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Screening and molecular identification of potential biosurfactant producing estuarine yeasts isolated from mangrove sediment samples of Vellalar estuary, Porto Novo, India

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Abstract

This study investigated the screening of potential biosurfactant producing estuarine yeast from mangrove sediment samples of Vellar estuary, Porto Novo, Tamil Nadu, India using a panel of biosurfactant screening methods *viz.*, hemolytic activity, oil displacement test, BATH assay and emulsification index. There were 97 morphologically distinct estuarine yeasts were isolated from the sampling source using 50% aged sea water prepared yeast malt medium and were screened for biosurfactant production. Among the isolates, only four strains showed potentiality in all the four screening methods. They were identified using 18S rRNA molecular sequencing method as *Saccharomyces cerevisiae* VEMS 13, *Candida tropicalis* VEMS 27, *Starmerella bombicola* VEMS 39 and *Yarrowia lipolytica* VEMS 51 which were submitted to NCBS gene bank with the accession numbers, MH822527, KT449837, MH820124 and MH822919, respectively. This study proved that the sampling site, estuarine mangrove sediments provides a good source for the isolation of potential biosurfactant producing yeasts using the adopted multiple screening methods for more extensive study on biosurfactants in future.

Keywords: Estuarine yeast; biosurfactant; emulsification; mangrove sediment

1. Introduction

Almost of the surfactants being currently produced are derived from petroleum derivatives. However these synthetic surfactants are toxic, hardly degraded by microorganisms and therefore, a potential source of pollution to the environment. These hazards associated with synthetic surfactants have in recent years; draw much attention to the microbial surfactants called biosurfactants^[1]. Biosurfactants are amphipathic molecules having both hydrophilic and hydrophobic moieties. Biosurfactant roles include enhance stable emulsification, reduce surface tension and enhance the bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis, quorum sensing and biofilm formation^[2]. Due to these properties, they are widely used for agricultural, food, cosmetic, pharmaceutical and bioremediation applications^[3].

Biosurfactants are also easy to produce from cheaper and renewable feedstock. The striking advantages of it over chemically synthesized surface active compounds includes their broad range of novel structural characteristics and physical properties and their capacity to be modified by genetic engineering, as well as by biotechnological or biochemical techniques^[4]. With the increasingly strict regulation on the use of environmentally compatible products, the use of biosurfactants in place of synthetic surfactants is increasing. Like synthetic surfactants, they exhibit high surface-active properties and their molecules can produce micelle or micelle-like aggregates and they are stable under extreme physicochemical conditions^[5]. Due to their good biocompatibility with the cell membrane, they are less toxic than synthetic counterparts and it is proved to be a good alternative^[6].

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. The tidal, sheltered waters of estuaries also support unique communities of flora and fauna especially adapted for life at the margin of the sea^[7]. 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, river deltas, tidal pools, and seagrasses; however there are only a few reports of biosurfactant of estuarine origin^[8].

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Moreover, mangrove forests are one among the best resources offering vast microbial diversity. On the worthwhile, they have not been investigated for biosurfactant producers.

Yeasts have a tradition in biotechnological applications and it has gained increased interest because of its diverse biotechnological role. It is found in many fermented food items and dairy products and has also been exploited for production of biochemicals and enzymes. Diversified yeasts were reported 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 environments [9]. Based on the importance and scarcity of research on yeast, this study undertook a study on isolation and screening of potential biosurfactant producing yeast from mangrove estuarine sediment sample procured from Vellar estuary, Porto Novo, Tamil Nadu, India and further, the strains were identified using standard molecular sequencing method.

2. Materials and Methods

2.1 Sample Collection

Surface sediment samples were aseptically collected from mangrove inhabiting site using Petersen grab sampler. Weekly sampling was done during the month of February 2015 and was analyzed for the isolation of estuarine yeast. The collected samples were transferred to pre-sterilized bottle containers and the 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 which were transferred and processed immediately in the lab.

