Low-cost Fermentative Medium

Achlesh Daverey · Kannan Pakshirajan

Received: 27 June 2008 / Accepted: 18 November 2008 /
Published online: 10 December 2008

Abstract The yeast *Candida bombicola* produces biosurfactant with properties akin to those of sophorolipid (SL) group of compounds. In the present work, the yeast was shown to produce 63.7 g l^{-1} SL when grown on a cheap fermentative medium containing sugarcane molasses, yeast extract, urea, and soybean oil. The partially purified SL was characterized and confirmed by Fourier-transform infrared (FT-IR) spectroscopy, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and liquid chromatography–mass spectroscopy (LC-MS) analysis. The critical micelle concentration (CMC) and minimum surface tension of the produced SL in aqueous solution were found to be 59.43 mg l^{-1} and 34.15 m Nm^{-1}, respectively. The emulsification activity and stability with kerosene oil and organic solvents viz. xylene, benzene, and hexadecane were also tested using the produced SL, which yielded better results compared to those reported in the literature.

Keywords Sophorolipids · Biosurfactant · Fermentation · *Candida bombicola* · Sugarcane molasses

Introduction

Sophorolipids (SL), a kind of extracellular biosurfactants, is reported to be secreted by yeasts of *Candida* sp., and *Wickerhamiella domercqiae* [1–4]. Typically, SL consists of a dimeric glucose (also called sophorose) linked by a glycosidic bond through a hydroxyl group located at the penultimate position of an 18-carbon fatty acid. This type of biosurfactant occurs as a mixture of macrolactone and open-chain (free acid) forms and may be acetylated at the primary hydroxyl positions of the sophorose sugars [3, 5]. Natural mixtures of SL, pure isolates of individual components and their derivatives have shown promise as therapeutic agents with spermicidal and virucidal properties [6], immunomodulators for treatment of antiendotoxic (septic) shock by cytokine downregulation [7] and

A. Daverey · K. Pakshirajan (✉)
Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati 781039, India
e-mail: pakshi@iitg.ernet.in

anti-cancer activity [8]. SL have also been implied for used in the treatment of skin diseases [9]. They also possess numerous potential for use as active ingredient for enhanced oil recovery, in cosmetics, germicidal preparations [10] and in detergents [11]. In the environment cleanup arena, surfactant properties of SL have been identified to be ideal for several applications, mainly for heavy metal removal from soil sediments [12] and in biodegradation of insoluble aromatic compounds [13]. Recently, sophorolipids and their derivatives are reported to decrease asthma severity in an in vivo asthma model [14] and shown lethal against human pancreatic cancer cells [15].

It is well known that the cost of raw materials generally contributes up to 75% of the selling price of bioproducts. Hence, in order to compete with a cheaper chemical surfactant, it is important to use a suitable low-cost fermentation medium for the production of sophorolipids [16]. In other words, biosurfactants can replace synthetic surfactants by way of maintaining the cost of the raw material in its production process at a minimum. So far, several renewable substrates from various sources, particularly from well-known industrial wastes have been intensively studied for microorganism cultivation and SL production in laboratory scale. For instance, in an attempt to reduce substrate costs, cheese whey—a co-product of dairy industry—was proposed as the hydrophilic carbon source [17]. Daniel et al. [17] investigated production of SL in a medium with deproteinized whey concentrate and rapeseed oil and reported a maximum SL production of 280 g l^{-1}. Solaiman et al. [18] used soy molasses, which is a co-product of soy oil processing, for the production of SL and they obtained 21 g l^{-1} SL from *Candida bombicola*. In yet another study, Deshpande and Daniel [19] used animal fat for the production of SL. Felse et al. [20] used industrial fatty acid residues and Shah et al. [21] used restaurant waste oil for the production of sophorolipids.

Molasses of different kinds viz., sugarcane molasses, blackstrap, and beet-molasses are widely used in the fermentation industry for the production of pullulan [22], succinic acid [23], and surfactin [24]. However, the production of SL using this raw material, which is considered much cheaper than others, has not been reported so far.

