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CHARACTERIZATION OF SOPHOROLIPID BIOSURFACTANT PRODUCED BY YEAST SPECIES GROWN ON DIESEL OIL

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ABSTRACT
The yeast species viz. *Rhodotorula muciliginosa and Candida rugosa* isolated from hydrocarbon contaminated sites were capable of producing biosurfactants in the presence of 2% (v/v) diesel as sole source of carbon and energy. The crude biosurfactants were purified using silica gel column chromatography followed by dialysis. Chemical structures of the purified biosurfactants were identified as diacetate acidic sophorolipid and mono acetate lactonic sophorolipid using FT-IR and GC-MS. The biosurfactants showed high physicochemical properties in terms of the surface activities when compared to synthetic surfactants, including Tween 80 and sodium dodecyl sulfate. Involvement of biosurfactant in physiological mechanism of diesel adsorption on yeast cell surface was characterized based on zeta potential measurement. When diesel oil was emulsified with biosurfactant, the surface charge of the diesel was modified resulting more adsorption of diesel on yeast cell surface. SEM analysis was used to monitor biosurfactants production by yeast species.

KEYWORDS: Biosurfactant; Diesel; Yeast species; Sophorolipid.

INTRODUCTION

Microbial biosurfactants are chemically diverse groups of extracellular surface active molecules containing both hydrophilic and hydrophobic moieties capable of reducing surface tension and facilitating hydrocarbon uptake and emulsification (Thavasi et al., 2009). They are the complex molecules, comprising different structures that include glycolipids, lipopeptides, polysaccharide protein complexes, fatty acids and phospholipids. Their superior properties are high biodegradability, low toxicity, ecological acceptability, and production from cheaper substrates (Nitschke and Pastore, 2004). Because of these properties, biosurfactants have potential application in food, pharmaceutical and cosmetic industries (Desai and Banat, 1997). The other advantages of biosurfactants are selectivity and specific activity at extreme temperatures, pH and salinity (Velikong and Kosaric, 1993). Structurally biosurfactants are polymers, totally or partially extracellular, amphipathic molecules containing polar and non polar moieties which allow them to form micelles that accumulate at interphase between liquids of different polarities such as water and oil (Desai and Banat, 1997).

The natural roles of biosurfactants are not clear but probably might increase the nutrient uptake of hydrophobic substrates, enhance the growth on hydrophobic surface, motility and biofilm formation (Van Hamme et al., 2006). Synthetic surfactants currently used are derived from petroleum and are usually toxic and hardly degraded by microorganisms causing damage to the environment. These hazards associated with synthetic surfactants drawn much attention to microbial biosurfactants (Kiran et al., 2009).

Various microorganisms such as bacteria, fungi and yeast are known to produce specific kind of biosurfactants (Kiran et al., 2009). This depends mainly on molecular composition of the type of biosurfactants produced. Among the bacteria, P*seudomonas* sp. was found to be the best known bacteria capable of degrading hydrocarbon and producing biosurfactants of glycolipid mainly rhamnolipid in nature (Kumar et al., 2008). Yeast species such as *Candida* sp. are known to produce sophorolipids, extracellular glycolipids when grown on carbon sources such as glucose, long chain fatty acids or nitrogen source (Nunez et al., 2001). Sophorolipids are the most important biosurfactants used in cosmetics and therapeutics (Maigault, 1999). The molecule consists of the disaccharide sophorose. (2- O-13-Dglucopyranosyl-13-D-glucopyranose) typically with the 6' and 6'' hydroxyl groups acetylated, that is linked to the fatty acid through a glycosidic bond between carbon 1' and the ~o-1 carbon of a long chain fatty acid (Rosenberg et al., 1999; Davila et al., 1997). The amphipathic structure of sophorolipids imparts to them surfactant type properties. These biosurfactants are readily isolated in high yield, nontoxic and biodegradable (Casas et al., 1999). Certain other biosurfactants like phospholipids, lipopolysaccharide, and polyol lipids are also known to produce by certain yeast species (Pattanathu et al., 2008).

Due to the increasing demand of microbial biosurfactants rather than synthetic ones, discovery of new biosurfactant producing strains and finding the optimum condition for biosurfactant production are vital to economically and effectively ameliorating contaminated soils and ground water. So the present work deals with the production and characterization of biosurfactants from yeast species isolated from hydrocarbon contaminated sites, having the capacity to produce extracellular glycolipids in the presence of diesel oil as sole source of carbon.