2.2 Isolation of estuarine yeast

The estuarine yeasts were isolated using Yeast Malt (YM) agar (HIMEDIA, Catalogue No. M424) in which the central portion of the sediment samples were taken and serially diluted using pre-sterilized 50% aged sea water and spread plated on the fresh YM agar petri plates prepared using 50% aged sea water. The medium composition was dissolved in IL pre-sterilized 50% aged sea water and acidic pH was maintained ($pH\ 6\pm0.2$) using 0.1N HCL. After four days incubation period at 30°C, individual colonies with distinct colony morphology were selected, pure cultured on fresh YM agar plates and axenic cultures were lyophilized for further studies.

2.3 Screening of potential biosurfactant producing estuarine yeast

All the axenic cultures were individually broth cultured on yeast malt (YM) broth (HIMEDIA, Catalogue No. M425) prepared in 50% aged sea water. After four days incubation, the strains were checked for potential biosurfactant producing estuarine yeast strains using different screening methods

2.3.1 Hemolytic activity

All the isolated strains were tested for hemolytic assay using blood agar [10]. Composition of blood agar per liter includes 20g agar, 15g pancreatic digest of casein, 5g papaic digest of soybean meal, 5g NaCl and 50ml defibrinated sheep blood and the pH was maintained at $pH\ 7.6\pm0.2$. Fifty microliter of cell free supernatant was inoculated into the wells made for well assay method. After 2 days incubation at 37 °C, the plates were inspected for zone of clearance (hemolysis) around the wells. The diameter of the zone is a qualitative

method used as an indicator of biosurfactant production.

2.3.2 Oil displacement test

The positive strains showed complete or β hemolysis in the test were further checked for the oil displacement activity. It is a method used to determine the surface activity by measuring the diameter of the clear zone, which occurs after dropping a biosurfactant-containing supernatant on a thin layer of oil on water. The zone of diameter allows an evaluation of the surface tension reduction efficiency of a given biosurfactant [11]. The oil displacement test was done by adding 20ml of distilled water to a Petri dish with a diameter of 8 cm in which 15 μ l of crude oil was dropped to form a thin oil layer on the surface of the water, and then 10 μ l of a centrifuged supernatant was added onto the surface of the oil. The test was conducted at room temperature (25–27°C). The maximum diameter of the clear zone was observed under light and measured. The larger the diameter of the clear zone, the higher the biosurfactant production of the strain.

2.3.3 BATH assay

The positive strains from the above screening methods were checked for cell surface hydrophobicity test [12]. The cells obtained from fermented broth were centrifuged at 3,000 rpm for 20 min to separate the cells. Those cells washed twice and suspended in a buffer salt solution (g/l 16.9 K₂HPO₄, 7.3 KH₂PO₄) to give an OD at 600 nm of ~ 0.5. The cell suspension (2 ml) along with 100 μ l crude oil was vortex-shaken for 3min. After shaking, crude oil and aqueous phase were allowed to separate for 1 hr. The OD of the aqueous phase was then measured at 600 nm in a spectrophotometer. Adherence was expressed as the percentage of cell adherence to crude oil and was calculated as follows:

$$\% \text{ Adherence} = \left(1 - \frac{\text{OD}_{400} \text{ of aqueous phase}}{\text{OD}_{400} \text{ of initial cell suspension}} \right) \times 100$$

2.3.4 Emulsification Index (E₂₄)

From the above three screening tests, emulsification index was finally analyzed as one of the multiple screening method and was measured following the method of Cooper and Goldenberg [13] in which 2ml of the crude oil was added to equal volume of cell free supernatant and homogenized in a vortex for 2min. The emulsification activity was measured after 24 hrs, and the emulsification index was calculated as per the formula given below.

$$E_{24} (\%) = \frac{\text{Total height of the emulsified layer}}{\text{Total height of the liquid layer}} \times 100$$

2.4 Molecular Identification of the potential estuarine yeast

The potential biosurfactant producing strains 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. The evolutionary distances were computed using the maximum-composite-likelihood method [14] and the evolutionary analyses was conducted using MEGA7 software [15]. The tree topologies were evaluated by bootstrap analyses based on 1,000 replicates and phylogenetic trees were inferred using the neighbor-joining method.