Molasses is a co-product of sugar production, both from sugarcane as well as from sugar beet, and it is characterized as the runoff syrup from the final stage of sugar crystallization, from which further crystallization of sugar is uneconomical. Sugarcane molasses contains 20% water, 62% sugar, 10% non-sugar contents and 8% inorganic salts as ash contents. Of the total sugar content, 35% *w/v* of sugarcane molasses contains sucrose as the major sugar with remaining being composed of glucose and fructose. A blackish homogenous liquid with high viscosity, it also contains minerals and ions such as Mg, Mn, Al, Fe, and Zn in variable ratios [25] that makes it suitable for use as an ingredient in fermentation medium.

The objective of the present work was, therefore, to investigate the production and properties of SL from *Starmerella bombicola*, an equivalent strain of *C. bombicola*, using sugarcane molasses in place of the much costly glucose as the carbon source. *S. bombicola*, a novel yeast species, is the 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 [26].

Materials and Methods

Microorganism and its Maintenance

The yeast used in this study *Starmerella bombicola* NRRL Y-17069, an equivalent strain of *C. bombicola* ATCC 22214, 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 (grams per liter): glucose, 10; yeast extract, 3; peptone, 5; and agar, 20 (GYP agar) slants. The microorganism was enriched in every two weeks and maintained at 4°C in a refrigerator.

Chemicals and Reagents

All the chemicals used in the study were purchased from Hi-Media Pvt. Ltd., India; and solvents were purchased from Merck India Ltd. Soybean oil and sugarcane molasses used in fermentation of the yeast were obtained from local market in Guwahati, India.

Seed Culture Preparation

The medium used for developing the seed culture contained (grams per liter): glucose, 20; yeast extract, 10; peptone, 20; pH 6.0 [4]. Erlenmeyer flasks (250 ml) containing 50 ml of the seed culture medium 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. The final biomass weight after 48 h was estimated to be 12 g l^{-1}.

Production of Sophorolipids from *C. bombicola* in a Fermentor

Sophorolipids production studies were carried out in a 5-l bioreactor (Biostat B, Sartorius, Germany) with 2-L working volume. The production medium contained (g l^{-1}): sugarcane molasses (hydrophilic carbon source), 100; soybean oil (lipophilic carbon source), 100; yeast extract, 10; urea, 1; pH 6.0. The sugarcane molasses was suitably diluted with distilled water to a final total carbohydrate content of 100 g l^{-1} of the medium. The medium was inoculated with 5% (*v*/*v*) seed culture, and the fermentation carried out for 5 days under batch-operated condition at a constant controlled temperature of 26°C; however, the pH was not controlled during the fermentation. Agitation and aeration were set at 700 rpm and 3 l min^{-1}, respectively. Samples were taken periodically for analyzing the concentrations of biomass, residual sugarcane molasses, oil, and SL. All analyses were carried out in triplicate and the results from each triplicate experiment were within ±2% standard deviation.

Prior to the fermentor study, preliminary investigations were carried out in 250-ml Erlenmeyer flasks containing 50 ml of the production medium mentioned above. Also, for comparison of SL produced from sugarcane molasses by the yeast, separate set of experiments in batch shake flasks containing 50 ml production medium containing glucose (100 g l^{-1}), soybean oil (100 g l^{-1}), yeast extract (10 g l^{-1}), and urea (1 g l^{-1}) were conducted.

Analytical Methods

Biomass and Sugarcane Molasses Estimation

For the yeast biomass measurement and estimation of sugarcane molasses, samples taken periodically along the 5-day fermentation were extracted twice with equal volume of ethyl acetate to remove unutilized oil and SL 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 washed twice with distilled water and dried to constant weight at 80°C for

determining the yeast biomass concentration. The resulting supernatant was utilized for analyzing residual sugarcane molasses concentration in the sample in terms of total carbohydrate content by anthrone method [27].

Sophorolipids Estimation

For SL analysis, the previously obtained ethyl acetate extract were vacuum-dried at 40°C to remove the solvent. The residue was twice washed with hexane to remove any remaining oil and any hydrophobic substances, viz. fatty acids and alcohols, formed during the fermentation [28]. Partially purified SL were thus obtained after vaporizing the residual hexane at 40°C under vacuum and its yield calculated from gravimetric analysis of the compound.

Soybean Oil Estimation

Concentration of unutilized soybean oil in the samples was determined gravimetrically from the hexane extract following extraction of SL by ethyl acetate and vacuum evaporation, as mentioned before [28].