MATERIALS AND METHODS

Identification of the yeast species

Rhodotorula mucilaginosa and *Candida rugosa* isolated from petroleum hydrocarbon contaminated soil in India were identified to the species level by Viktek 2 Compact Yeast card reader with the software version V2C 03.01 from CFRD (Center for Food Research and Development),

Kerala, India. The isolated strain was maintained in YEPD (Yeast extract, Peptone, Dextrose) agar slants at 28 °C and subcultured every two weeks

Diesel oil and other chemicals

Diesel substrate used in this study was obtained from local petrol pump in Vellore, TamilNadu, India. This diesel oil was filter sterilized and used throughout the studies. All other chemicals used in the present study were of high purity in grade.

Isolation and optimization of biosurfactant production in shake flasks

Bushnell- Haas medium with diesel oil was used for optimization and production of biosurfactant by yeast species. The yeast species were cultured at different temperatures (25 ºC to 55 ºC), substrate concentrations (0.5% - 5% *v/v* of diesel) and pH (3.5-9.5). All the experiments were carried out in 250 ml conical flasks containing 50 ml mineral salt medium (Bushnell- Haas medium). The culture was maintained in a water bath shaker at 120 rpm for a period of 14 days. The culture broth was centrifuged at 10, 000 rpm for 10 min and extracted with Chloroform and methanol (2:1 *v/v*). The solvents were removed by rotary evaporation and the resultant residue obtained was crude biosurfactant. Weight of the biosurfactant was expressed in terms of milligrams per milliliter (dry weight).

Purification of biosurfactant

Purification of the biosurfactant was done according to Thavasi et al. (2009). The culture broth prepared under optimized parameters were centrifuged at 10, 000 rpm for 10 min and extracted with Chloroform and methanol (2:1 *v/v*). The solvents were removed by rotary evaporation and the residue purified on a silica gel (60-120) mesh column eluting with a chloroform/methanol gradient ranging from 20:1 to 2:1, collecting ten fractions. Then fractions eluted were pooled and the solvents evaporated; the resulting residue was dialyzed against distilled water and lyophilized as reported by Li et al. (1984). Weight of the biosurfactant was expressed in terms of milligrams per milliliter (dry weight).

Physiochemical characterization

To study the biosurfactant production and activity, yeasts were grown in mineral salt medium with diesel as carbon source (2% v/v final concentration) for period of 14 days and cultures withdrawn intermittently and extracted. The physicochemical properties of the crude biosurfactant was investigated and compared with those of two commercial surfactants, Sodium dodecyl sulphate (SDS) and Tween 80. SDS is a well known anionic surfactant and Tween 80 is a non ionic surfactant and emulsifier derived from polyethoxylated sorbitan and oleic acid.

Microbial adhesion to the hydrocarbon (MATH)

Cell surface hydrophobicity was assessed by microbial adhesion to the hydrocarbon method (MATH) according to Rosenberg et al. (1980). Yeasts cells were harvested from 7 days and 14 days grown culture by centrifugation at 10,000 x g for 10 min at 4 ºC and washed twice with PUM buffer (buffer salt solution (pH 7.0) containing KH_2PO_4 - 7.26 g l^{-1}, K_2HPO_4 - 19.7 g l^{-1}, urea - 1.8 g l^{-1} and $MgSO_4$. 7 H_2O - 0.2 g l^{-1}). The cells were again suspended in PUM buffer to fit an optical density of ca.1.0 (A_0). Optical density was measured at 600 nm on UV-Visible Spectrophotometer (Shimadzu). Diesel (500µl) was added to 5 ml of microbial suspension and vortexed for 2 min. The optical density of aqueous phase was measured (A_1) after 10 min. The degree of hydrophobicity is calculated as $[1-(A_0-A_1)/A_0.100\%]$. The experiment was repeated thrice.

Drop collapse test

Qualitative drop collapse test was performed according to Bodour and Maier (1998). 2 µl of diesel oil was added to the 96 well microtitre plates. The plates were equilibrated for 1 h at 37 ºC and 5µl of culture supernatant was added to the surface of the oil. The shape of drop on oil surface was observed after 1min. The culture supernatant that make the oil drop collapsed was indicated as positive result and that drops remain beaded were scored as negative which is examined with distilled water as control.

Oil displacement test

The oil displacement test is a method used to measure the diameter of the clear zone, which occurs after dropping a surfactant-containing solution on an oil-water interphase. The binomial diameter allows an evaluation of the surface tension reduction efficiency of a given biosurfactant. The oil displacement test was done by adding 50 ml of distilled water to a petri dish with a diameter of 15 cm. After that 20 µl diesel oil was dropped on to the surface of the water, followed by the addition of 10 µl of cell culture supernatant. The diameter and the clear halo visualized under visible light were measured after 30 s (Rodrigues et al., 2006).