3. Results

3.1 Isolation of estuarine yeasts from mangrove sediment samples

Estuarine yeast isolates were isolated from sediment samples of Mangrove, collected from Vellar estuary Porto Novo, Tamil Nadu, India. After incubation, the YM agar plates were inspected for distinct morphological colonies and totally 97 colonies were isolated during the four sampling period. The selected colonies were pure cultured using fresh YM agar plates and the obtained pure cultures were kept in 50% aged sea water prepared YM agar slants for further work. For retrieving convenience, the strains were initially named by their station's first letters of Velar Estuarine Mangrove Sediment (VEMS) followed by the strains number specified by Arabic numerals (eg, VEMS1 – VEMS97).

3.2 Screening of most potential biosurfactant producing estuarine yeast

All the collected and purified strains were screened for the hemolytic activity using well assay method, however, only 48

strains showed complete hemolysis which were taken to the next screening test, oil displacement test. During the analysis, there were only 27 strains showed positive response and were taken to the next tests cell surface hydrophobicity which has showed 15 positive strains followed by emulsification index revealed only 7 positive strains. Based on the results obtained from the above screening procedures, there were only 7 strains among the 97 yeast isolates selected which have showed positive results in all the screening procedures (Table 1). Among those strains, only four strains showed appreciable activity in the multiple screening methods, they were strain numbers, VEMS 13 (β hemolysis, 2.2 cm zone of clearance in oil displacement test, $61\pm2.3\%$ in BATH assay and $44.1\pm1.7\%$ emulsification activity), VEMS 27 (β hemolysis, 2.7 cm in oil displacement test, $72\pm4.3\%$ in BATH assay and $55\pm1\%$ emulsification activity), VEMS 39 (β hemolysis, 2.3 cm in oil displacement test, $59\pm2.5\%$ in BATH assay and $49.4\pm0.7\%$ emulsification activity) and VEMS 51 (β hemolysis, 1.7 cm in oil displacement test, $65\pm3.1\%$ in BATH assay and $51.7\pm1.1\%$ emulsification activity), respectively.

Table 1: Screening results of potential biosurfactant producing yeast

S. NO	Strain name	Hemolytic activity	Oil displacement test (cm)	Cell Surface hydrophobicity (%)	Emulsification Index (%)
1.	VEMS 7	β	1.8	63 ± 3.3	42.9 ± 1.2
2.	VEMS 13	β	2.2	61 ± 2.3	44.1 ± 1.7
3.	VEMS 27	β	2.7	72 ± 4.3	55 ± 1
4.	VEMS 39	β	2.3	59 ± 2.5	49.4 ± 0.7
5.	VEMS 51	β	1.7	65 ± 3.1	51.7 ± 1.1
6.	VEMS 71	β	1.3	47 ± 1.7	47.6 ± 1.5
7.	VEMS 78	β	1.9	49 ± 2.2	51.2 ± 1.1

3.3 Molecular identification of potential estuarine yeasts

Based on the obtained results for potential biosurfactant producers, there were four strains *viz*, VEMS 13, VEMS 27, VEMS 39 and VEMS 51 identified using molecular methods with the help of 18S rRNA partial sequencing. The phylogenetic position of these strains was determined by amplifying the 18S rRNA region and sequences were examined by BLAST analysis. Based on the BLASTN homology of the 18S rRNA gene sequence of the strains against the nucleotide sequence collection of the NCBI

Genbank sequence database, they were identified as *Saccharomyces cerevisiae*, *Candida tropicalis*, *Starmerella bombicola* and *Yarrowia lipolytica*, respectively. Further, these obtained sequences were submitted in the NCBS Gene Bank with the accession numbers, MH822527, KT449837, MH820124 and MH822919, respectively. Based on the evolutionary relationships, the phylogenetic tree for these strains was plotted with reference to closest NCBI (BLASTn) strains based on 18S rRNA gene sequence (Fig 1, 2, 3 and 4).

