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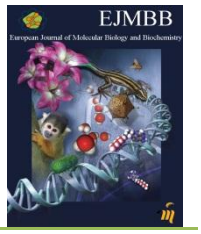

MOLECULAR CHARACTERIZATION, OPTIMIZATION AND EFFICIENT PRODUCTION OF A BIOSURFACTANT FROM AN ESTUARINE YEAST *CANDIDA TROPICALIS*

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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 using multiple screening methods which showed appreciable results of β hemolytic activity, 2.7cm in oil displacement test, 72±4.3% activity in cell surface hydrophobicity and 55±1% emulsification activity. The potential yeast was identified as *Candida tropicalis* based on 18S rRNA sequencing method and blast homology search. The potential strain showed its enhanced production at 42nd h with the production medium supplemented with 2% of glucose, 1.5% of ammonium nitrate as carbon and nitrogen sources, ionic supplements of 0.3% $MgSO_4$, 0.2% $FeSO_4$, 0.1% of K_2HPO_4, $ZnSO_4$ and $CaCO_3$ along with the cultural conditions of 7 pH, 35°C temperature and 10pp salinity. Produced biosurfactant from the optimized conditions was isolated with acid precipitation method and purified using silica gel column chromatography. The final dry weight of the purified biosurfactant was accounted as 4.85g/L. The above findings show the possibilities of this biosurfactant production for industrial scale fermentation.

INTRODUCTION

Biosurfactants are amphipathic molecules with both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases that have different degrees of polarity and hydrogen bonding, such as oil and water or air and water interfaces. Biosurfactant roles include increasing the surface area and bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis, quorum sensing and biofilm formation[1]. Beside their regular roles in enhanced oil recovery, bioremediation and industrial emulsification, in recent years, microbial surfactants have been found to possess several properties of therapeutic and biomedical importance, e.g. antibacterial, antifungal and antiviral properties.

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They also have antiadhesive action against several pathogenic micro-organisms [2]. Due to these properties, biosurfactants are widely used for industrial, agricultural, food, cosmetic and pharmaceutical applications [3].

Estuarine environments are among the most productive on earth, creating more organic matter each year than comparably-sized areas of forest, grassland, or agricultural land. Many different habitat types are found in and around estuaries, including shallow open waters, freshwater and salt marshes, swamps, sandy beaches, mud and sand flats, rocky shores, oyster reefs, mangrove forests, river deltas, tidal pools, and seagrasses; however there are only a few reports [4] of biosurfactant bearing antimicrobials of estuarine origin. Yeasts have a tradition in biotechnological applications, and *Saccharomyces* species are the most dominating representatives. Among the yeast species, *Candida* species has been isolated from different habitats, and in recent years, it has gained increased interest because of its diverse biotechnological

role. *Candida tropicalis* represented 4% of yeasts obtained from seawater, sea sediments, mud flats, marine fish intestine, mangrove plants and marine algae, as well as shrimp, indicating its wide distribution in tropical and subtropical marine environment [5].

The genus has been reported earlier for efficient production of biosurfactant by *C. bombicola, C. lipolytica, C. glabrata, C. antarctica, C. sphaerica,* and *C. tropicalis* from terrestrial origin. To our knowledge, *C. tropicalis* was unexplored earlier from estuarine habitats. So the present study, reported the production and purification of biosurfactant from a estuarine yeast *C. tropicalis*. The study dealt with the following objectives (a) Comprehensive isolation of estuarine yeast from mangrove sediment samples, (b) screening of potential biosurfactant producer using multiple screening method (c) identification of potential marine yeast using 18S rRNA partial sequencing, (d) media formulation for enhanced biosurfactant production (e) isolation and purification of biosurfactant.

MATERIALS AND METHODS

Sample Collection

Surface sediment samples were aseptically collected using Petersen grab sampler from mangrove sites of estuarine habitats (Lat. 11^{o} 24' N; long. 79^{o} 46' E), Vellar estuary, Tamil nadu, India. Weekly sampling was done during the month of February 2015 and was analyzed for the isolation of marine yeast. The collected samples were transferred to pre-sterilized bottle containers where precautionary measures were taken to minimize the contamination while handling the samples. The collected sediment samples were kept in an ice box maintained at 4˚C and samples were transferred and processed immediately in the lab.

