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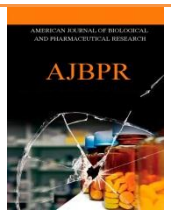

PURIFICATION AND CHARACTERIZATION OF GLYCOLIPID BIOSURFACTANT FROM AN ESTUARINE YEAST *Candida tropicalis*

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ABSTRACT

A potential biosurfactant producing estuarine yeast *Candida tropicalis* was isolated from the mangrove sediment of Vellar estuary, Porto Novo, Tamilnadu, India. The biosurfactant was produced using yeast malt broth and purified using acid precipitation followed by silica gel column chromatography. The purified biosurfactant was found to be a glycolipid surfactant characterized using TLC, FT-IR and NMR. Stability testing of biosurfactant revealed that it had broader range of stability to over pH 4-9, temperature up to 80ºC and salinity from 0 ppt to 50 ppt. The glycolipid biosurfactant showed excellent antimicrobial activity against many human bacterial and fungal pathogens viz., bacterial strains of *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Vibrio cholerae, Bacillus subtilis, Salmonella paratyphi* and *Staphylococcus aureus* and fungal strains of *Cryptococcus neoformans, Candida albicans* and *Aspergillus niger.* Thus in the present study, the glycolipid biosurfactant produced by *Candida tropicalis*, an estuarine yeast was found to be an ideal product for the possible development of antimicrobial therapeutic drugs.

INTRODUCTION

Biosurfactants are amphipathic molecules with both hydrophilic and hydrophobic moieties. It has different localizations viz., intracellular, cell surface bound and extracellular, and they play divergent physiological roles in the various producing microorganisms [1].

It comprises a wide range of chemical structures, such as glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids and neutral lipid. Usually, different microbial genera synthesize structurally different classes of biosurfactants whereas strains belonging to a same species produce structurally similar biosurfactants.

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However, small differences in the molecular structures can have major impact on the functions and potential industrial applications of microbial surfactants [2].

Almost all surfactants being currently produced are derived from petroleum. However these synthetic surfactants are usually toxic themselves and hardly degraded by microorganisms. They are therefore, a potential source of pollution and damage to the environment. These hazards associated with synthetic surfactants have in recent years; draw much attention to the microbial production of surfactants [3]. The well known biosurfactants includes surfactin produced by *Bacillus subtilis*, iturin produced by *B. subtilis* [4], mannosylerythritol lipids from *Candida antarctica* [5] and rhamnolipids from *Pseudomonas aeruginosa* [6]. Estuarine environments are among the most productive on earth, however there are only a few reports [7] of biosurfactant bearing antimicrobials of estuarine origin.

The yeast *Candida tropicalis* has been reported earlier for efficient production of biosurfactant from terrestrial habitats [8]. To our knowledge, *C. tropicalis* was unexplored earlier from estuarine habitats, so the present study with the following objectives (a) biochemical estimation of the purified biosurfactant (b) functional group analysis (c) Stability conditions and (d) antimicrobial potential of against different human pathogens

MATERIALS AND METHODS

Yeast strain

The biosurfactant producing yeast *Candida tropicalis* was isolated from the mangrove sediment sample of Vellar estuary, Porto Novo, Tamil Nadu, India. The yeast strain was identified based on 18s rRNA molecular identification using the universal set of the primers ITS1 (5´TCC GTA GGT GAA CCT TGC GG 3´) and ITS4 (5´TCC TCC GCT TAT TGA TAT GC 3´) were used. Sequence homologies were examined using BLAST version 2.2.12 of the National Center for Biotechnology Information [9] and it was submitted in the NCBS Gene Bank

Production and purification

Candida tropicalis was cultured in sea water prepared yeast malt broth for the biosurfactant production. After four days incubation, cultures were centrifuged at 3,000 rpm for 30min. and the cell free supernatant was subjected to acid precipitation using 6N HCl until pH 2 was attained [10]. The treated broth was kept at 4°C overnight for precipitation of biosurfactant. Yellowish white precipitate was collected by centrifugation at 10,000 rpm for 30 min.

The collected biosurfactant was then re-suspended in phosphate buffer at pH 7 and neutralized. The re-suspended residue was purified in a silica gel (60–120 mesh) column and the elutions were made with chloroform and methanol ranging from 20:1 to 2:1 v/v in a gradient manner and 10 fractions were obtained [11]. The fractions showing maximum emulsification index [12] were pooled and the solvents were evaporated, the resulting residue was dialyzed against distilled water and lyophilized.