Surface tension measurement

The surface tension of the distilled water at different surfactant concentrations (biosurfactants+ commercial surfactants like SDS and Tween 80) was measured by using a du Nouy ring type tentiometer (Kruss, K10T) and compared with surface tension of the distilled water without biosurfactant. The surface measurement was carried out at 25 ± 1 ºC after dipping the platinum ring in the solution for a while in order to attain equilibrium conditions. The measurement was repeated three times and an average value was obtained. For calibration of the instrument, the surface tension of the pure water was measured before each set of experiment.

Measure of emulsification activity

Emulsification ability of biosurfactant towards diesel as well as other hydrocarbons was studied. A mixture of 6 ml of studied hydrocarbons and 4 ml, 1 mg/ml of the crude biosurfactant were vortexed at a high speed for 2 min. The emulsion activity was investigated after 24 h and the emulsification index (E_{24}) was calculated by dividing the measured height of the emulsion layer by the total height of the mixture and multiplying by 100 (Cooper and Goldenberg, 1987). The higher the emulsification indexes the higher the emulsification activity of tested biosurfactant.

Stability characterization

Stability studies were carried out using the cell-free broth obtained by centrifuging the cultures at 5,000 g for 20 min. The pH of the biosurfactant (4 ml) was adjusted to 2.0-10 using NaOH or HCl after which E_{24} was determined. To test the heat stability of the biosurfactant, the broth was heated at 10–100 °C for 15 min, cooled to room temperature and emulsification index were

determined. The effect of NaCl on the biosurfactant was also assayed at NaCl concentrations of 0–10%, w/v.

Biochemical characterization of biosurfactant

Chemical composition of the biosurfactant was analyzed following standard methods. Carbohydrate content of the biosurfactant was determined by the anthrone reagent method using 620 nm (Spiro, 1966). D-glucose used as standard. Protein content was determined by the Lowry et al. (1951) using bovine serum albumin as a standard. Lipid content was estimated adopting the procedure of Folch et al. (1956).

Fourier transform infrared spectroscopy

One milligram of lyophilized purified biosurfactant was ground with 100 mg of KBr and pressed with 7, 500 kg for 30 s to obtain translucent pellets. The infrared spectra were recorded on Mattson 1, 000 FT-England FTIR system within the range of 500-4000 cm^{-1} wave number. All measurements consisted of 500 scans, and KBr pellet was used as background reference.

Gas chromatography – Mass spectrophotometry

The methyl ester derivates of the fatty acids were prepared by mixing the purified biosurfactant (10 mg) with 5% HCl- methanol reagent (1 ml). After the reaction was quenched with water (1 ml), the methyl ester derivatives were extracted with n-hexane and then injected (1 µl) into a Hewlett Packard (HP, Wilmington, DE, USA) gas chromatograph (GC) model 5890 Series II Plus equipped with a capillary inlet and an HP Mass Selective Detector (MSD) model 5972 set to scan from *m/z* 45 to *m/z* 600 at a scan rate of 1.2 scans per second. The capillary column used was an SP-2340 (60m x 0.25 mm) (Supelco, Bellefonte, PA, USA). The oven temperature was programmed from 130 ºC to 230 ºC at 2 ºC min^{1}. The temperature of the injector port was 230 ºC and the detector transfer line temperature was 240 ºC. The carrier gas was He at a flow rate of 1 mL min^{-1} and a split ratio of 50:1.

Measurement of zeta potential

Surface charges on the yeast cell surface, diesel and emulsified diesel with biosurfactant were measured at room temperature at different pH (3.5-9.5) with a Brookhaven 90+ zeta analyzer (Brookhaven Instrument Corp., NY, U.S.A.). Emulsified diesel was prepared by mixing three times for 3 min 97% distilled water and 1% v/v surfactant and 2% diesel at a power level of +200 V by an ultrasonic generator (Sonics-Vibracell ultrasonic processor).The stock emulsions were diluted 10 fold in phosphate (0.01 M, pH 7.5) buffer before use.

Biosurfactant monitoring

Yeast cells grown in Bushnell haas medium with 2% diesel for 72 h were subjected to SEM (JOEL JSM 5600LV) analysis to determine the biosurfactant production. Sample for SEM was prepared by dipping 1 cm^2 coverslips in 1 ml of 72 h grown yeast cultures with 5 × 10^8 CFU ml^{-1} and placed for 90 min of adhesion phase at 37 °C. The coverslip was then washed with sterilized phosphate buffer saline (PBS) to remove loosely adherent cells. One millilitre of Bushnell haas medium with diesel was added to the washed pieces and incubated at 37°C for 72 h. Again the coverslip was washed with phosphate buffer, dried and fixed with 3% gluteraldehyde and dehydrated with a series of ethanol solutions (50, 75, 95, and 100%) and subjected to SEM analysis