Identification and Characterization of the Sophorolipid

The SL was first identified and characterized by Fourier-transform infrared spectroscopy (FTIR) with a Perkin-Elmer Spectrum-One spectrophotometer, USA. Further characterization of the biosurfactant was carried out using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) using $CDCl_3$ with Mercury Plus 400 NMR Spectrometer, Varian, USA. Final characterization of the compound was performed by liquid chromatography–mass spectroscopy (LC-MS) with Q-TOF Premier™, Waters, USA, using a 3.5-μ Symmetry C18 column of length 50 mm and diameter 2.1 mm. The LC-MS analysis method was due to that of Nunaz et al. [29]; however, ESI probe connected to a Micromass ZMD served as the positive ion mode in the analysis.

Surface Tension and CMC of the Produced Sophorolipid

The minimum surface tension and critical micelle concentration (CMC) of the SL mixture were estimated using a surface tensiometer (DCAT 11, Dataphysics Instruments, Germany) by the standard Du Nuoy ring method with a platinum ring of diameter 18.7 mm. Different concentrations (0 to 250 mg l^{-1}) of the partially purified SL in distilled water were prepared and its surface tension measured at 25°C. CMC value and minimum surface tension were calculated from the relationship between SL concentration and corresponding surface tension of distilled water.

Assay of Emulsification Activity and Stability with the Produced Sophorolipid

To investigate the properties of the produced SL, emulsification activity was measured using a modified method of Cirigliano and Carman [30, 31]. Briefly one ml sample containing the SL at 0.5 mg/ml was mixed with one ml of non-aqueous phase liquid, which was either kerosene oil or any of the organic solvents like xylene, benzene, or hexadecane. Thereafter, the mixture was shaken vigorously in a vortex mixer for 2 min and allowed to

sit for 10 min before measuring its absorbance at 600 nm. Emulsification activity was therefore expressed as the absorbance of the mixture at 600 nm (A_{600}) [30, 31] and stability of the resulting emulsion was expressed as the decay constant (k_d) estimated from the following linear relationship between absorbance (A_{600}) and time (days):

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

Results and Discussion

Sophorolipid Production in Batch Shake Flasks

Results from batch shake flasks study revealed that the yeast *C. bombicola* produced more biomass when grown on sugarcane molasses–soybean oil (18.9 g l^{-1} biomass) compared to that grown on glucose–soybean oil (16.5 g l^{-1} biomass). On the other hand, the SL concentration was found to be less at 9 g l^{-1} using sugarcane molasses–soybean oil compared to 15 g l^{-1} of SL when grown on glucose–soybean oil in batch shake flasks after 5 days of fermentation. Similar results have been reported by other authors [17, 18] when glucose was replaced with deproteinized whey or soy molasses in the fermentation medium. This preliminary result in batch shake flasks, however, showed the ability of the culture to utilize sugarcane molasses as carbon source in producing SL.

Sophorolipid Production in a 5-l Fermentor

In order to further evaluate the cheap raw material in producing the SL, scale up study in a laboratory fermentor under necessary continuous monitoring of pH, dissolved oxygen (DO), and temperature was carried out. The profiles of SL production, substrate utilization, biomass, pH, and DO during the fermentation were monitored over time in the bioreactor. The SL production and substrate utilization profile by the yeast in the bioreactor experiments is presented in Fig. 1, which shows a maximum concentration of the SL mixture to be 63.7 g l^{-1} at 60 h of fermentation and after this time period, its concentration declined quite gradually up to 34.8 g l^{-1} at the end of 5 days. Deshpande and Daniel [19], in their study, also found that the maximum SL production (97 g l^{-1}) decreased after 72 h of batch fermentation when the yeast was grown on animal fat in a 10-l fermentor without any pH control during the experiments. They suggested a decrease in the product yield due to substrate limitation in the fermentation medium thereby necessitating a need for harvesting the product before this time period. Kim et al. [32] reported a maximum sophorolipids production of 80 g l^{-1} when the same yeast was grown on glucose–soybean oil containing medium in a 5-l fermentor. But, when soy molasses and oleic acid were used in addition to yeast extract and urea in the fermentation medium, Solaiman et al. [18, 33] observed that the yeast produced only 21 g l^{-1} of sophorolipids, and its yield slightly improved to a value of 53 g l^{-1} when grown on medium containing only soy molasses and oleic acid. Compared to these yield values of sophorolipids found in the literature, its production using sugarcane molasses in the study seems more feasible and more attractive as well.