Chemical characterization

Biochemical composition

The partially purified biosurfactant was biochemically determined using silica gel TLC plate which was separated using CH_3Cl: CH_3 OH: H_2O (65/15/2(v/v/v)) as developing system. Visualizing reagents used were ninhydrin reagent (0.2 g ninhydrin in 100 mL ethanol) to detect peptides, anthrone reagent (1 g anthrone in 5 mL sulfuric acid mixed with 95 mL ethanol) to examine sugars and lipid portion was evidenced using rhodamine B reagent (0.25g in 100 ml ethanol).

Functional group analysis of purified biosurfactant

FT-IR can be most probably used to elucidate components of an unknown mixture. One milligram of freeze-dried biosurfactant was grounded with 100 mg of KBr. Infrared absorption spectra were recorded on a Thermo Nicolet, AVATAR 330 FTIR system with a spectral resolution and wave number accuracy of 4 and 0.01 cm^{-1}, respectively and a KBr pellet was used as the background reference. ^1H NMR spectra of the purified biosurfactant were recorded on a Bruker AV600 NMR spectrometer using D_2O as the solvent. Chemical shifts were expressed in parts per million down field from an internal standard of tetra methylsilane (TMS).

Stability studies

The purified biosurfactant samples was taken at a concentration of 4mg/ml distilled water and heated in a boiling water bath at various temperatures for 30min. and cooled gradually to room temperature [10]. The pH stability was checked by adjusting the initial pH of the medium to different pH ranges [13] between 2 to 10. For studying the effect of salinity on biosurfactant, different concentrations ranged from 5 to 50ppt was prepared using synthetic sea salt (Hi-Media). After 24hrs of incubation the activity was checked using emulsification index.

Antimicrobial activity

The antimicrobial activity of purified biosurfactant was studied on Muller-Hinton agar (MHA) against a panel of different human pathogens using antimicrobial disk susceptibility tests. The human bacterial pathogens viz. *Escherichia coli, Salmonella typhi, S. paratyphi, Klebsiella pneumoniae, K. oxytoca, Vibrio parahemolyticus, V. cholera, Proteus mirabilis, Streptococcus pneumoniae, Bacillus subtilis, B. cereus* and *Staphylococcus aureus* and human fungal pathogens were *Aspergillus niger, A. flavus, Candida albicans, Cryptococcus neoformans* and *C. gattii* were gifted from Rajah Muthiah Medical College Hospital, Annamalai University, India. MHA plates were cultured with 100µl of individual pathogenic strains and the wells were impregnated with 50µl of purified different concentration (2µg- 128µg/ml) of biosurfactant dissolved in phosphate buffer (pH 7) to obtain the minimum inhibitory concentration (MIC). After 24hrs of incubation period at 37°C, the plates were examined for zone diameter of inhibition using an antibiotic zone scale.

RESULTS

Chemical characterization of the purified biosurfactant

The purified biosurfactant was eluted in between 4:1 to 2:1 fraction with 56% emulsification index (E_{24}) and it was qualitative assessed for its biochemical composition using thin Layer Chromatography which has showed the presence of two biochemical compound lipid and carbohydrate on TLC plates with Rf value 0.73 and

0.45.The purified biosurfactant was further analyzed for its functional groups through FT-IR and NMR spectrum (Fig. 1(A)). The characteristic aliphatic long hydrocarbon chain functional groups which was evinced from the vibrations of 1377 cm^{-1}, 1458cm^{-1}, 2854 cm^{-1}, 2870 cm^{-1}, 2924 cm^{-1} and 2953 cm^{-1}, intense stretch vibration band located at 3401 cm^{-1} predicted the hydroxyl groups in the chemical structures of the biosurfactant and 1628 cm^{-1}, 1652 cm^{-1} and 1733 cm^{-1} indicated the presence of alkene (C=C) and carbonyl group (C=O) and the 1105 cm^{-1} vibration revealed the presence of rhamnose sugar. From the above results, the presence of long chain aliphatic groups and rhamnose sugar represented the rhamnolipid structure. Stretch vibration located at 833 cm^{-1} was matched with α pyroryl structure of dirhamnolipid**.** The characteristic chemical shifts indicated that the sample had the molecular structure of aliphatic chain in the region of 1.934ppm, 1.315ppm, and 1.151ppm, an intense shift at 4.696ppm indicating the presence of OH bond and the fractions observed at 2.592ppm, 2.254ppm indicated the presence CH_2 C=O, CH_2 C=C functional groups at dirhamnolipid (Fig. 1 (B))**.** From the above results predicted using TLC, FTIR and NMR showed the presence of glycolipid chemical complex.

