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Isolation and characterization of *Candida vishwanathii* strain TERI MS1 for degradation of petroleum hydrocarbons in marine environment

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ABSTRACT

In an ongoing prospecting project for developing a bioremediation strategy for marine oil spills, a strain of yeast was isolated from petroleum crude oil contaminated sample. The strain, TERI MS1 was initially characterized biochemically, subsequently identified as *Candida vishwanathii* based on the sequence analysis of D1/D2 domain of 26S rRNA, and further confirmed by sequencing of internal transcribed spacer. The strain *C. vishwanathii* was able to utilize petroleum crude oil in natural seawater with 49 percent degradation in 72 h. The strain has high affinity toward degradation of polyaromatic hydrocarbons with degradation of 83% of pyrene (0.1% w/v) and 69% of naphthalene (0.1% w/v). With the specific growth rate of 1.25 h^{-1} and doubling time of 33.5 min, TERI MS1 showed potential for its use in implementing a bioaugmentation strategy for restoring oil spills in marine environment.

Keywords: Biodegradation; *Candida vishwanathii*; Petroleum crude oil; Polyaromatic hydrocarbon (PAHs); Seawater

1. Introduction

Despite the best efforts of petroleum industry, petroleum hydrocarbon contamination resulting from both upstream and downstream activities is one of the major environmental hazards. Accidental spills occur regularly during the exploration, production, refining, and transport of petroleum products. The Alaskan Spill in Nakhodka and the most recent BP oil spill spewed 4.1 million barrels of oil in the Gulf of Mexico making it the biggest oil spill in the history of the petroleum industry [1]. The environmental impacts of these incidents are well documented and there have been a continual pursuit among researchers for strategies to mitigate such hazards.

With the previous successes in restoring contaminated sites with crude oil in terrestrial environment, bioremediation can be considered as one of the promising methods for restoring ecosystems damaged due to crude oil spills in marine environment [2,3]. The large number of bacterial strains has been reported for the degradation of crude oil [4,5]. Yeasts cells are considered significantly robust and have higher biomass a property that is apt for an augmentation strategy; studies on yeast bioremediation are considerably limited.

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However, bioremediation is yet to be proven as an effective counter measure in an open sea environment. Tests done in laboratory as well as field trials have shown that bioremediation can enhance oil biodegradation on contaminated shorelines [3,6]. For effective bioremediation, it is essential for microbes to have direct contact with the hydrocarbon substrate and this would require considerable amount of biomass in the dynamic marine environment [3,7]. Thus, a selected microbe(s) with the desirable set properties for remediation of petroleum crude oil, through a bioaugmentation strategy, may prove effective to initiate and carry out the bioremediation process in a contaminated marine environment.

Yeast species such as *Candida lipolytica*, *Rhodotorula mucilaginosa* and *Geotrichum* sp. and *Trichosporon mucoides* isolated from contaminated water were reported to degrade petroleum compounds [8].

Emulsifiers, surfactants, and organic acids are relevant substances that the organism produces. These features promote yeast strain to interact with pollutants such as hydrocarbons (nitro, halogenated and organophosphates), metals, and different types of wastes. In addition, yeast cell displays tolerance toward physical parameters such as low temperatures, presence of salt, acidic and alkaline conditions that may be significant in different remedial applications. Marine yeasts have been reported to degrade 78% of aliphatic fraction of Bombay crude oil together with n-alkanes, isoprenoids, and aromatic compounds (naphthalene, phenanthrene and their derivatives) [9,10]. Hydrophobicity and emulsification ability are the two important factors of yeast cell in the treatment of oil contamination. *Candida* has been reported for both hydrophobicity and high emulsification ability promoting enhanced degradation of petroleum crude oil [9,10].

The present study describes the isolation, characterization, and assessment of the biodegradation potential of a yeast strain under marine conditions. Growth and degradation kinetics were also evaluated indicating the yeast strain as a potential candidate for an effective bioaugmentation strategy in restoring marine oil spill sites.

