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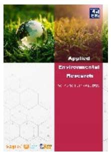

Research Article

Screening, Isolation and Hydrocarbon Degradation Characteristics of Yeasts from Mangrove Sediments of Kerala

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Abstract

The aim of the present work was to examine the hydrocarbon degradation potential of yeasts isolated from the mangrove sediments. Mangrove sediments around the world were considered to be heavily polluted with oil contaminants and the microbes dwelling in them were believed to be highly active in metabolizing and detoxifying the oil fractions. In the present study, 70 yeast isolates were screened for their lipolytic activity qualitatively and 35 of them which showed comparatively higher lipolysis were tested for their crude oil degradation ability. Screening of hydrocarbon utilizing isolates was performed on Bushnell Haas agar media overlaid with 1% crude oil and the appearance of colonies were considered a positive. Three isolates were selected based on this and were sequenced using ITS gene of the 18S region of the rDNA and identified to be *Candida tropicalis*. The extent of crude oil degradation was assessed by growing the isolates in basal medium supplemented with heavy crude oil as the carbon source for 21 days. The residual crude oil fractions were then analyzed using GC-MS to quantitatively measure the degradation potential of each isolate. Our results showed that the yeast strains under study could actively metabolize the crude oil including the higher chain fractions which make them efficient hydrocarbon degraders that can be used in the bioremediation processes.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are environmental contaminants present widely in air, water and soil. They are formed either naturally by incomplete combustion of organic matter or by human activities including petrochemical processing and oil refining [1–2]. Majority of PAHs are extremely toxic or exist as complex mixtures which causes mutations and cancers [3–4]. Exposure to PAHs possesses considerable risks to human health and aquatic life which leads to growing environmental concerns [5]. In the environment, they undergo abiotic transformations like oxidation, volatilization and biotic processes like microbial degradation and bioaccumulation [6]. Further, they get absorbed on to particulate matter due to their hydrophobicity and eventually sink in to coastal and marine sediments [7]. Biodegradation of PAHs has gained research attention lately and microbes have been identified as the most efficient degraders by virtue of their metabolic pathways [8–9]. Das and Chandran [10] reported that the biodegradation capacity of microbes ranged from 0.003% to 100% for marine bacteria, 6% to 82% for fungi and 13% to 50% for soil bacteria. In marine ecosystem, those microbes which can degrade hydrocarbons appear to be low in number and abundance. But the occurrence of hydrocarbon pollutants triggers their growth, changes their community structure and metabolic activities in the particular contaminated area [11]. Recognizing the microbes which are pivotal in the biodegradation of these pollutants and understanding their mode of action is a crucial requirement in developing various bioremediation strategies. Though bacteria have been studied as the best hydrocarbon degrading microbes

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[12–15], some yeasts were also found to have exceptional ability to break down such compounds [16–19]. They have high growth rate in contaminated soils and activation of their enzymes like lignin peroxidase can degrade complex total petroleum hydrocarbons (TPH), 5-ringed benzo(a)fluoranthene and benzo(a) pyrene and 6-ringed benzoperylene [20].

Mangrove sediments are bestowed with high organic carbon levels and debris composition which in turn cause for the deposition and accumulation of various contaminants particularly PAHs. Hence, these sediments would harbor potential microbes that have the capacity to metabolize and degrade hydrocarbon pollutants which can be further employed in bioremediation processes [21–22]. Though marine yeasts were studied to have enzymatic metabolic pathways that can use oil as source of carbon and energy to break down hydrocarbon compounds, only very few works have been conducted to explore their potential as hydrocarbon degraders [23–24]. Moreover, mangrove yeasts are least explored among the other marine yeasts on their hydrocarbon degrading properties [25–26]. There were no previous reports on similar aspect from the mangrove sediments of the sites selected for our current research. In this context, the objectives of our study was to screen, isolate, and identify the yeast strains with hydrocarbon degradation ability from the sediments of two selected mangroves along the coast of Kerala and to investigate their biodegradation potential.

Materials and methods

1) Collection of mangrove sediment

Sediment samples were collected from the mangroves Kannur (12.10°N 75.22°E; North Kerala) and Ernakulam (9.92°N 76.32°E; Central Kerala) along the Kerala Coast, India during the year 2021(Figure 1). Approximately 20 g of sub-surface sediment collected using a hand corer were transferred aseptically into sterile polythene bags, transported in ice boxes and processed within 4 hours of collection. Five sub-samples were collected from each location and then they were homogenized to form one composite sample per sampling site.