Figure 2 shows the relationship between pH, DO, and biomass during SL production by *C. bombicola* in the bioreactor under batch mode. It could be seen that during the exponential growth phase of the yeast, pH dropped drastically from 6.0 to 3.6, probably due to consumption of nitrogen source together with generation of fatty acids; however, during the later phase of fermentation, the pH was stable after reaching a value of 4.0. With respect

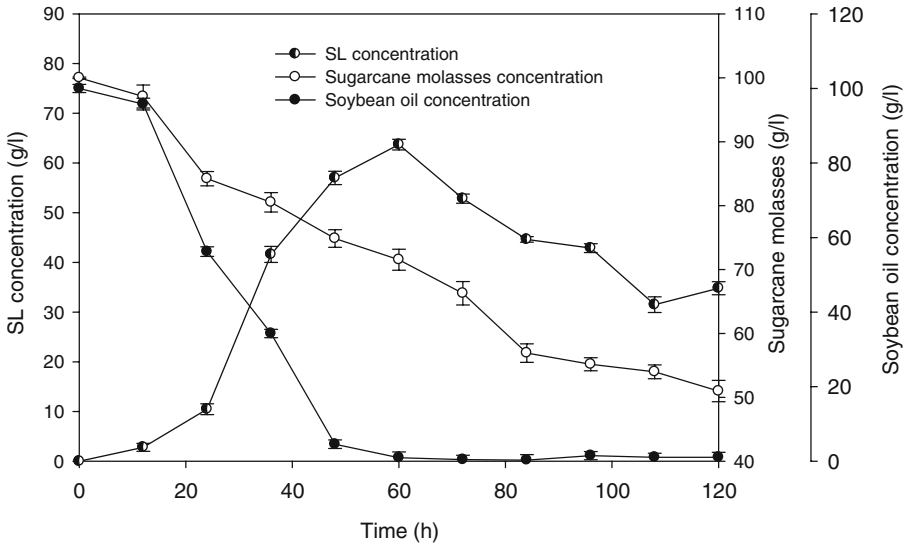

Fig. 1 Time course of sophorolipid production and substrate utilization by *C. bombicola* under batch-operated conditions in the fermentor

to the yeast biomass, the amount increased very rapidly within the first 36 h and then decreased slightly till it assumed a constant value. These changes in this biomass profile corresponded well with the medium DO, but negatively. The observations on these parameters were also found to be consistent with those of Deshpande and Daniel [19] on SL production by *C. bombicola* in their study.

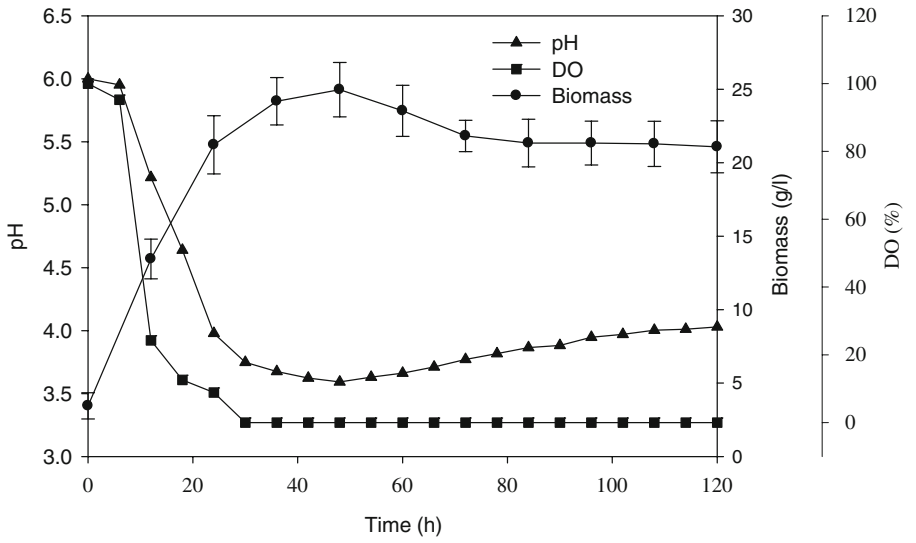

Fig. 2 pH, biomass, and DO profiles during sophorolipid production by *C. bombicola* in the fermentor