2. Materials and methods

2.1. Micro-organisms and culture condition

Microbes used in the present study were isolated by enrichment technique from seawater and soil samples contaminated with petroleum crude oil from Vishakhapatnam coast (17° 41′ 18′′ N, 83° 13′ 7′′ E) Andhra Pradesh and Mehsana (23.60°N, 72.40°E), Gujarat, India respectively [11]. Petroleum crude oil used in the present study was obtained from the oil fields of Mehsana, Gujarat. Degradation experiments were performed in Artificial Sea Water (ASW) media [12] with petroleum crude oil (1% w/v) as the sole source of carbon. Model aliphatic and aromatic compounds were procured from Sigma–Aldrich (USA) and were used to evaluate individual hydrocarbon degradation. Growth kinetics was done in 3 l bioreactor with 2 l fermentation media consisting of ASW and 0.5% (w/v) sucrose as carbon source. Natural seawater from R.K beach, Vishakhapatnam (17° 41′ 18′′ N, 83° 13′ 7′′ E), Bay of Bengal was used for mesocosm studies in lab.

2.2. Isolation of hydrocarbon degrading microbes

The contaminated samples were subjected to a modified enrichment protocol described previously for isolation of marine and terrestrial isolates [5,11]. Soil (1 g) or water (1 ml) samples were added to 250-ml Erlenmeyer flasks with 100 ml of artificial seawater media (ASW) described by Kester et al. [12], with total NaCl of (3%) and petroleum crude oil (1%) as sole source of carbon. It was incubated at 30°C on a rotary shaker (180 rpm) for 7 d. After five cycles of enrichment, 1 ml of the enriched culture was diluted 10^5 folds, and 100 µl was plated on ASW plates containing petroleum crude oil (1% w/v) [11]. The isolated strains were routinely subcultured and the stock cultures were stored in 25% glycerol at −70°C.

The efficiency of the isolated strains to utilize petroleum crude oil was checked in 250-ml Erlenmeyer flasks containing 100 ml of artificially designed seawater (ASW) media as described by Kester et al. [12]. Petroleum crude oil (1% w/v) was used as the sole carbon source inoculated with 4% (v/v) inoculum and incubated on a rotary shaker (180 rpm) at 30°C for 72 h. Inoculum was prepared by growing the selected isolates in LB broth to a cell density of 10^8 cfu ml^{-1}. Un-inoculated ASW media flasks with petroleum crude oil were used as a control. After 72 h of incubation, the residual petroleum crude oil was extracted and the remaining oil was compared with un-inoculated control by gravimetric estimation [5]. All the experiments were performed in duplicate and the data points are average of the duplicate ± standard deviation (less than 5% of average).

2.3. Identification and characterization of the selected isolate

Based on the initial morphological study, TERI MS1 was suspected to be yeast and was selected for

the current study. TERI MS1 was grown on GYP, Malt agar media to examine its morphological features. YM broth was used to test biochemical parameters. Phenotypic characterization was performed using standard method for yeast taxonomy that included tests for assimilation of different sugars, starch formation, and urea hydrolysis as described previously by Yarrow [13]. Surfactant activity measurements were done by tests such as drop collapse and emulsification index (E24) [14,15]. Surface tension measurement of culture broth supernatant was done according to method by Gudiña et al. [16], using a CSC Du Nouy Tensiometer (Cole Parmer India) equipped with platinum ring. An average of triplicates was determined to increase the accuracy of the measurements. All the measurements were performed at room temperature (25℃ ± 1℃).