2) Screening and isolation of lipolytic yeast

About 5 g of sediment were inoculated into 100 mL enrichment broth (Mineral Basal Salt (MBS) Medium: 5% peptone, 0.3% yeast extract, 0.1% $MgSO_4$, 0.1% $NaNO_3$, 0.01% phenol red; pH 6) supplemented with 1%(v/v) olive oil as substrate. The cultures were grown for 72 h at 100 rpm, 28±2°C in a shaking incubator. The enriched cultures were then serially diluted and plated on to MBS media with agar containing 1% olive oil and incubated at 28±2°C for 48 h to screen for yeasts that can produce extracellular lipase. The colonies with clear/halo zones were selected, purified by quadrant streaking and rechecked for their lipolytic activity on same media (1% olive oil). The selected isolates were transferred to malt extract agar slants for further studies.

3) Screening for hydrocarbon degradation ability

The lipolytic yeasts were tested for their capacity to degrade hydrocarbons by growing them on Bushnell-Haas agar media containing 0.5% (v/v) crude oil [27]. The plates were incubated at 28±2°C for one week and appearance of colonies confirmed the utilization of crude oil as sole source of carbon which results in degradation.

4) Qualitative oil degradation efficacy of potential isolates

The potential isolates which were found to have hydrocarbon degradation capacity in the screening process were then checked for their ability to degrade crude oil by using suspended cells [15]. The isolates were inoculated into 100 mL MBS supplemented with heavy crude oil (1% v/v) in 250 mL Erlenmeyer flasks and the experiment was done in triplicates. A control flask with crude oil and without inoculum was also maintained. The flasks were incubated at 28±2°C in a rotary shaker at 100 rpm for three weeks and the degradation of the crude oil in the medium was assessed by visual observation.

(a) (b)

Figure 1 Sites of collection of mangrove sediments for the isolation of yeast. (a) Kannur (b) Eranakulam.

5) Quantitative oil degradation efficacy

5.1) Extraction of oil components for gas chromatography analysis

Inoculated and control flasks were harvested by centrifuging the culture broth at 10,000 rpm for 15 min. The supernatant was recovered after discarding the pellet and then extracted in a separating funnel using 1:1 n-hexane. The separating funnel with the mixture was shaken vigorously for 5 min and then kept stationary for 10 min for the separation. Top layer with oil residues were taken in a clean beaker and the moisture content was removed using anhydrous ammonium sulphate. The solvent hexane was evaporated in a fume hood and the residual oil were then shaken with 5 mL of n-hexane to precipitate the asphaltene fraction. These samples were then subjected to gas chromatography to estimate the degradation of hydrocarbon.

5.2) Gas Chromatographic analysis of residual hydrocarbon components

The quantitative analysis of crude oil in control and the residual oil after biodegradation were performed with the help of gas chromatography and mass spectrometry (GC-MS)system (Perkin Elmer GC 2400™, USA) operating in the split less mode. HP – 5MS (30 m×0.2 mm×0.25 µm) fuse – silica capillary column was used with Helium as the carrier gas. 1 µL samples (1:10 alkane fraction: n- hexane) were injected at an injection temperature of 151°C and the composition of various petroleum hydrocarbons were recorded as peaks. The column temperature was set for 1 min at 100°C hold and linearly increased to 160°C at the rate of 15°C min^{-1} and then to 300°C at 5°C min^{-1} and finally at 300°C hold for 7 min. The detector temperature was set at 550°C and degradation was assessed by comparing the peak area in the chromatogram of the samples with that of control.

6) Molecular identification of yeast isolates

The isolates with hydrocarbon degradation ability were identified by sequencing ITS region with forward ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse ITS4 (5'TCCTCCGCTTA TTGATATGC-3') primers [28]. The amplified fragment of approximately 580 bp, containing the ITS 1, 5.8 S and ITS 2 regions was used for the sequence similarity search using NCBI BLAST. The gene sequences thus obtained were then submitted to NCBI database to get the accession numbers.

Results

1) Isolation of lipolytic yeast

The enriched cultures from the sediments of Kannur and Ernakulam which were plated on to 1% olive oil containing agar plates showed 15×10^4 and 5×10^5 colony forming units per gram (CFU g^{-1}) of sediment, respectively. After screening for lipase activity based on the formation of halo zones, 25 isolates from Kannur mangrove sediments and 45 isolates from Ernakulam mangrove sediments were selected for further studies. These isolates were chosen on the basis of their colony morphology and the size of hydrolytic zones on agar plates. The lipolytic activities of all the 70 isolates were again confirmed on agar plates containing 1% olive oil substrate (Figure 2) and were named as VPY 1 – 70.