RESULTS AND DISCUSSION

Isolation and optimization of biosurfactant production

Biosurfactant production on diesel was found to be maximum at pH 7.5, at a temperature of 35 ºC and substrate concentration of 2% (*v/v*) diesel substrate (Fig. 1). In all culture conditions tested, biosurfactant concentration was highest at the early stationary phase, 10^{th} day. Higher concentration of biosurfactant at the early stationary phase may be due to the release of cell-bound biosurfactant in to the culture broth which led to the raise in extracellular biosurfactant concentration (Goldman et al., 1982). It was found that biosurfactants produced by yeast species on diesel were growth associated similar to that for some bacterial species like *Bacillus stearothermophillus* VR-8 (Gurjar, 2001), *Pseudomonas aeruginosa* (Ilori and Amund, 2001) and *Aeromonas* sp. (Ilori et al., 2005).

Screening for biosurfactant production

The yeast species *Rhodotorula muciliginosa* and *Candida rugosa* showed high cell surface hydrophobicity (82% and 79%) over a period of 10 days and almost constant for a period of 14 days. An increase in adherent propery of diesel was found in all the three yeast cultures with advancement of time and reaching the saturation after seven days. The cell surface hydrophobicity was related to the biosurfactant secreted on the cell surface, helping adhesion of bacteria to the hydrocarbons, and resulting in the effective degradation (Rosenberg et al., 1980; Maneerat, 2005).

The drop collapse test and oil displacement test were also conducted for the primary screening of biosurfactant production. These qualitative tests are indicative of the surface and wetting activities (Youssef et al., 2004). The oil displacement test is an indirect measurement of surface activity of a surfactant sample tested against oil; a larger diameter represents a higher surface activity of the testing solution (Rodrigues et al., 2006). In the present study surface activities of the crude biosurfactant was investigated in comparison with that of Tween 80 and SDS. Drop collapse test and oil displacement test were highly positive for crude biosurfactants than commercial surfactants, Tween 80 and SDS, which indicated high surface activity (Table 1).

The ability of the crude biosurfactants as well as Tween 80 and SDS to reduce the surface tension of distilled water was compared (Table 1). In case of biosurfactant produced by *Rhodotorula muciliginosa* and *Candida rugosa* reduced the surface tension of distilled water to a minimum value with low value of CMC. As shown in Table 1 Tween 80 and SDS were found to reduce the surface tension but with high CMC values. The results suggested that the biosurfactant from the yeast species provided excellent properties in terms of reduction of surface tension and a low value of CMC.

FIGURE 1. Biosurfactant production by *Rhodotorula muciliginosa* and *Candida rugosa* at different pH (B1a, B2a), at different temperature (B1b, B2b), at different diesel concentrations (B1c, B2c) in Bushnell- Hass medium over a period of 14 days.

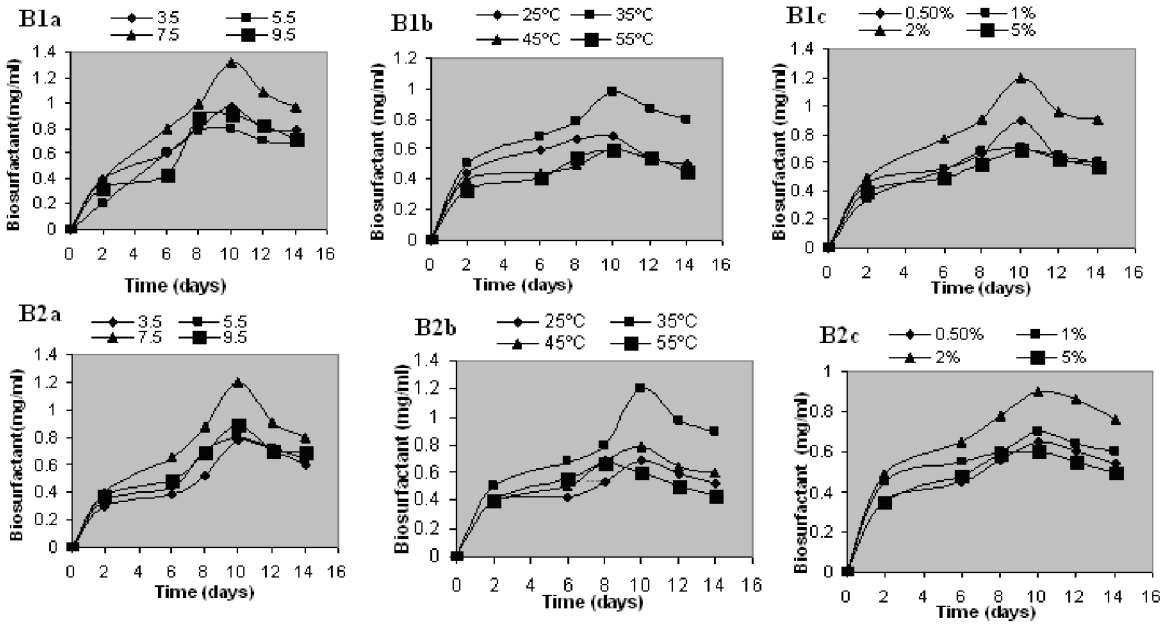


TABLE 1. Physicochemical characterization of biosurfactant produced by yeast species, *Rhodotorula muciliginosa* and *Candida rugosa*