For genomic characterization, total RNA free genomic DNA was isolated from overnight grown culture using yeast DNA extraction kit (Epicenter Technologies) as per the manufacturer's instruction. The D1/D2 region of 26S rRNA of large ribosomal subunit (LSU) was amplified with NL-1 forward primer and NL-4 reverse primer. Internal transcribed spacer (ITS) region containing ITS1–5.8S rRNA and ITS2 was amplified using ITS1 and ITS4 primers [17]. Purified amplicons of the genes were sequenced with ABI big dye terminator cycle sequencing ready reaction mix kit (Applied bio systems). Nucleotide sequence was compared by BLASTn program [18].

2.4. Hydrocarbon degradation

The ability of *C. vishwanathii* strain TERI MS1 to degrade selected aliphatic and aromatic hydrocarbon compounds as the sole carbon source was studied. The experiment was performed in 25 ml of natural seawater in 100 ml of flask for 72 h. Four aliphatic hydrocarbons (C17, C20, C25, C30) and three aromatic hydrocarbons (fluorene, naphthalene and pyrene) were selected and 0.1% (w/v) concentration of the individual compounds was used as carbon source. Un-inoculated flasks served as control for each set of compounds [11]. After 72 h of incubation, the culture flasks with aliphatic compounds were extracted with hexane while aromatic compounds were extracted by toluene with equal volume respectively [5,11]. Gas Chromatography was done to estimate the respective residual compounds as described in analytical section. Degradation analysis was on the basis of the retention time with the authentic standards as described in analytical section. All the experiments were performed in duplicate and the data points are average of the duplicate ± standard deviation (less than 5% of average).

2.5. Degradation kinetics

Mass culture of TERI MS1 was studied in 2 l ASW media in 3 l working volume bioreactor (BIOFLO 3000, New Brunswick Scientific Co. Inc. Edison, N.J. USA) containing 0.5% of sucrose with growth condition as follows: temperature 30℃, aeration 1.0 volume of air per volume of medium per min, agitation, 150 rpm. The microbial growth was determined spectrophotometrically by measuring the optical density (OD) at 600 nm. Enumeration of cells was done by plating on YPD agar media [13]. Specific growth rate and generation time were calculated from the graph. Degradation kinetics was also studied simultaneously in natural seawater with 1% (w/v) crude oil as sole carbon source. Culture of varying cell density was taken as inoculum and degradation of crude oil was studied (as described in analytical section) to determine the best time of harvesting for maximum degradation. All the experiments were performed in duplicate and the data points are average of the duplicate ± standard deviation (less than 5% of average).

3. Analytical methods

The TPH (extractable) was dissolved in 10-ml toluene and 1microlitre was injected in Gas chromatography (GC Hewlett Packard 6890) with Flame Ionization Detectors (FID) fitted with and DB 5 column (30 m × 0.25 mm × 0.25 μm). Similarly, for the model aliphatic and aromatic compounds one microliter of the individual aliphatic fractions previously dissolved in 10-ml hexane and 10-ml toluene respectively were analyzed. Oven temperature was programmed from 55℃ for 1 min to 290℃ @ 5℃ min^{-1} for 20 min with total run time of 70 min, injector port temperature 250℃ and detector temperature 300℃. Helium was used as a carrier gas with a flow rate of 2 ml min^{-1} with split ratio of 1:50. The individual model compounds present in the aliphatic and aromatic fractions were determined on the basis of the retention time with the authentic standards [5,11]. Microbial growth was measured by spectrophotometric analysis by standard method [19].

4. Results and discussion

Development of bioremediation strategies for cleanup of oil spills in marine environment is a challenge due to the dynamic conditions of the sea as well as the difficulty to contain the microbial strains that can carry out remediation within the expected zone of activity. Thus, a strategy focused on strains that are robust enough to sustain the roughness of the sea and

with higher surface area of contact with the substrate would be ideal. Thus, the objective of the present study was to isolate microbes capable of utilizing petroleum crude oil under marine conditions with the mentioned criterion.