2) Screening for hydrocarbon degradation ability

Out of 70 yeast isolates, 32 isolates those showed comparatively higher lipolytic activity (larger halo zones) on plates were then screened for their hydrocarbon degradation property on Bushnell-Haas agar media (BHA) containing 0.5% (v/v) crude oil. 10 isolates were confirmed to have medium to very high crude oil degradation capacity based on the appearance of colonies on BHA plates (Figure 3). VPY 3, VPY 8 and VPY 12 were the isolates with highest crude oil degradation ability and were then considered for further studies.

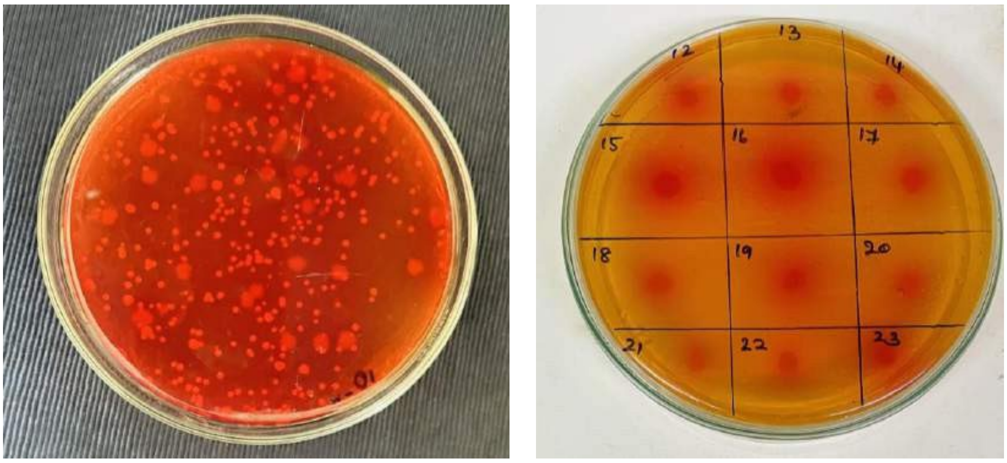

Figure 2 MBS plates with 1% olive oil as substrate (a) yeast colonies showing yellow zones indicating lipase activity and (b) purified yeast isolates showing zones confirming lipase activity.

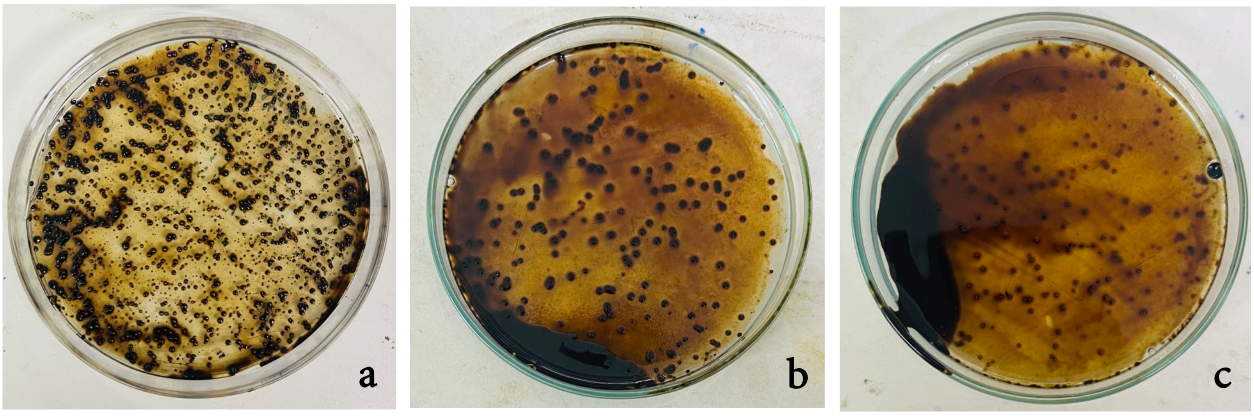

Figure 3 BHA agar plates showing growth of yeast colonies indicating crude oil degradation (a) VPY12 (b) VPY8 (c) VPY3.