Identification and Characterization of the Sophorolipids

FTIR Analysis

The product obtained using sugarcane molasses–soybean oil in the fermentation of the yeast was identified and characterized by FTIR. Figure 3 shows the FTIR spectra of the SL, which reveals a broad band at 3,433 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 2,926 and 2,854 cm^{-1}, respectively. Further, since lactones and esters have two strong absorption bands arising from C–O and C–O stretching, the C–O absorption band at 1,744 cm^{-1} may include contributions from these groups (lactones, esters, or acids). The stretch of C–O band of C(–O)–O–C in lactones exists at 1,157 cm^{-1}, while that from the acetyl esters was found to be at 1,247 cm^{-1}. Moreover, sugar C–O stretch of C–O–H groups was found to be at 1,048 cm^{-1} and the band at 1,445 cm^{-1} corresponded to the C–O–H in-plane bending of carboxylic acid (–COOH) in the structure of the product. All these structural details of the product were found similar to those reported in the literature [34], which therefore confirmed the fermentation product to be SL group of compounds.

NMR Analysis

According to the ^{1}H NMR spectrum of the produced sophorolipids, illustrated in Fig. 4, a signal at 2.063 ppm in the spectra confirmed the presence of $–COCH_3$ group in its structure. Further, while a signal peak at 5.316 ppm confirmed the presence of –CH=CH– group, multiple signals of protons between 1.20 and 1.40 ppm revealed the existence of a fatty acid chain moiety in the sophorolipid structure. Figure 5 is a ^{13}C NMR spectrum of the