Characterization	Surface tension (mN/m)	CMC (mg/l)	Oil displacement (cm^2)	Drop collapse test
Distilled water	72 ± 0.5	Negative	Negative	Negative
Crude extract [a]	33 ± 0.6	250	72 ± 1.5	[c] + + +
Crude extract [b]	34 ± 0.7	300	74 ± 0.9	+ + +
Tween 80	39 ± 0.8	1250	65 ± 0.9	+ +
SDS	36 ± 0.5	380	50 ± 0.7	[d] + +

All values are means ± SD for triplicate cultures
Crude extract [a] crude form of biosurfactant from *Rhodotorula muciliginosa*
Crude extract [b] crude form of biosurfactant from *Candida rugosa*
[c] + + + indicate highly positive drop collapse test
[d] + + indicate moderate positive drop collapse test

Rhodotorula muciliginosa and *Candida rugosa* could effectively emulsify (E_{24}) and stabilize emulsions with diesel (86 ± 0.7 and 78 ± 0.7%) as well as various types of hydrocarbon substrates (Fig. 2). Formation of emulsion usually results from the dispersion of liquid phase as microscopic droplets in another liquid continuous phase (Desai and Banat, 1997). From the point of view of microbial degradation, dissolution and emulsification of hydrocarbons appear to have positive effect on degradation rate (Amund and Adebiyi, 1991).

The stability of formed emulsions was found to be more than one month in room temperature without changing emulsification activity. Interestingly, the crude biosurfactants gave the highest emulsification activity (E_{24}) on diesel, probably because it was produced by diesel oil as a carbon source. The emulsification index values of biosurfactants were also measured at different temperatures, pH and NaCl concentrations (Fig.3) The optimum temperature for emulsification activity of biosurfactants was at room temperature (28 ºC), even showing emulsification activity at temperature of 10 -100 ºC. All biosurfactants isolated from the yeast species showed emulsification activity over a pH range from 2 to 10. (Fig. 3a). This suggests that biosurfactants isolated from yeast species might be useful in acidic and alkaline environment conditions. The retension of over 60% emulsification activity at 10 ºC and 100 ºC suggests that this biosurfactants might be useful in extreme environments such as temperate marine compartments and industrial systems where extremes of temperature are

integral elements. The emulsification index of the biosurfactants produced were found to be inversely proportional to the NaCl concentration, but still retained its activity at high concentration of NaCl (8-10%). This suggests that these biosurfactants might be useful in marine environments and other systems where salt concentration is above physiological level. Previous reports have also shown stability of some biosurfactants of bacteria in presence of high salt concentration (Obayori et al., 2009; Sarrubbo et al., 2007).

FIGURE 2. Emulsification activity of biosurfactants produced by yeast species *R..mucilaginosa* and *C.rugosa* in diesel towards different hydrocarbon substrates.