3) Molecular identification of oil degrading yeasts

IsolatesVPY3, 8 and 12 were subjected to DNA isolation, PCR amplification and sequencing of the ITS region with the primers ITS1 and ITS 4. They were identified as *Candida tropicalis* yeast strains when compared with the GenBank database with 100% sequence homology. The sequences obtained were deposited in the GenBank and accession numbers were procured (Table 1).

4) Biodegradation studies

After 21 days of growth in mineral medium with crude oil, the biodegradation capacity of the 3 selected isolates were determined by the visual observation of the medium in the flasks (Figure 4). Flasks inoculated with isolates VPY3, and 12 were found to have tiny oil particles dispersed in the medium which resulted in the browning of the medium. The flask inoculated with isolate VPY 8 produced small oil droplets dispersed with slight browning of the medium while the control flask did not show any change in the medium (Table 2). The results indicated that all the 3 isolates are good oil degraders in which VPY 3 and 12 were with comparatively higher efficiency. The hydrocarbon degradation capacity of the yeast strains were then quantitatively assessed with the help of GC on extracted residual oil residues.

5) GC-MS analysis of the crude oil and residual oil

The GC results showed that the yeast strains in our study were efficient degraders of crude oil (Figure 5). There was reduction in the intensity of hydrocarbon peaks of the yeast degraded crude oil fractions in comparison with the control crude oil which was taken as 100%. The suspended cells of strain VPY 3 showed complete degradation of C15 fractions and almost total degradation of C17 and C18 fractions. VPY 12 showed complete degradation of C16fraction while they almost fully degraded C15 and C18 fractions, along with higher fractions from C25-C32. The cells of strain VPY8 also showed considerably complete degradation of hydrocarbon fractions including C12, C16 and C25-C28 fractions. The degradation of crude oil fractions C12 to C14 were meager and gradual degradation of C19-C24 was observed. The suspended cells of VPY12 showed the highest crude oil degradation potential followed by VPY3 and VPY8, respectively.

Figure 4 Crude oil degradation by yeast isolates in flasks (a) control flask showing no hydrocarbon degradation and (b) flask with *Candida tropicalis* (VPY12) showing efficient hydrocarbon degradation.

Table1 NCBI GenBank accession details of yeast strains with biodegradation capacity

Isolates	GenBank ID	Organism	GenBank accession
VPY 3	CUMBVP –KNR-03	*Candida tropicalis*	OP077321
VPY 8	CUMBVP –EKM-02	*Candida tropicalis*	OP108843
VPY 12	CUMBVP –KNR-12	*Candida tropicalis*	OP108842

Table 2 Visual observation of crude oil degradation by yeast in media

Isolates	Visual characteristics in medium	Degradation potential
VPY 3	Small oil particles dispersed in medium, browning of the medium	+++
VPY 8	Small oil droplets dispersed in the medium, slight browning of the medium	++
VPY 12	Small oil particles dispersed in medium, browning of the medium	+++

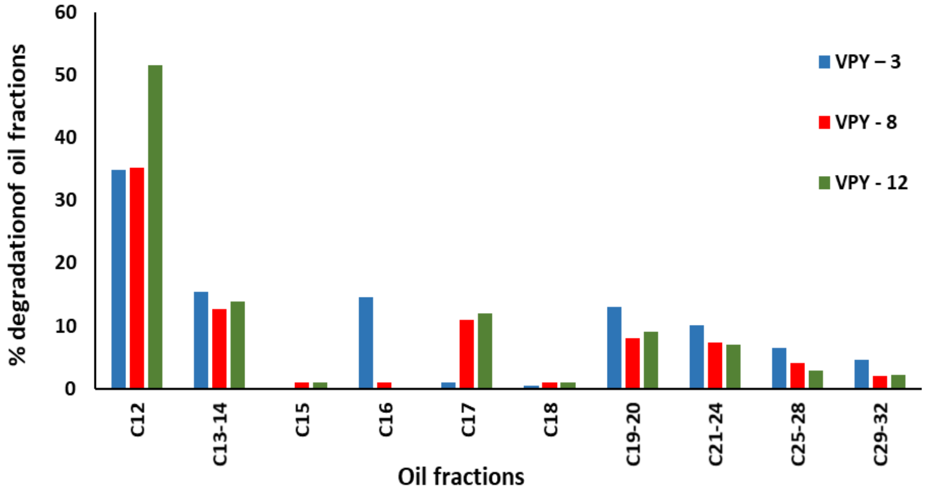

Figure 5 Graph showing percentage of crude oil degradation by selected isolates compared to control based on Gas Chromatographic analysis.