Stability studies

Emulsification activity was used as a criterion for determining its stability. The purified biosurfactant had an emulsification of 80% EA was used and the activity was compared in various pH, temperature and salinity. Biosurfactant showed good stability over a wide range of pH 4-9 with 72-80% EA. On temperature heat stability ranged up to 80ºC, beyond the temperature it was started to denature and showed no emulsification activity above 100 ºC. Effect of salinity did not harm the emulsification stability of the biosurfactant, it was checked between 0 to 50 ppt the emulsification activity found to be the same in every ranges. Based on the results we had checked on pH, temperature and salinity, results showed that this biosurfactant has the ability to withstand at different physical conditions.

Antimicrobial activity

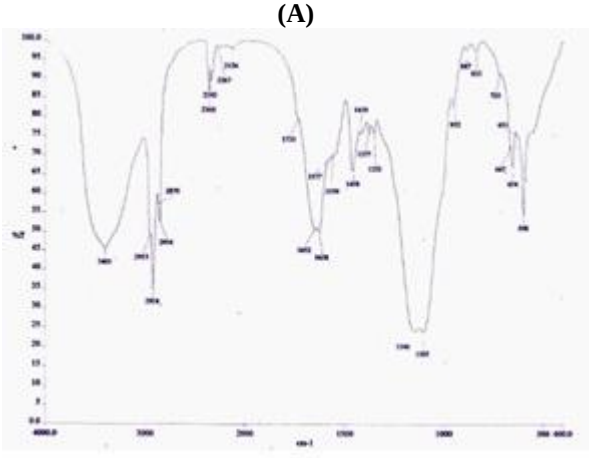
Out of the twelve tested bacterial human pathogens, the isolated biosurfactant showed antimicrobial activity against seven strains. The maximum zone of inhibitory activity was recorded with *Klebsiella pneumoniae* (23 mm) followed by *Escherichia coli* (20 mm), *Pseudomonas aeruginosa* (20mm), *Vibrio cholerae* (18mm) *Bacillus subtilis* (15mm), *Salmonella paratyphi* (13mm) and *Staphylococcus aureus* (11mm) (Table 1) (Fig. 5). The most valued minimum inhibitory concentration was observed with *Vibrio cholerae* (64µg/ml) followed by *Bacillus subtilis* (48 µg/ml), *Pseudomonas aeruginosa* (32µg/ml), *Salmonella paratyphi* (32µg/ml) *Escherichia coli* (12 µg/ml), *Staphylococcus aureus* (12µg/ml) *and Klebsiella pneumonia* (4µg/ml). Among the tested pathogens, the inhibitory activity was seen in both gram positive as well as negative strains with minimal MIC values which showed its antimicrobial potency. Regarding antifungal activity of the tested five different human pathogens, isolated biosurfactant showed activity against three strains with maximum activity on *Cryptococcus neoformans (22 mm)* followed by *Candida albicans* (21 mm) and *Aspergillus niger* (15 mm) with the MIC values of 32 µg/ml, 32 µg/ml and 16 µg/ml respectively. From these observations, the isolated biosurfactant showed its board range of potential antimicrobial activity with both bacterial and fungal pathogenic strains

Table 1. Antimicrobial activity of lipopeptide biosurfactant against different human pathogenic strains

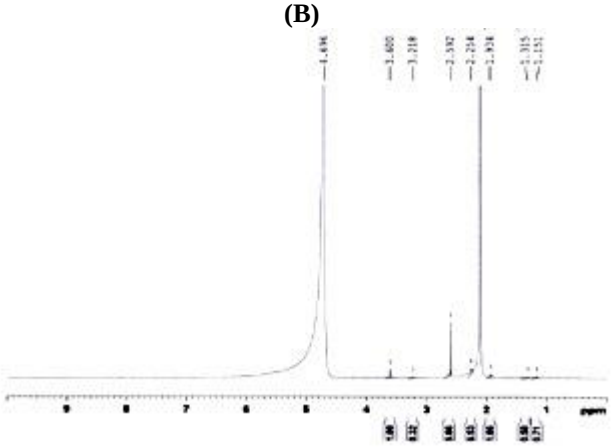
S.No.	Bacterial pathogens	Zone of inhibition	Minimum inhibitory concentration (MIC)
1	*Escherichia coli*	20 mm	12 µg/ml
2	*Salmonella typhi*	-	-
3	*S. paratyphi*	13 mm	32 µg/ml
4	*Klebsiella pneumoniae*	23 mm	4 µg/ml
5	*K. oxytoca*	-	-
6	*Vibrio cholerae*	18mm	64 µg/ml
7	*Vibrio parahemolyticus*	-	-
8	*Proteus mirabilis*	-	-
9	*Pseudomonas aeruginosa*	20mm	32 µg/ml
10	*B. cereus*	-	-
11	*Bacillus subtilis*	15mm	48 µg/ml
12	*Staphylococcus aureus*	11mm	12 µg/ml
Fungal Pathogens			
13	*Aspergillus niger*	15 mm	16 µg/ml
14	*A. flavus,*	-	-
15	*Candida albicans,*	21 mm	32 µg/ml
16	*Cryptococcus neoformans*	22 mm	32 µg/ml
17	*C. gattii*	-	-