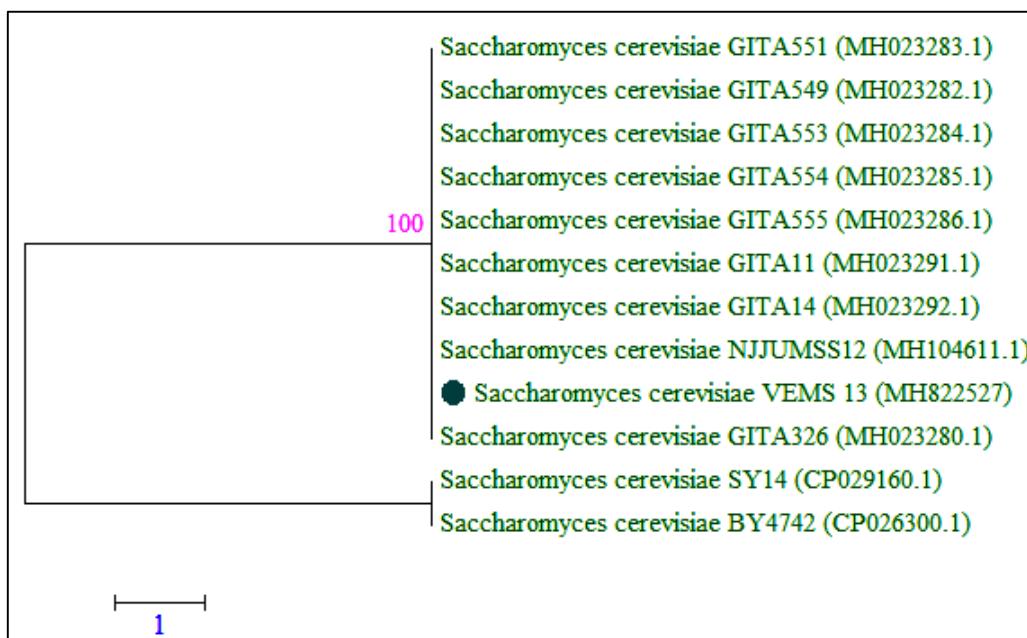
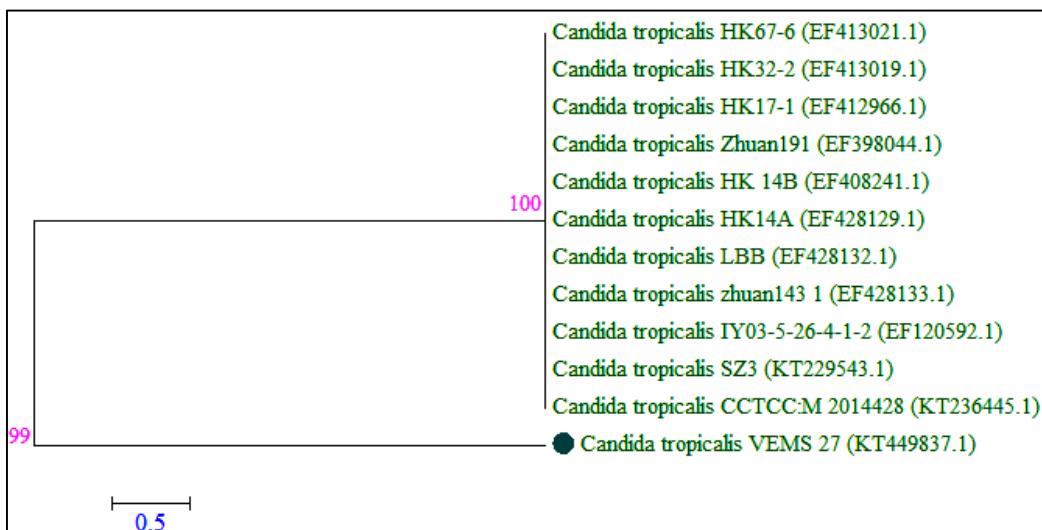