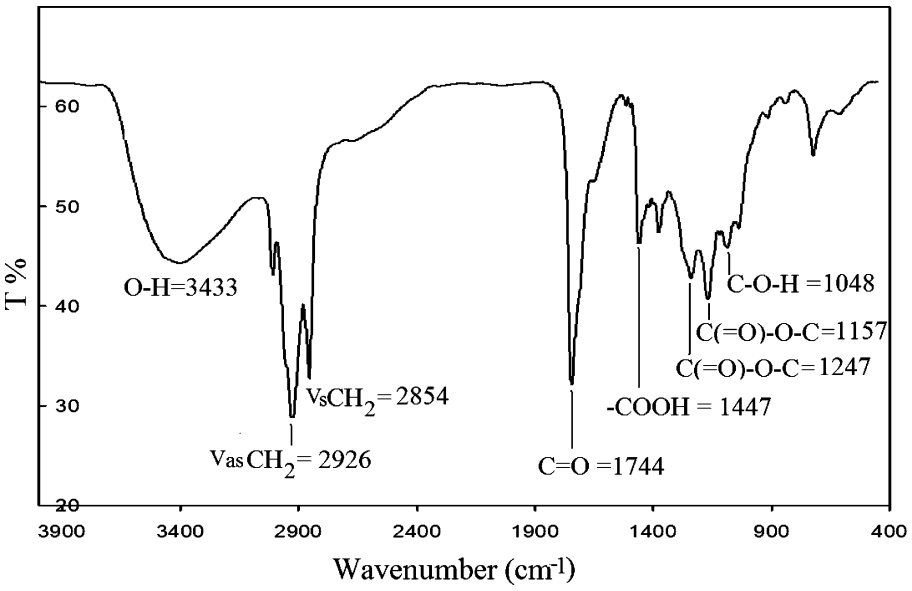

Fig. 3 FTIR spectra of the produced sophorolipid from the yeast

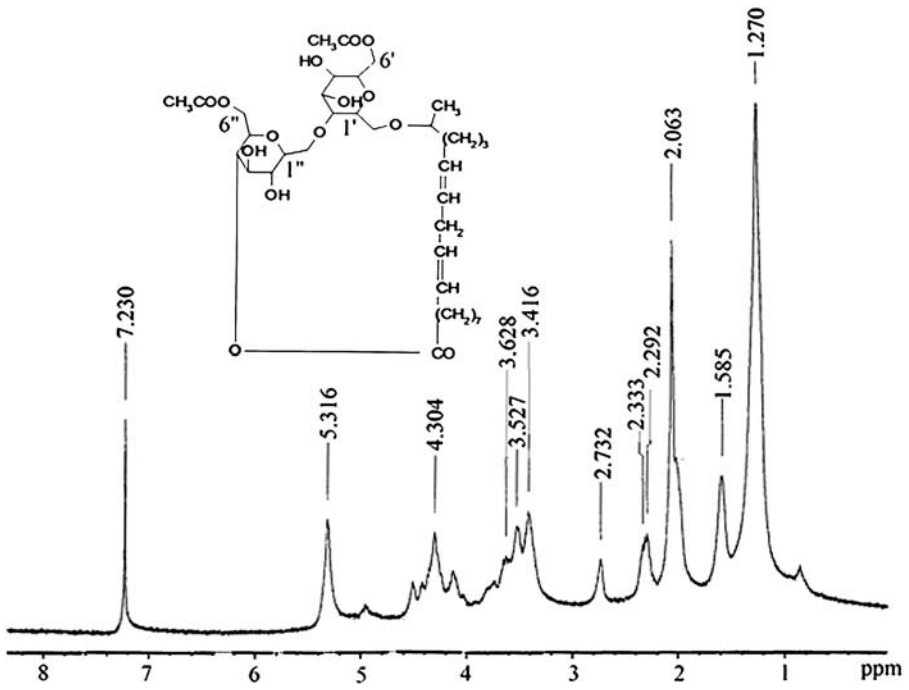

Fig. 4 ^{1}H NMR spectra of the produced sophorolipid from the yeast

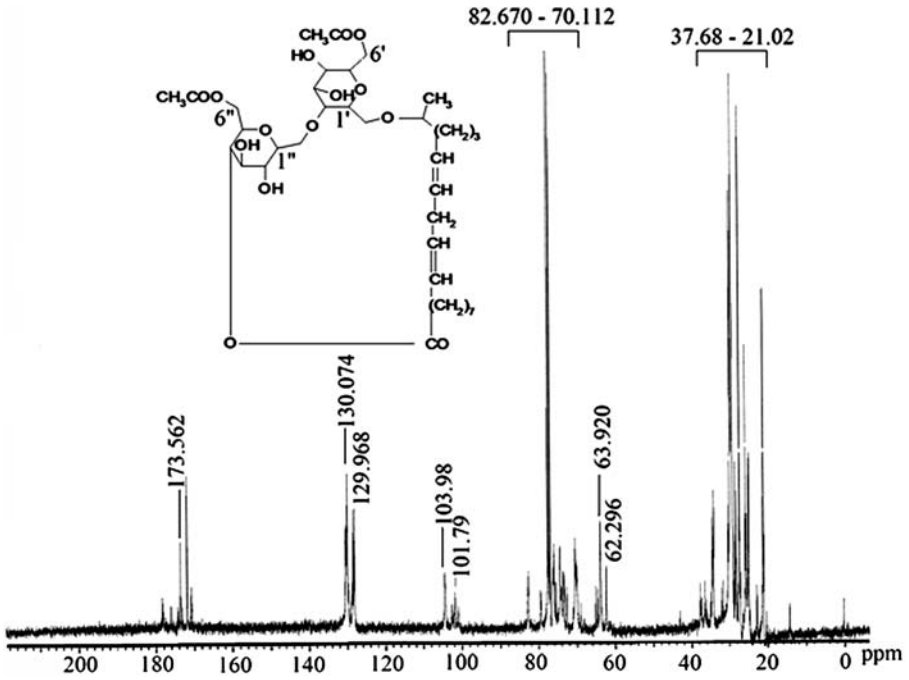

Fig. 5 ^{13}C NMR spectra of the produced sophorolipid from the yeast

biosurfactant, which shows the presence of two =CH– groups in the fatty acid chain moiety corresponding to signals at 130.07 ppm and 129.96 ppm; however, five more =CH– groups in the fatty chain moiety were resonated between 128 and 130 ppm, which may be probably due to contribution from other interfering group of sophorolipids in the sample. In addition, several $–CH_2–$ groups in the fatty chain moiety were also resonated at 21.02–37.68 ppm. The spectrum also revealed signals of glucose-C-1′ at 103.98 ppm, glucose-C-1″ at 101.79 ppm, glucose-C-6′ at 63.92 ppm and glucose-C-6″ at 62.29 ppm; the other carbon atoms of glucose were resonated between 70.11 and 82.67 ppm [4]. Chen et al. [4] also reported similar spectra of SL obtained in their study. Akin to the results of FT-IR analysis, all these structural features further confirmed sophorolipid group of compounds in the fermented product.