Isolation of marine yeast

The marine yeast were isolated using Sea Water prepared Yeast Malt Agar (SWYMA) in which the central portion of the sediment samples were taken and serially diluted using pre-sterilized natural sea water and spread plated on the fresh SWYMA plates. Composition of Yeast Malt Agar includes Peptic digest of animal tissue 5g, Yeast extract 3g, Malt extract 3g, Dextrose 10g and Agar 20g. All ingredients were dissolved in IL pre-sterilized natural sea water and acidic pH was maintained (pH 6±0.2) using 0.1N HCL. After four days incubation period at 30ºC, individual colonies with different colony morphology were selected, pure cultured on fresh SWYMA plates and axenic cultures were lyophilized for further studies.

Screening of potential biosurfactant producing yeast

All the axenic cultures were individually broth cultured on sea water prepared yeast malt broth (SWYMB). After four days incubation, the strains were checked for potential biosurfactant producing yeast using multiple screening methods. They are hemolytic activity [6], Oil displacement test [2], BATH assay [7] and Emulsification Index (E_{24}) [8].

Molecular Identification

The most potential 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]. Multiple sequence alignments were carried out using ClastalW, and a consensus neighbor-joining tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software (version 4.0) [10].

Optimization of physic-chemical parameters for enhanced biosurfactant production

The optimization was done by adopting search technique i.e., varying parameters one at a time. The experiment were conducted in 250ml Erlenmeyer flask using the mineral salt medium, Bushnell Haas broth supplemented with 0.1% crude oil [9] at 30°C with 7 pH, as the basal media conditions and all the experiments were carried out in triplicate and the average values were calculated. The enhanced biosurfactant production was determined with emulsification activity of the cell free supernatant using emulsification index method [8]

Time course on production

Incubation was carried out for a time period ranging from 0hr to 120hrs with a 12hrs interval in which the peak production time was estimated.

Carbon and nitrogen sources

The effect of various carbon sources like glucose, fructose, maltose, sucrose and starch with different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5%) of substrates was tested. The influence of different nitrogen sources like peptone, yeast extract, beef extract, tryptone and ammonium nitrate were prepared in different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5%) was examined.

Ionic Supplements

The effect of various mineral ions such as magnesium sulphate ($MgSO_4$), dipotassium hydrogen phosphate (K_2HPO_4), zinc sulphate ($ZnSO_4$), calcium carbonate ($CaCO_3$) and ferrous sulphate ($FeSo_4$) prepared in different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5%) were tested for maximum production.

pH, temperature and salinity

For the evaluation of optimum pH range, the culture medium BH broth was prepared in pH range from 5 to 9 with an interval of pH 1. The effect of temperature on emulsification activity was tested over a range from 25˚C to 45˚C with an interval of 5˚C. Medium was prepared

with ranges of salinity from 0ppt to 35ppt with an interval of 5ppt and inoculated to test the effect of salinity since the strain is of marine origin**.**

Isolation and purification of biosurfactant

After optimal 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 [11]. 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 [12]). The fractions were pooled and the solvents were evaporated, the resulting residue was dialyzed against distilled water and lyophilized.

RESULTS

Screening of most potential biosurfactant producing estuarine yeast

Estuarine yeast strains were isolated from sediment samples of estuarine mangrove sediments collected from Vellar estuary. After incubation period, the SWYMA plates were inspected for different morphological colonies and totally 97 colonies were isolated during the four sampling period. The selected colonies were purified cultured on fresh SWYMA plates and the obtained pure cultures were kept in SWYMA slants for further work**.** For convenience, the strains were named by their station's first letters Vellar Estuarine Mangrove Sediment (VEMS) followed by the strains number specified by Arabic numerals (eg., VEMS1 – VEMS97). All the pure cultured strains were screened for multiple screening methods. Based on the results, there were only 7 yeast isolates were selected which have showed appreciable results in the multiple screening procedure. Strain no VEMS 27 showed the most appreciable results in all the tests with consistent values viz., β hemolysis, 2.7 cm zone formation in oil displacement test, 72±4.3 BATH and 55±1 emulsification activity.