The selective enrichment protocol was modified and designed for the isolation of strains suitable for use in subsequent marine environment. Initially, a set of 10 strains were isolated and screened which could utilize petroleum crude oil in the range of 12–41% (w/v) (Fig. 1). Although TERI MS2 and TERI MS3 showed potential for degrading crude oil initial morphology studies indicated TERI MS1 to be a yeast strain [11]. Yeast strain with unique physiological traits and enzymatic features has been associated with a vast variety of pollutants. The frequent incidence of yeast species in contaminated soils, seawater and wastes implies that it may be playing a major role in detoxifying hydrocarbons even under natural conditions. Hydrophobicity and emulsification ability are the two important factors of the yeast cell promoting enhanced degradation of petroleum hydrocarbons. There have been reports of yeasts, which have been isolated from hydrocarbon-contaminated sites and utilize crude oil as the sole source of carbon and energy [8–10]. Thus, strain TERI MS1 was taken up subsequently for this study. Detailed biochemical characterization was done and efficiency of the selected yeast was evaluated for bioaugmentation in marine environment.

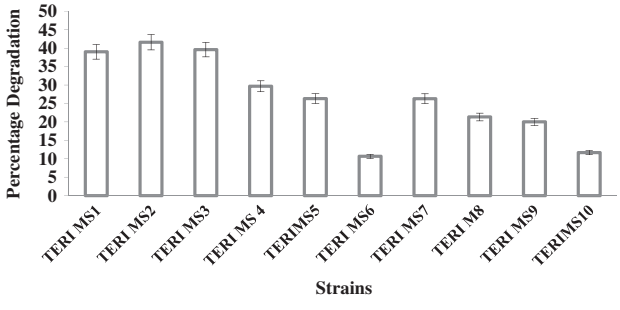

Fig. 1. Degradation of total petroleum crude oil by isolated strains TERI MS1 to TERI MS10. Values are mean of three samples. Values are the mean of three samples ± SD (Standard deviation less than 5% of average).

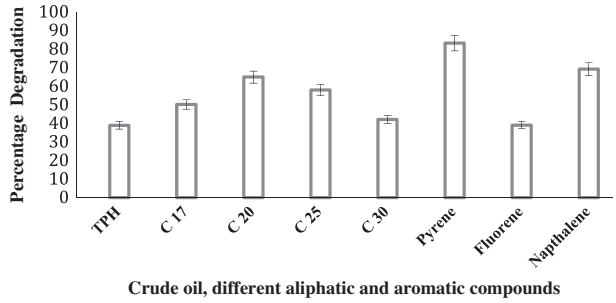

Fig. 2. Degradation of aliphatic and aromatic hydrocarbons by TERI MS1.Values are average of three samples ± SD (Standard deviation less than 5% of average).

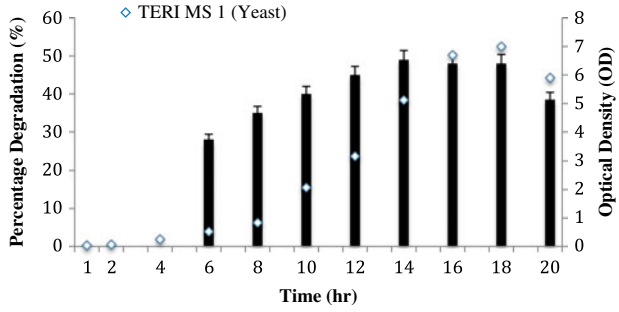

Fig. 3. Growth of *C. vishwanathii* TERI MS1 with time. Degradation of crude petroleum vs. growth (OD) of TERI MS1 at different time intervals. Values are mean of three samples ± SD. (Standard deviation less than 5% of average).