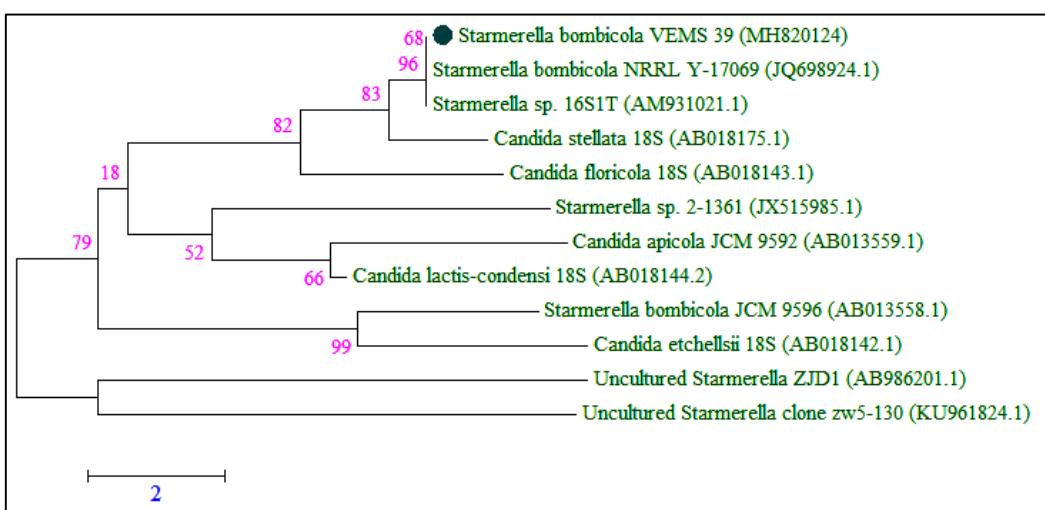
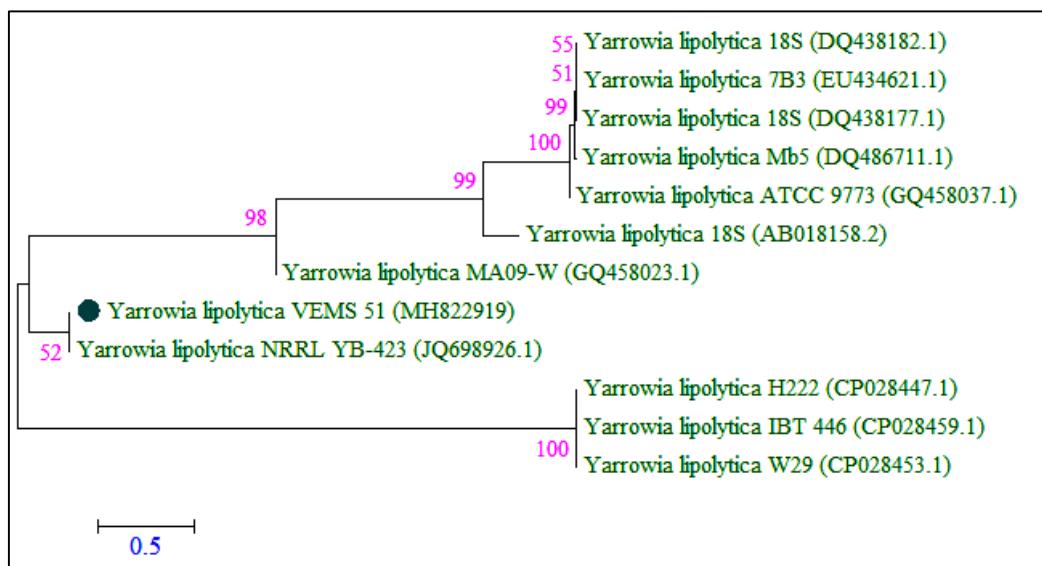