Molecular identification of most potential estuarine yeast

Based on the obtained results, VEMS 27 was chosen for the detailed further study. The most potential strain was identified as *Candida tropicalis* with the help of 18S rRNA partial sequencing (Fig. 1) and it was submitted in the NCBS Gene Bank with the accession no and based on the sequence similarity significant alignment obtained from BLAST tool, PS7 strain showed the maximum similarity with *Candida tropicalis* KT449837.

Optimization of physico-chemical parameters for maximum biosurfactant production

Optimization of various physic-chemical parameters was carried out with the most potential yeast isolate using one factor at a time method. Among the various parameters, the optimal level for maximum biosurfacatant production was estimated and maintained it for consecutive determinations carried out.

Time course on biosurfactant production

The right time cultivation of the product plays an important role in maximizing product quantity. A sample of 3ml broth was taken from cultured media intermediately with 3h interval from 0h to 72h and analyzed for emulsification activity. The samples were aseptically taken, centrifuged and assayed immediately. The peak production was achieved after 42h (55.5% EA) of incubation up to 60h it was maintained (Fig. 2).

Influence of different carbon and nitrogen source on biosurfactant production

Different carbon sources with varied concentration were used in the determination of best carbon source with optimal concentration for the maximum biosurfactant production (Fig.3 (A)). There were monosaccharide, disaccharides and polysaccharides involved in our study, monosaccharides especially 2% of glucose having 55.3% EA showed maximum production. Various nitrogen sources were estimated with different concentration for determining the optimal level of nitrogen substrate for the maximum biosurfactant production. Higher level of nitrogen source influences the growth of the organism but not the production of the biosurfactant. Ammonium nitrate with the concentration of 1.5% showed higher yield of biosurfactant with 55.7%EA (Fig.3 (B)).

Effect of different Ionic supplements on biosurfactant production

Various ionic supplements were given at different concentrations and its effect on maximum biosurfactant production was studied. Among this, increased emulsification activity was recorded in the case of 0.3% $MgSO_4$ with 55.7%EA and 0.2% $FeSO_4$ with 56.8%EA. However the other optimized ionic supplement 0.1% of K_2HPO_4, $ZnSO_4$, $CaCO_3$ with 53.6%, 51.2% and 52.9% EA showed their presence might be important in the biosurfactant production (Fig.4 (A)).

Effect of different pH, temperature and salinity on biosurfactant production

One of the important characteristics of most organisms is their strong dependence on the pH for cell growth and production of metabolites. The strain showed the highest yield of biosurfactant production at pH 7.0 with 64.9% EA (Fig.4 (B)). Optimization for determining optimum temperature of bacterial culture for the maximum biosurfactant production also plays a key role. Every

bacterial culture has its own optimum temperature for maximum growth as well as for maximum production of product. The peak value was observed between 35 to 40˚C temperature with 64.9% EA (Fig. 5 (A)) and it was estimated as 35˚C for energy conception. The isolated bacterial strain is off from marine origin so optimization of salinity makes an important estimation about the affinity of the organism to the salt concentration. Strain showed maximum production of biosurfactant at 10ppt with 64.2% EA (Fig.5 (B)).

Isolation and purification of biosurfactant: After predicted the optimal conditions of the potential strain for maximum biosurfactant production, the strain was freshly cultured on optimal conditions and the biosurfactant was isolated by centrifugation and acid precipitation method. The precipitate was subjected for purification using silica gel chromatography using different solvent ratio of methanol and chloroform method. Ten fractions were taken and each fraction was studied using emulsification activity and found that biosurfactant was eluted between 4:1 to 2:1 fraction. The purification was checked and conformed by TLC method. The eluted biosurfactants were rotary evaporated and dried which showed dry weight concentration of 4.85g/L.