Discussion

The ability of microbes to degrade hydrocarbons was influenced by their previous exposure to the same. Those microbes which have pre exposure to hydrocarbon pollutants attain selective growth pattern and modify their genetic mechanisms to bring about additional coherent metabolism to accelerate the hydrocarbon degradation in the ecosystem they dwell [29]. Studies on mangrove ecosystems around the world have shown that mangrove sediments are widely contaminated with high to very high concentration of PAHs due to various anthropogenic effects including urbanization and deposition of industrial effluents [30–31]. Hence the microbial community in mangrove sediments has been acclimatized to degrade these contaminants with higher efficacy [32–33].

In this study three yeast strains isolated from mangrove sediments, namely VPY 3, 8 and 12 identified as *Candida tropicalis* were shown to have good lipolytic activity and hydrocarbon degradation capacity. A number of yeast strains were reported to have the ability to make use of hydrocarbons as their sole carbon source [34–35]. Strains like *Cryptococcus laurentii*, *Candida tropicalis*, *Rhodotorula mucilaginosa*, *Trichosporon asahii*, *Candida rugose*, *Yarrowia lipolytica* were studied to have exceptional biodegradation capacity. Also, some strains belonging to genera Candida, Clavispora, Pichia, Sporobolomyces, Sporidiobolus, Stephanoascus, Debaryomyces, Lodderromyces, Leucosporidium, Metschnikowia, Rhodotorula, Rhodosporidium, Trichosporon and Yarrowia were also reported to have the ability to utilize hydrocarbons and metabolize them to non-toxic forms [19, 29, 36]. This property of yeasts to metabolize hydrocarbons was found to be aided by the extracellular enzymes importantly lipase produced by them [37]. In our study also, those strains with higher lipolytic ability were found to be the ones with higher crude oil biodegradation capacity. The crude oil hydrolysis was detected qualitatively by the presence of turbidity in the medium compared to the control. There was removal of oil layer in the medium with the formation of oil droplets as a result of the breakdown of crude oil by yeast cells. *Candida tropicalis* was identified as the strain responsible for the hydrolysis of crude oil in mangrove sediments under study. Previous works on the same have stated that *Candida tropicalis* is one of the efficient hydrocarbon degrading yeasts and have been extensively used for the bioremediation of petroleum products [38, 10, 39].

Crude oil is a mixture of different hydrocarbon compounds with varying complexity. The biodegradability of hydrocarbons is different from each other such that aliphatic, straight chain aliphatic, saturated and long chain aliphatic hydrocarbons are easily degraded than aromatic, branched chain aliphatic, unsaturated and short chain hydrocarbons, respectively [25, 40]. It has been studied that yeasts can utilize most of the hydrocarbons especially alkanes with intermediate carbon chain lengths which are used as food source for their growth and multiplication [26, 41]. The GC-MS analysis of the residual hydrocarbon after their breakdown by yeast showed complete degradation of certain components while some where partially degraded. The strain

Candida tropicalis VPY12 was observed to be efficient in degrading crude oil fractions ranging from lower to higher fractions followed by strains VPY3 and VPY8 respectively. Considering the previous reports and the present study, it could be deduced that *Candida tropicalis* is a versatile candidate of hydrocarbon degradation in mangrove sediments. Since, the degradation of oil can be accelerated by mixing more than one strain, a consortium of efficient *C. tropicalis* strains can be developed for the bioremediation of hydrocarbon polluted environments.

Conclusion

Seventy yeast isolates from mangrove sediment samples were screened for their lipolytic activity and hydrocarbon degradation ability. Three isolates namely VPY3, VPY8 and VPY12 identified as Candida tropicalis were found to have the highest ability to degrade crude oil under the standard conditions. GC-MS analysis of the residual crude oil fractions after degradation by yeast showed that the isolate VPY12 (OP108842) was superior for oil degradation followed by isolates VPY3 (OP077321) and VPY8 (OP108843). It is to be noted that these strains could metabolize and degrade even higher chain alkanes which makes them efficient hydrocarbon degradators. Hence, our results suggest the use of *C. tropicalis* strains isolated from mangrove sediments for the development of a biodegradation consortium along with the optimization of parameters for the bioremediation of hydrocarbon contaminated environments.

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