4.1. Characterization and Identification of the selected isolate

Morphologically TERI MS1 is cream color, non-mucoid discrete colonies on malt agar suspected to be a yeast strain belonging to genus *Candida*. Microscopic examination showed pseudo-hyphae forming yeast cells, which are characteristic for *Candida* sp. Budding was clearly observed when grown with petroleum crude oil as carbon source. Slight variation in the morphology was observed in the form of pseudo-hyphae in hydrocarbon containing media. Understanding of the morphological features of yeast in the presence of hydrocarbons is significant in applications of the yeast for remedial purposes. Hyphae formation is also observed in the presence of pollutants such as triglycerides containing saturated fatty acids. In the presence of other hydrophobic pollutants such as alkanes, the yeast morphology is predominant. There are reports of the formation of mycelia when inoculated into alkane-containing media, a speedy transition to the yeast form was observed [9,10]. This rapid change indicated the suitability of the yeast form in alkane degradation. Yeast strains tend to respond to the environmental condition by change in their morphology, which helps them in adapting to new physiological conditions [20]. Mycelial forms are important in scavenging oxygen and enabling the organism to tide over unfavorable conditions [9,10].

Identification of yeast is generally by its cellular morphology, fermentation, and assimilation of carbon and nitrogen source [17,21]. The strain TERI MS1 is physiologically similar to *Candida tropicalis*. It is similar to other yeasts like, *C. blankii* and *C. auringiensis*, in its inability to assimilate melibiose and inulin. It can grow at a wide range of temperature ranging from 25 to 42˚C. In the phenotypic characterization, TERI MS1 showed some differences with *C. tropicalis*, whereas it showed almost identical phenotypic profile with *C. vishwanathii* and *C. tropicalis* (Table 1).

The BLAST search of 26S RNA D1/D2 region shows 99% nucleotide sequence similarity with *Candida viswanathii* strain ATCC 38835 and *C. viswanathii* CBS 7923. Yeast species are generally characterized using 26S LSU [17]. According to the report and characterization done by the mentioned primer pair, the divergence between yeast species varies just in few base pairs. The secondary confirmation using nucleotide sequence of ITS1, 5.8S rRNA and ITS2 also proved that the strain is *Candida vishwanathii*. The nucleotide sequences of 26S RNA gene and ITS region of TERI MS1 were submitted to NCBI nucleotide database under the accession KC315783 and KC608220, respectively [11]. Maximum similarity of TERI MS1 was with the type strain ATCC38835. The type strain has not been reported for hydrocarbon degradation, however Hesham et al. [22] has reported *C. viswanathii* for its ability to utilize polyaromatic hydrocarbon in terrestrial environments.

4.2. Hydrocarbon utilization profile

The selected yeast was cultured with aliphatic hydrocarbons, namely C17, C20, C25, C30 and aromatic hydrocarbons such as pyrene, fluorene, and naphthalene as sole carbon source. Among aliphatic hydrocarbons, maximum degradation was observed in C20 followed by C25, C17, and C30 (65, 58, 50 and 42% w/v, respectively) (Fig. 2). Among aromatic hydrocarbons, maximum degradation was observed in pyrene followed by naphthalene and florene (83, 69 and 39% w/v, respectively) (Fig. 2). Large number of bacteria is capable of degrading aromatic hydrocarbons but there are few reports for the growth of yeast with pyrene and naphthalene as the sole source of carbon [21,23]. Among aromatic compounds, the degradation of pyrene was maximum which is considered to be high molecular weight poly aromatic hydrocarbon [23]. This supports the previous findings with *C. tropicalis* degrading both short and long chain hydrocarbons of the crude oil, which may be due to the presence of effective degrading enzyme system or the resistance of the species from various inhibitors [24].