Figure 1. 18S rRNA Phylogenetic tree of *Candida tropicalis*

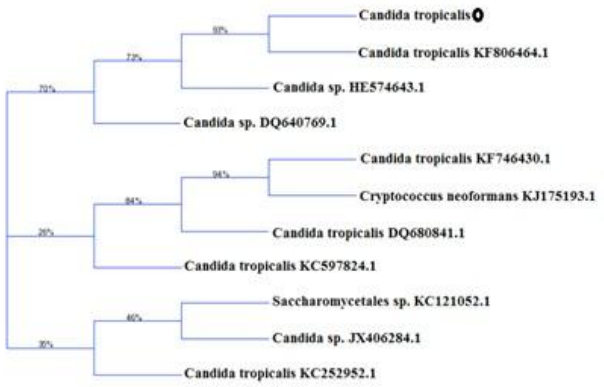


Figure 2. Optimization of incubation period for maximum biosurfactant production

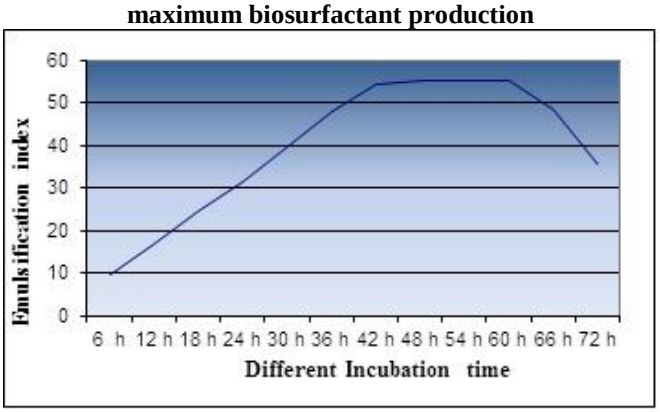
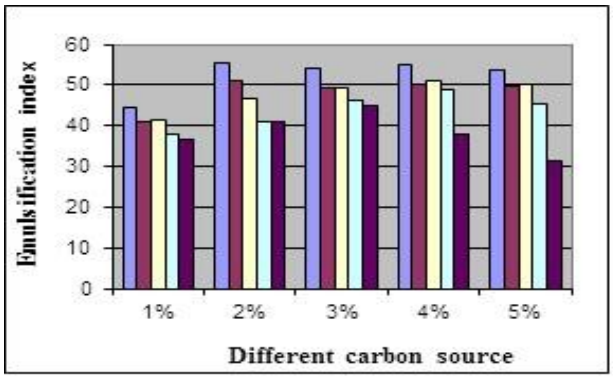


Figure 3. Optimization of different carbon and nitrogen source for maximum biosurfactant production

A



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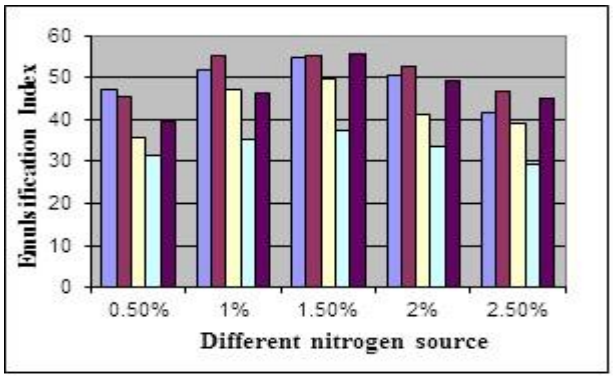
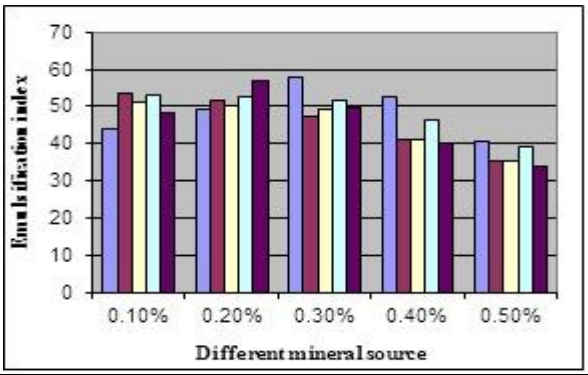