LC-MS analysis

To elucidate the structure of the sophorolipids produced in the study, LC-MS analysis was further performed. The mass spectrum of the compound is presented in Fig. 6, which indicates the presence of a mixture of sophorolipids with varying molecular weights. However, the mass to charge ratio (*m*/*z*) at 717.36 (M+H) in the spectra revealed that the mixture predominantly contains a sophorolipid molecule with 716 Da molecular weight that is different than the molecule produced by the yeast using glucose and soybean oil as the two different carbon sources [28]. Further, a decrease of *m*/*z* from 717.36 to 673.34 and then to 629.31 clearly indicated the fact that the SL molecule was diacetylated (Fig. 6). In addition, presence of two sugar moieties in the sophorolipid molecule was ascertained by a respective decrease in the *m*/*z* at 453 and at 295 of the spectrum (Fig. 6). Moreover, peak at 295 *m*/*z* in the spectrum revealed a C18:2 lipidic moiety in the sophorolipid molecule.

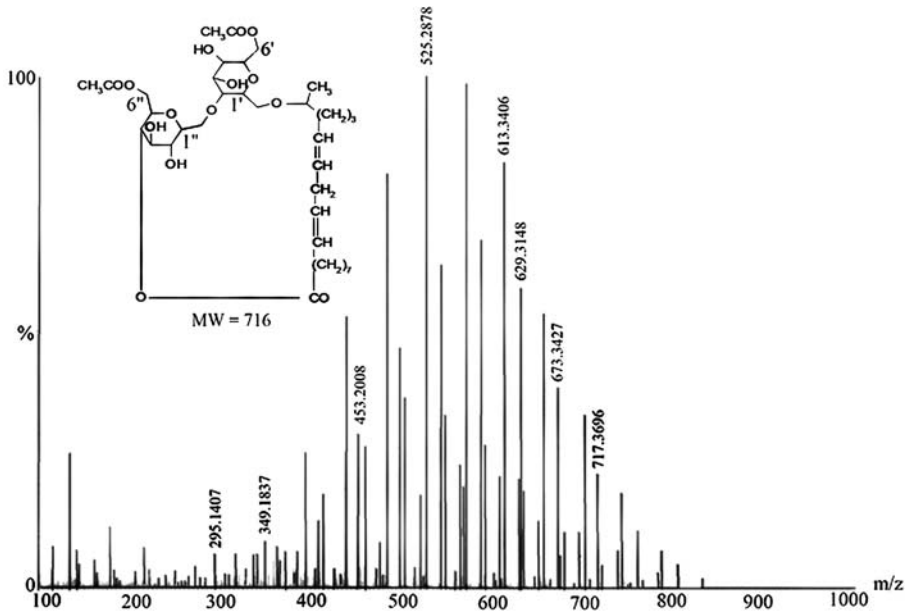

Fig. 6 Mass spectra of the produced sophorolipid from the yeast

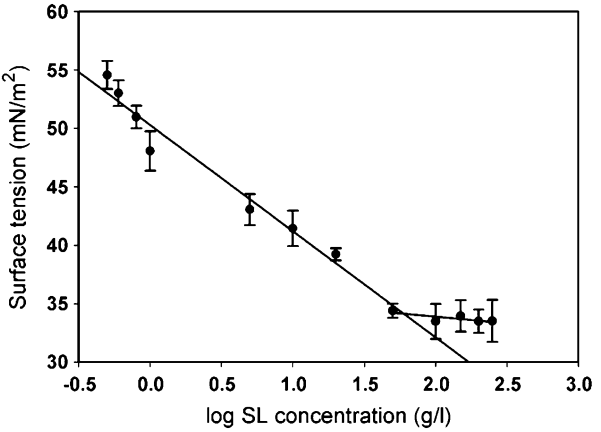

Fig. 7 Results of surface tension measurements and determination of CMC of the produced sophorolipid in water

Similarly, in the literature, Hu and Ju [28] reported, from the results of LC-MS analysis of sophorolipids produced by *C. bombicola* using soybean oil as the second carbon source, that the compound was a mixture of both lactonic (C18:0, C18:1, C18:2, and C16:0) and acidic sophorolipids (C18:0, C18:1, C18:2, and C16:0).