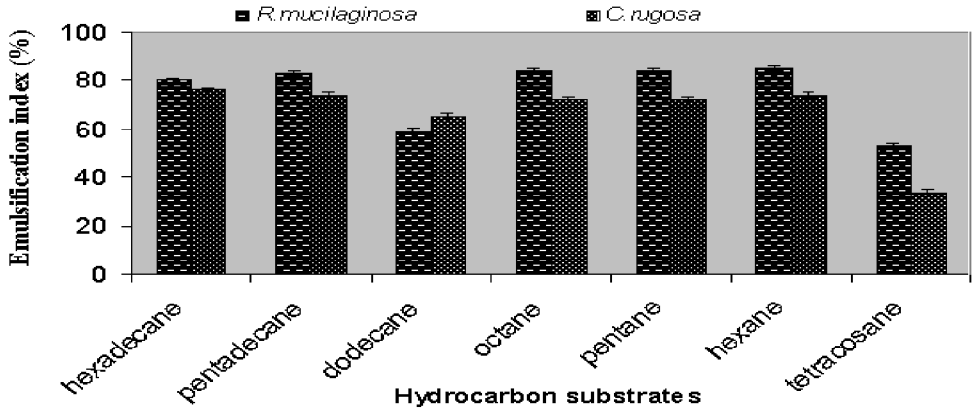
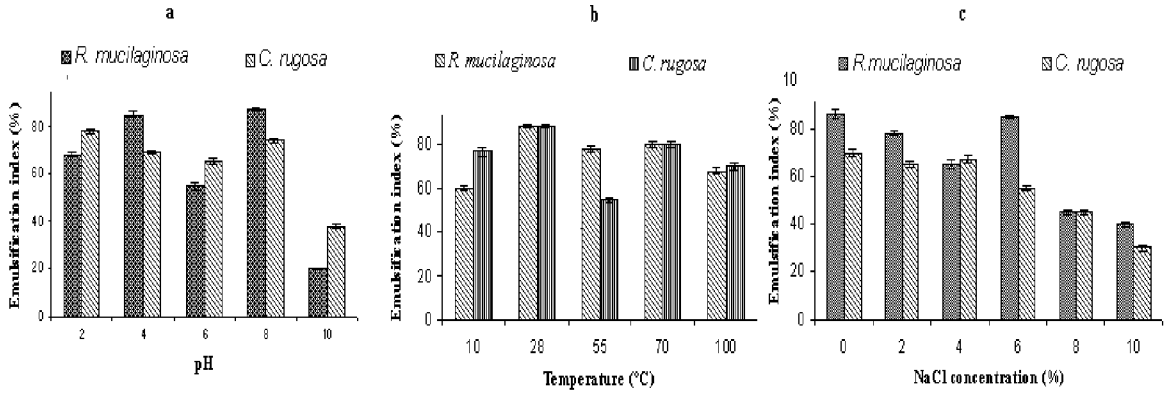


FIGURE 3. Effect of pH (a), temperature (b), NaCl concentration (c) on the emulsification activity of biosurfactants produced by yeast species *R.mucilaginosa* and *C. rugosa* in diesel.



Biochemical and analytical characterization of biosurfactant

Carbohydrate, Protein and lipid estimation of the biosurfactants produced by the yeast species were classified as a glycolipid with carbohydrate and lipid as major constituents (Table 2).

TABLE 2. Biochemical characterization of biosurfactants produced by the yeasts species

Chemical analysis	Carbohydrate	Lipid	Protein
Purified extract [a]	30.78	60.23	Nil
Purified extract [b]	40.56	58.32	Nil

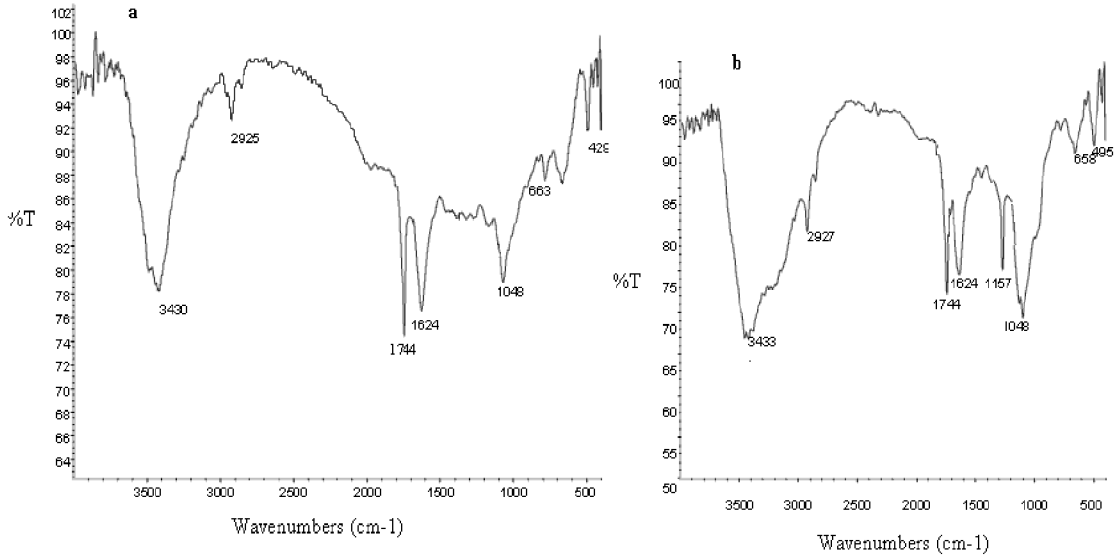
Purified extract [a] – Purified form of biosurfactant from *R.mucilaginosa*