Figure 4. Optimization of different mineral source and pH parameter for maximum biosurfactant production

A



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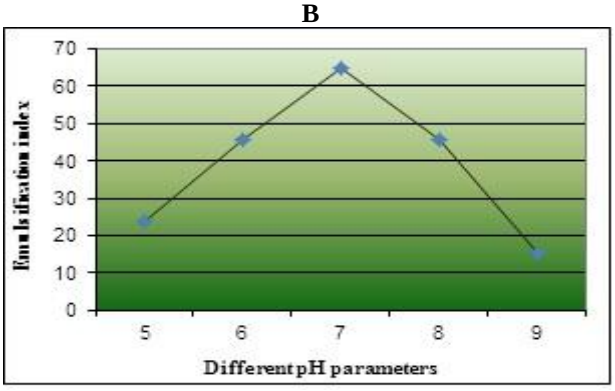
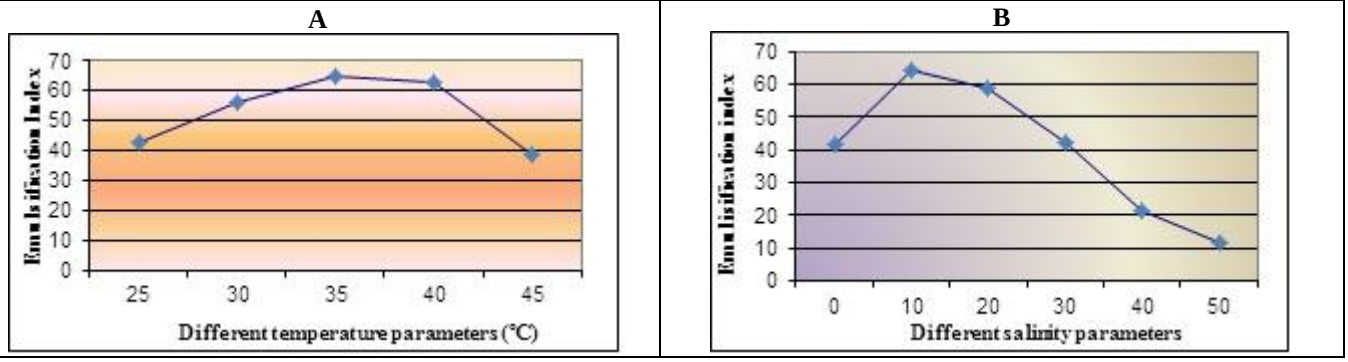


Figure 5. Optimization of different temperature and salinity parameters for maximum biosurfactant production



DISCUSSION

In the present study, estuarine mangrove sediment was studied for the isolation of biosurfactant producing yeast isolates. However, researchers concentrated on hydrocarbon contaminated terrestrial environments [13-14] for the isolation of biosurfactant producing strain and there were studies available from marine environments for the effective screening and isolation of potential biosurfactant producers [15]. But very few researchers concentrated on estuarine niche for the efficient biosurfactant producers, [4] estimated the total heterotrophic bacteria in the water sample from an estuarine ecosystem, Nigeria, Africa was 2.7×10^5 CFU/ml. To our knowledge, this is the first report for the screening and production of biosurfactant from estuarine yeast of mangrove sediment.

In this study, biosurfactant producers were first screened by hemolytic assay in the multiple screening. [6] recommended the blood agar method as a simple screening method which reveals the biosurfactant production based on surface activity measurements. Similarly, the oil displacement method developed by [16] 2000 is a simple, reliable, quick method and does not require any specialized equipment and strains that gave larger diameter of clearance also were reduces surface tension to a greater extent and does not relies on critical micelle concentration. Emulsification index is one among of the standard screening methods for assessing emulsification property of biosurfactants in which the emulsion is formed when one liquid phase is dispersed as microscopic droplets in another liquid continuous phase [17]. Cell surface hydrophobicity testing method has a direct correlation with biosurfactant production. Generally, microbes which can take hydrocarbon by direct uptake mode mediates high surface hydrophobicity and on the other hand, demonstrated that microbes show low surface hydrophobicity mediates extracellular biosurfactant [15]. *Candida tropicalis* was selected as the most potential biosurfactant producer using multiple screening methods. The study also proved that the sampling site provides good source for biosurfactant producing yeast apart from confirming the effectiveness of using multiple screening procedures.