Properties of the Produced Sophorolipid

Surface Tension and CMC of the Produced Sophorolipid

Critical to any surfactant are its properties such as surface tension and critical micelle concentration (CMC). CMC is normally referred to the concentration of surfactant at which micelles begins to form and at this point, surface tension of the medium in which the surfactant is dispersed takes up a minimal value. A lower CMC indicates that less surfactant is needed to saturate interface between either air–liquid or liquid–liquid and form micelles. Figure 7 depicts the results of determination of CMC and minimum surface tension in water due to the SL produced in the fermentor. The values of these two properties of the produced SL were determined to be 34.15 mNm^{-1} and 59.43 mg l^{-1}, respectively. Kim et al. [35] in their study, employing glucose and soybean dark oil as the carbon sources, found that the sophorolipids produced by the yeast *C. bombicola* yielded a CMC value of 150 mg l^{-1} and minimum surface tension of 48 m Nm^{-1}. Similarly, Otto et al. [36] reported a high value of 130 mg l^{-1} for CMC and 39 m Nm^{-1} for minimum surface tension due to sophorolipids produced by the yeast using deproteinized whey and rapeseed oil as the carbon sources in their study. Compared to these literature-reported values for the SL, the results obtained in the present study suggest that the partially purified biosurfactant provided excellent properties in terms of the reduction of surface tension and a low value of the CMC.

Table 1 Emulsification activity and stability of SL produced from the yeast *S. bombicola* with different non-aqueous phase liquids.

Non-aqueous phase liquids	Emulsification activity (A_{600} nm)	Decay constant (k_d) (day^{-1})
Kerosene 0.716	1.584	−1.584
Xylene 0.727	2.016	−2.016
Benzene 0.533	26.784	−26.784
Hexadecane 0.709	2.88	−2.88

Emulsification Activity and its Stability with the Produced Sophorolipid

Emulsification activity and its stability with any biosurfactant truly indicate its potential for practical applications and was therefore carried out in this study. Table 1 presents the results of emulsification activity and its stability with the produced SL using various organic solvents and kerosene oil as the substrate. Among the different non-aqueous phase liquids tested, all the substrates showed comparable emulsification activity; and in case of the emulsion stability, the stability due to kerosene oil was the best followed by xylene and hexadecane. Compared to these three substrates, the SL exhibited, however, slightly poorer emulsification activity and stability with benzene (Table 1).

SL produced by the yeast *C. bombicola* is generally reported to be a poor emulsifying agent [37, 38]. Cooper and Paddock, [37] tested the emulsification activity and stability of the SL produced by the yeast with hydrocarbons and vegetable oils and reported that the SL was not able to stabilize emulsions containing water and either the hydrocarbons or the vegetable oils. However, the present study shows that the SL produced by the yeast when grown on sugarcane molasses as a hydrophilic carbon source alternative to costly glucose has better emulsification activity and stability as compared to those reported by others in the literature [37–40]. To further reveal the potential of the produced SL from the yeast using cheaply available sugarcane molasses, it would be interesting to test the product in pre-treatment of high fat and oil containing industrial wastewaters.

Conclusions

Conventional synthetic media were replaced by a cheaper alternative medium containing sugarcane molasses as the main carbon source in producing sophorolipid (SL) by the yeast *C. bombicola*, in both batch shake flasks and in a 5-l fermentor. The yield of the partially purified product from the cheap raw material was only slightly less compared to that using glucose as the hydrophilic carbon source. The spectra obtained from FTIR spectroscopy, NMR, and LC-MS confirmed the presence of SL in the samples. The properties of the produced SL in terms of its CMC and minimum surface tension in water, emulsification activity against several organic solvents and kerosene oil and the emulsion stability revealed very high potential of the biosurfactant for further environmental applications.

Acknowledgment This work was supported by the research funds from the Department of Science and Technology (DST), India. The authors wish to thank Dr. T. Punniyamurthy, for his help in the analyses of LC-MS and NMR spectra.

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