Table 1
Different Phenotypic Traits for TERI MS1 (+ Positive, − Negative)

Traits	+/−	Traits	+/−	Traits	+/−
D-Glucose fermentation	+	α-Trehalose growth	+	DL-Lactate growth	
D-Galactose fermentation	−	Cellobiose growth	−	Succinate growth	−
Maltose fermentation	+	Salicin growth	−	Citrate growth	−
α-D-glucoside fermentation	−	Arbutin growth	−	Methanol growth	−
Sucrose fermentation	+	Melibiose growth	−	Ethanol growth	+
α-Trehalose fermentation	−	Lactose growth	−	Growth at 25˚C	+
Melibiose fermentation	−	Raffinose growth	−	Growth at 30˚C	+
Lactose fermentation	−	Melezitose growth	+	Growth at 37˚C	+
Cellobiose fermentation	−	Inulin growth	+	Growth at 42˚C	+
Melezitose fermentation	−	Starch growth	−	0.1% Cycloheximide growth	+
Raffinose fermentation	−	Glycerol growth	+	50% D-Glucose growth	+
Inulin fermentation	−	Erythritol growth	−	60% D-Glucose growth	−
Starch fermentation	+	Ribitol growth	+	Starch formation	−
D-Galactose growth	+	Xylitol growth	+	Urea hydrolysis	−
L-Sorbose growth	−	L-Arabinitol growth	−	Pink colonies	−
D-Glucosamine growth	+	D-Glucitol growth	+	Budding cells	+
D-Ribose growth	−	D-Mannitol growth	+	Splitting cells	−
D-Xylose growth	+	Galactitol growth	−	Filamentous	−
L-Arabinose growth	−	myo-Inositol growth	−	Pseudo hyphae	+
D-Arabinose growth	−	D-Glucono-1, 5-lactone growth	+	Septate hyphae	+
L-Rhamnose growth	−	2-Keto-D-gluconate growth	+	Arthroconidia	−
Sucrose growth	+	D-Gluconate growth	+	Ballistoconidia	−
Maltose growth	+	D-Glucuronate growth	−	Ascospores	−

Degradation of aliphatic and aromatic compounds within 72 h under marine environment by TERI MS1 *C. vishwanathii* is being reported for the first time.

The distributions of the strain *C. vishwanathii* have been reported in petroleum-contaminated sites, which confirms its ability in utilization of petroleum hydrocarbons [22,23]. The biodegradation of hydrocarbon is quite a complex metabolic process that involves several metabolic pathways taking place in different subcellular compartments. The capability of yeasts species to use n-alkanes as a sole source of carbon is mediated by the existence of multiple microsomal Cytochrome P450 family CYP52. These cytochrome P450 enzymes have been reported in various yeast species such as *Candida maltosa*, *C. tropicalis*, *Candida apicola*, and *Yarrowia lipolytica* [25]. Two classes of CYP450 are involved in this catabolic pathway. The first is the CYP450-ALKs, which are involved in the aliphatic hydrocarbons (n-alkanes). The metabolic functions of CYP52s in these yeasts have been illustrated to be involved in the terminal oxidation of long-chain n-alkanes to fatty alcohols, as the first step of the n-alkane degradation pathway. The second is non-characterized epoxide/hydroxy-forming CYP450s, which catalyse the oxygenation of poly aromatic hydrocarbons [25] Yeast cells with dimorphic behavior have an added advantage for application in hydrocarbon remediation. These contaminants are not miscible with water hence their uptake also requires morphological and physiological modifications, notably in cell adhesion properties (surface hydrophobicity) or in the production of emulsifiers (surfactants) [26]. The cell adhesion properties for the selected strain TERI MS1 have been described in Table 2. There was a reduction in surface tension from 70 to 40 dynes cm^{-1} with an increase in emulsification index in natural seawater condition (Table 2). Yeast strains have the ability to produce surfactants aiding in the degradation of hydrocarbons [23].

4.3. Degradation kinetics

For a successful bioaugmentation strategy, mass production of the selected strain is an important point for consideration. Successful bioaugmentation requires catabolically active inoculum together with microbes that can survive in target environment [27]. The minimum inoculum range of 10^7–10^9 cfu ml^{-1} has been documented for the utilization of petroleum crude oil for field applications [28]. Most of the studies done on yeast are focused on its fermentation products rather than on the production of its biomass [23]. Thus, in the present study the growth of TERI MS1 was studied to achieve actively growing culture, which can be effectively used for the degradation of petroleum crude oil [1,5].