One of the main factors for the bulk production of metabolites is incubation period. The study demonstrating that the accumulated biosurfactant was stable and growth associated, because a good correlation is observed between maximum yield of biosurfactants and bacterial growth phase which formed a consistent biosurfactant production between 42h – 60h during the stationary growth phase of the culture. A study has agreed our observation that no significant difference in the yield of biosurfactant is noticed between 48 h and 96 h of bacterial stationery growth phase, suggesting that lipopeptide biosurfactant produced by *B. subtilis* DM-03 and DM-04 strains are showing growth dependent production [18].

Numerous reports reveal that the type of carbon substrates markedly affected the production yield of rhamnolipid [19]. In this work, five carbon sources, including glucose, fructose, maltose, sucrose and starch were used. The results showed that isolated strain was able to grow in medium containing any of the five substrates used. The performance of glycolipid production with different carbon sources is depicted and reached a maximum value with 2% of glucose with 55.3% EA which is in good agreement with the results of Das and Mukherjee, 2007 using *B. subtilis.* Similar result was observed that glucose and glycerol were effective carbon substrates for rhamnolipid production with 136.4 and 71.8 mg/L/h through *P. aeruginosa* [20].

Limiting multivalent cation concentrations also causes over production of biosurfactant [21]demonstrated that limiting the concentrations of ionic supplements of magnesium, calcium, potassium and trace elements resulted in a better yield of rhamnolipid in *P. aeruginosa* DSM2659. In our study, supplementation of metal ions increased the biosurfactant production using 0.3% of $MgSO_4$ (57.7%EA) followed by 0.2% of $FeSO_4$ (56.8% EA). Similar observation depicted that Mg^{2+}, K^{+},Mn^{2+} and Fe^{2+} are all important factors affecting cell growth and surfactin production of *B. subtilis* ATCC 2133 [22].

To explore the influence of temperature on biosurfactant production strain was grown between the ranges 25–45˚C. As indicated, biosurfactant production increased with temperature from 30 to 40˚C, close to this, the optimal temperature for maximum rhamnolipid production by *P. aeruginosa* J4 strain begins from 30 to

37˚C (Wei *et al*, 2005). Similarly pH also exhibits a main role in the growth and formation of biosurfactant, results showed that it would be pH 7 with 64.9% EA the production of biosurfactant was high. From the results pH of the media highly influence the production of glycolipid biosurfactant similar result was observed by [23] 2008 in biosurfactant production was maximum at pH 7 by *B. subtilis.* Salinity is a major factor in which the isolated *C. tropicalis* is off from estuarine environment, so the need of optimizing salinity also plays a key role for better biosurfactant production. In this study, the potential isolate showed maximum biosurfactant production at 10ppt with 64.2% EA. However [24] 2009 observed that salinity with 30ppt showed maximum biosurfactant production in *Aspergillus ustus* MSF3. Biosurfactant purification from the crude compound is an essential step and it was done using silica gel chromatography using chloroform and methanol solvents. Chandran and Das, 2010 purified glycolipid biosurfactant from *Trichosporon asahii* and Chandran and Das, 2011 purified sophorolipid biosurfactant produced by *Rhodotorula mucilaginosa* and *Candida rugosa* using the same solvents as well as silica gel mesh (60-100) size.

CONCLUSION

The present investigation isolated a potential biosurfactant producing estuarine yeast using multiple screening methods and it was identified as *Candida tropicalis*. On optimization, the potential strain showed significant response to the culture medium for enhanced production and purified using simple chromatographic technique which showed its possibilities on industrial scale production and application studies.

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CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest.

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