Figure 1. FTIR and ^{1}H NMR spectrum of purified glycolipid biosurfactant from *C. tropicalis*

(A)



(B)



DISCUSSION

The biochemical composition of purified biosurfactant showed 26% of carbohydrate and 74% of fatty acid. However, [11] described the glycolipid biosurfactant produced by *B. megaterium* with carbohydrate and lipid combination of 28:70%. Further TLC results confirmed the glycolipid nature of the biosurfactant produced in the present investigation. The functional group of glycolipid was evaluated with the help of FT-IR spectroscopy. The presence of characteristic adsorption bands at 1377 cm^{-1}, 1458cm^{-1}, 2854 cm^{-1}, 2870 cm^{-1}, 2924 cm^{-1} and 2953 demonstrated the presence of aliphatic long hydrocarbon chain which was endorsed by [14]. Peaks observed in the region 1628 cm^{-1}, 1652 cm^{-1} and 1733 cm^{-1} indicated the presence of alkene (C=C) and carbonyl group (C=O) and the 1105 cm^{-1} vibration revealed the presence of rhamnose sugar [14] and stretch vibration located at 833 cm^{-1} was matched with α pyroryl structure of dirhamnolipid [15].

The characteristic chemical shifts present in the ^{1}H NMR spectra confirmed that the presence of long hydrocarbon chains indicated by the appearance of the characteristic chemical shifts at 1.315ppm, and 1.151ppm [15]. The presence of chemical shift at 4.696ppm depicted OH bond of dirhamnose sugar [16]. The present result evaluated the biosurfactant as a glycolipid in nature with the presence of dirhamnose sugar with long chain fatty acid. [17] described that rhamnolipids is one of the potential glycolipid biosurfactants employed in the industries and have been used in health care products in several different formulations, for example, insect repellents, antacids, acne pads, anti-dandruff products, contact lens solutions, deodorants, nail care products and toothpastes.The stability of purified biosurfactant was tested against varying pH temperature and salinity concentration. The emulsification activity of purified biosurfactant was 80%. It retained its activity almost in the same range between pH 4 to 9. Similar result was observed by [13] in the emulsifier obtained from the yeast *Y. lipolytica* NCIM 3589 cultivated in n-hexadecane. Likewise in the present study the biosurfactant showed stable emulsification in the temperature range of 20 to 80ºC. [1] reported that the biosurfactant produced by bacterial isolates were stable at a higher temperature range of 60–120ºC. Salinity variation up to 50ppt was checked which showed no variation on the emulsification activity and the emulsification was stable in the range of 0 to 50 ppt salinity concentration.

Research done by [18] on the emulsification of the strain *Rhodococcus erythropolis*, showed appreciable emulsification was produced at 2.5% of NaCl concentration. In the present study, the isolated biosurfactant was evaluated for antimicrobial activity against a panel of bacterial and fungal human clinical pathogens. The results showed its board range of activity against nearly 60% of the tested bacterial and fungal strains. According to [19], lipopeptides biosurfactants are most widely reported for antimicrobial action. Among the lipopeptides, surfactin, produced by *Bacillus subtilis* is the first and the most well-known member [4]. Other antimicrobial lipopeptides include fengycin, iturin, bacillomycins and mycosubtilins produced by *B. subtilis* [20]. Most of the biosurfactants having antimicrobial properties till date have been obtained from organisms isolated from terrestrial and marine sites.

CONCLUSION

The purified glycolipid biosurfactant from the estuarine yeast *Candida tropicalis* revealed significant commercial property of stable emulsification activity under different physic-chemical conditions. Furthermore, the biosurfactant showed promising antimicrobial activity against many human pathogens which was noteworthy for the development of antimicrobial drug in possible industrial and clinical applications.

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CONFLICT OF INTEREST:
The authors declare that they have no conflict of interest.

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