Sucrose is a cheap substrate and can be readily used to achieve a good biomass in the bioreactor for industrial applications. It has been reported that cell density of 10^6–10^9 cells favored the production of biosurfactant enhancing petroleum crude oil degradation [28]. Varying concentration of sucrose was checked for initial studies (data not shown). Sucrose concentration of 0.5% (w/v) was used to study the growth, which gave the cell density of 10^9 cells (OD 7) in 16 h.

Culture of varying cell density of different time intervals was taken for degradation kinetics using crude oil as the sole source of carbon. TERI MS1 showing OD of 0.528 (10^5 cfu ml^{-1}) used 28% of crude oil after 6 h. TERI MS1 has long log phase of 10 h with range 10^6–10^9 cfu ml^{-1} showing degradation range of 40–49% in natural sea water (Fig. 3). Hydrophobicity and emulsification ability are the two unique factors of yeast cell in the treatment of petroleum crude oil contamination. Biosurfactant production and crude oil degradation is a growth related phenomenon as high emulsification ability of the surfactants makes easier for yeast cells to access hydrophobic contaminants [9,29].

Although there are reports of yeast strains for crude oil degradation and emulsification ability (Table 3), none of them has been taken up for successful field application in marine environment. TERI MS1 having generation time of 33 min and specific growth rate of 1.25 h^{-1} can be effectively mass cultured at industrial level for bioaugmentation and subsequent cleaning of oil spills in marine environment.

Table 2
Measurement of biosurfactant production (drop collapse), emulsification activity (%, E24) and the decrease in the surface tension (Dynes cm^{-1}) for the yeast strain. Respective control for ASW media (69 dynes cm^{-1}), YM (62 dynes cm^{-1}), Seawater (70 dynes cm^{-1}) was found. Experiment was done in triplicate and the data points are average of the triplicate ± standard deviation (less than 10% of the average value)

Strain	YM media	ASW media	Natural sea water
Drop collapse (Shape of drop compared to blank)	++++	+++	++++
E 24 (%)	45	40	47
Surface Tension (Dyne cm^{-1})	42	51	40

Table 3
Studies in literature on marine yeast for degradation of petroleum hydrocarbons (modified from Zinjarde et al. [10])

Strain	Source of isolation	Features	Refs.
NCIM 3589 (*Y. lipolytica*)	Dockyard sea water	Degraded n-alkanes (C10–C18) and aliphatic fraction of crude oil	[9,10]
PG-20 (*Y. lipolytica*)	Sediment/seawater	Degraded crude oil, n-alkanes (C9–C16), produced emulsifiers	[15]
AEH (*P. anomala*)		Degradation of Naphthalene, Phenanthrene, Chrysene and Benzo-pyrene	[22]
PFS-95 (*C. tropicalis*)	Ebubu oil polluted soil of Rivers state	Crude oil degradation	[24]
IMUFRJ 50682 (*Y. lipolytica*)	Estuarine water	Assimilated n-alkanes (C11–C19), isoprenoids, aromatics	[30]
TERI MS 1 (*C. vishwanathii*)	Sediment/Seawater	Degradation of crude oil, Pyrene, Naphthalene in natural sea water	Present Study

5. Conclusions

The primary aim of this study was to isolate a microbe and develop a strategy for mitigation of crude oil spills in marine environment. TERI MS1 has high potential to degrade pyrene (83%), naphthalene (69%) and also showed potential to utilize wide range of hydrocarbons degrading 49% of petroleum crude oil in natural seawater. The developed strain has high emulsification activity and unique phenotypic properties favoring degradation of PAHs in marine environment. Growth and degradation kinetics revealed that it has a long log phase of 10 h and doubling time of 33 min thus giving a good biomass and showing potential for cleaning of oil spills in marine environment.

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