Purified extract [b]– Purified form of biosurfactant from *C. rugosa*

Molecular compositions of biosurfactants were evaluated by FT-IR. Figs. 4a and b represents the spectra's of the purified freeze dried biosurfactants from *C. rugosa* and *R.mucilaginosa.* Biosurfactants isolated from the yeast species had mostly similar absorption bands. The broad band observed in biosurfactants isolated from yeast species *Candida rugosa* and *Rhodotorula muciliginosa* was at 3410-3434 cm^{-1} corresponds to the O-H stretch. The asymmetrical stretching (V_{as} CH_2) of methylene occur at 2920-2926 cm^{-1}. The band 1620-1627 cm^{-1} is from stretching of unsaturated C═C bonds. Lactones and esters have two strong absorption bands arising from C═O and C─O stretching. The C═O absorption band at 1744 cm^{-1}

may include contributions from that of lactones, esters, or acids (Silverstaein and Webster 1998) and this characteristic band can be observed in spectra of biosurfactants of three yeast species. The stretch of C─O band of C (═O) ─O─C in lactones appear at 1157 cm^{-1} which can be observed in the spectra of biosurfactant isolated from *Candida rugosa*. The sugar C─O stretch of C─O─H groups is at 1048 cm^{-1} and this characteristic sugar stretch was observed in the spectra of all biosurfactants. From the FT-IR data it is evident that lactonic form of biosurfactant is dominant in *Candida rugosa.* It was reported that sophorolipids are the only surfactants produced in larger quantities by yeast species and similar FT-IR spectra results of sophorolipids from *Candida bombicola* were obtained previously (Shah and Prabhune 2007).

FIGURE 4. FT-IR spectra of the purified biosurfactants of (a) *R.mucilaginosa* and (b)*C.rugosa*



GC-MS analysis of purified biosurfactants were done to elucidate their structures and MS spectra of biosurfactants isolated from three yeast species *Candida rugosa* and *Rhodotorula muciliginosa* is shown in Figs 5a and b . The significant ions occurred at m/z 728 (Fig. 5a) on mass spectra of biosurfactant isolated from *Rhodotorula muciliginosa* and corresponding chemical structures were determined as diacetate acidic sophorolipid with fatty acid moiety C18:1. At m/z 668 significant ions of mass spectra of biosurfactants from *Candida rugosa* the chemical structure identified as monoacetate lactonic sophorolipids with fatty acid moiety C18:1 (Fig. 5b). The yeast species *Candida rugosa* and *Rhodotorula muciliginosa* were found to be the potent producers of sophorolipids which are in monoacetate, diacetate, lactonic and acidic forms. Since sophorolipids are useful in oil recovery, removing hydrocarbon contaminated sites and decontaminating soils and groundwater tables polluted with hydrocarbons (Pesce 2002; Ducreux et al., 1997), the sophorolipids produced by the three yeast species have potential application in all the above fields.

Surface charge measurement

The zeta potentials of the yeast species, biosurfactant emulsified diesel and diesel emulsion at different pH are shown in Fig. 6. Mostly all the microbial cells are negatively charged at neutral pH. Here the yeast species exhibited negative zeta potential in all the pH ranges. The biosurfactant emulsified diesel showed highly positive zeta potential up to pH 7.5 whereas the zeta potential turned to less positive when pH is above 7.5. Therefore, the maximum degradation can be observed up to pH 7.5, due to the attachment of highly positive charge biosurfactant emulsified diesel to the negative charge yeast cell surface. Whereas diesel emulsion showed negative charge in all the pH, so there will be weak attachment of diesel to yeast cell surface. These results showed that biosurfactant is involved in the surface charge modification of diesel and enhancing the adsorption of diesel to yeast cell surface.

SEM analysis for biosurfactant production

The biosurfactant or exopolymer production by the yeast species was well observed in SEM photograph (Fig. 7). Yeast cells with diesel oil resulted the formation of sticky colourless matrix of exopolymers interconnecting individual cells into an intricate network of coherent mass after 72 hours of incubation Microencapsulation of yeast cells in their exopolymers was also noted in SEM image.

CONCLUSION

The two yeast species *Rhodotorula muciliginosa* and *Candida rugosa* isolated from hydrocarbon contaminated sites are found to be potent producers of sophorolipids having higher hydrophobicity, emulsification activity, surface tension reduction and wide range of hydrocarbon emulsification activity which could be feasibly used during *in situ* bioremediation of ground water due to their oxygen requirement nature. The stability of biosurfactants in wide range of pH, temperature and salinity also enable these compounds to be used in extreme environments.