Fig 1: Phylogenetic tree of *Saccharomyces cerevisiae* VEMS 13

Fig 2: Phylogenetic tree of *Candida tropicalis* VEMS 27Fig 3: Phylogenetic tree of *Starmerella bombicola* VEMS 39Fig 4: Phylogenetic tree of *Yarrowia lipolytica* VEMS 51

Further, the evolutionary history was inferred using the Neighbor-Joining method and the evolutionary distances were computed using the Maximum Composite Likelihood method which were in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences and

all ambiguous positions were removed for each sequence pair and the evolutionary analyses were conducted in MEGA7.

4. Discussion

In the present study, estuarine mangrove sediment was

concentrated for the isolation of biosurfactant producing yeasts. However, many studies concentrated on hydrocarbon contaminated environments 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 [16, 17]. But very few researchers concentrated on estuarine niche for the efficient biosurfactant producers. Similar to this study, Adebusey *et al.* [8] estimated the total heterotrophic bacteria in the water sample from an plain 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. Biosurfactant can cause lysis of erythrocytes and the principle in this assay was developed by Mulligan *et al.* [10] He recommended the blood agar method as a simple screening method which should be supported by other techniques based on surface activity measurements. The blood agar method is often used for a preliminary screening of microorganisms by many researchers for the ability to produce bio surfactants on hydrophilic media [18].

The oil displacement method was used as one of the screening methods to detect the potential biosurfactant producer [19]. The method is a simple, reliable, quick method and does not require any specialized equipment. Strains that gave larger diameter of clearance also were reduces surface tension to a greater extent and does not relies on critical micelle concentration [20]. In the present study biosurfactant producing yeast were screened by emulsification index which is one of the standard screening methods for assessing biosurfactant production and was followed by many researchers [21, 22]. According to Desai and Banat [23] an emulsion is formed when one liquid phase is dispersed as microscopic droplets in another liquid continuous phase which is mediated only by amphiphatic molecules possess both hydrophobic and hydrophilic moieties, often called biosurfactant.

Cell surface hydrophobicity testing method used in this present study as one among the multiple screening methods which has a direct correlation with biosurfactant production. Depending upon the hydrocarbon uptake behavior, microorganisms may have high and/or low surface hydrophobicity. Generally, microbes which can take hydrocarbon by direct uptake mode mediated shows high surface hydrophobicity. On the other hand, Bouchez-Naitali *et al.* [24] demonstrated that microbes show low surface hydrophobicity when biosurfactant are released extracellularly. Mani *et al.* [25] and Balan *et al.* [26] described that the combination of different methods is appropriate for a successful screening of biosurfactant producing bacteria. In this investigation, four yeast strains were selected as the most potential biosurfactant producers using the multiple screening methods. Further, this study identified the four potential yeasts strains isolated using 18S rRNA molecular sequence method which is a standard identification method for the fungal cultures [27].

5. Conclusion

This study screened biosurfactant producers using a panel of different screening procedures exhibiting various biosurfactant properties. From the multiple screening methods, this investigation isolated four different estuarine yeasts viz, *Saccharomyces cerevisiae* VEMS 13, *Candida*

tropicalis VEMS 27, *Starmerella bombicola* VEMS 39 and *Yarrowia lipolytica* VEMS from mangrove sediment sample which are highly potential for biosurfactant production. Further, this study also highlighted that the estuarine mangrove sediment evidenced a potential source for biosurfactant producing yeast.

6. Acknowledgements

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