FIGURE 5. Mass spectrum of purified biosurfactant (sophorolipid) produced by *R.mucilaginosa* and *C.rugosa* grown on diesel using silica Column chromatography.(a) Mass spectrum of diacetate acidic sophorolipid (MW 728) produced from diesel by *R.mucilaginosa*. (b) Mass Spectrum monoacetate lactonic sophorolipids (MW 668) produced from diesel by *C.rugosa*

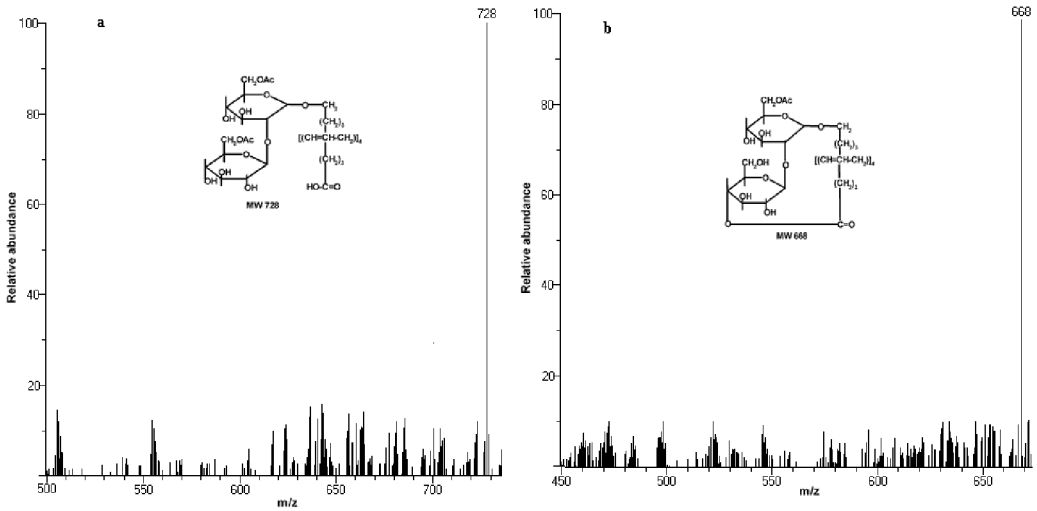


FIGURE 6. Zeta potential and electrostatic interaction between *R.mucilaginosa* (Y3), *C.rugosa* (Y4), biosurfactant emulsified diesel (B4, B5), Diesel emulsion (D), at different pH.

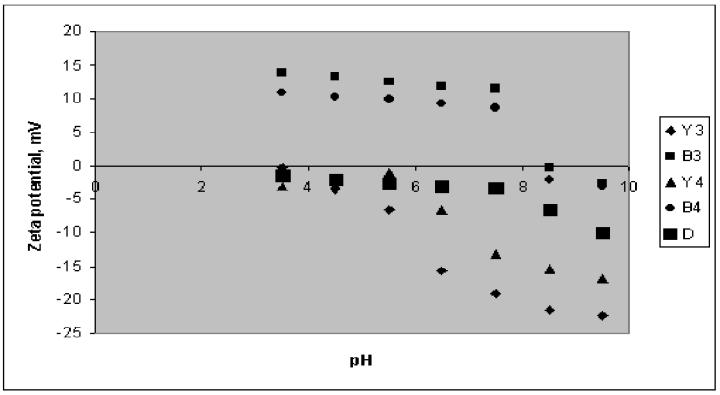
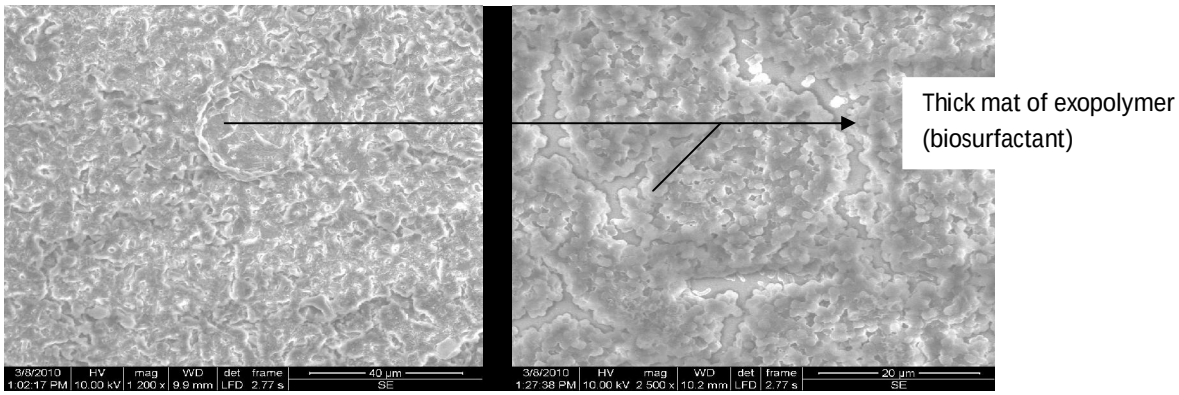


FIGURE 7. SEM image showing exopolymer production (sticky colorless matrix) by *R.mucilaginosa* and *C.rugosa* grown in Bushnell haas medium containing diesel after 72 h of incubation.

a) b)



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