Annexure

Minimal Salts Medium

Solution B1

- MgSO$_4$.7H$_2$O: 0.5 g
- KNO$_3$: 0.5 g
- NH$_4$Cl: 0.5 g
- Trace element solution: 10 ml
- Vitamin solution: 1 ml
- Distilled water: 900 ml
- pH: 7.2

Solution B2

- KH$_2$PO$_4$: 1 g
- K$_2$HPO$_4$: 1 g
- Distilled water: 100 ml

Solution B1 and B2 are autoclaved separately and then mixed aseptically after cooling.

Various steam sterilized hydrocarbons were added as the carbon sources to this medium.

Composition of trace element solution

- Nitrilotriacetic acid*: 1.5 g
- Mg$_3$SO$_4$.2H$_2$O: 3.0 g
- Mn SO$_4$.2H$_2$O: 0.5 g
- NaCl: 1.0 g
- FeSO$_4$.7H$_2$O: 0.1 g
- CoCl$_2$: 0.1 g
- CaCl$_2$.2H$_2$O: 0.1 g
- ZnSO$_4$: 0.01 g
- CuSO$_4$.5H$_2$O: 0.01 g
- Alk(SO$_4$)$_2$: 0.01 g
- H$_3$BO$_3$: 0.01 g
Annexure

Na$_2$MoO$_4$ 0.01 g
Distilled water 100 ml

* Dissolved in distilled water by adjusting the pH to 6.5 and then add the other salts.

Composition of vitamin solution

Biotin 2.0 mg
Folic acid 2.0 mg
VitaminB12 0.1 mg
Pyridoxine HCl 10.0 mg
Thiamine HCl 5.0 mg
Riboflavin 5.0 mg
Nicotinic acid 5.0 mg
Calcium Pentothionate 5.0 mg
p-aminobenzoic acid 5.0 mg
Lipoic acid 5.0 mg
Distilled water 100ml

Glucose Yeast Extract medium

Glucose 1 g
Yeast extract 1 g
Peptone 1 g
Distilled Water 100 ml
pH 3.0

The pH was adjusted with 11N sulfuric acid.

Agar plates

The agar medium plates for respective media were prepared with
Agar powder (HiMedia) 2 %

In the case of media with pH3, the media and agar solution were prepared separately and mixed after autoclaving.

Luria Bertani broth (LB)

Luria Bertani broth powder (HiMedia) 2 g
Distilled water 100 ml
pH 7.2
Annexure

**Luria Bertani Agar (LA)**

Luria Bertani agar powder (HiMedia) 3.5 g  
Distilled water 100 ml

To prepare LA with pH3, the pH of LB was adjusted to 3 using 11N sulphuric acid. Agar was autoclaved separately and added aseptically to the LB medium.

**Gram’s staining reagents**

**Grams iodine**

Gram’s iodine: 10 g  
Potassium iodide 20 g  
Distilled water 1000 ml

**Crystal violet**

Crystal violet 5 g  
Distilled water 1000 ml  
Filter the solution with Whatman filter paper no. 1 before use

**Saffranine solution**

Saffranine 0.5 g  
95% Alcohol 20 ml  
Make up the final volume to 200ml distilled water

**Glutaraldehyde**

Glutaraldehyde 2.5 ml  
Distilled water 100 ml

**Fatty Acid Methyl Ester Analysis reagents**

**Reagent 1:**

45 g NaOH, 150 ml methanol and 150 ml distilled water.
Dispensing is through the use of autopipette to assure reproducibility and to allow for a large number of assays in a day.

Reagent 2:
325 ml of certified 6.0 N HCl and 275 ml of methanol.
This drops the pH of the solution below 1.5 and causes methylation of the fatty acid.

Reagent 3:
200 ml hexane and 200 ml of MTBE.
This will extract the fatty acid methyl esters into the organic phase for use in the GC.

Reagent 4:
10.8 g of NaOH in 900 ml distilled water.
For reducing the contamination of injection port liner, column and detector.

**PCR reagents**

Taq polymerase buffer composition
10X buffer composition

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<th>Component</th>
<th>Concentration</th>
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</thead>
<tbody>
<tr>
<td>KCl</td>
<td>500 mM</td>
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<tr>
<td>Tris HCl (pH 8.3 at room temperature)</td>
<td>100 mM</td>
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<tr>
<td>MgCl₂</td>
<td>15 mM</td>
</tr>
<tr>
<td>Gelatin</td>
<td>0.01 % (w/v)</td>
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Tris Acetate EDTA solution
Stock: 50X

<table>
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<th>Amount</th>
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<tr>
<td>Tris chloride</td>
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<tr>
<td>Glacial acetic acid</td>
<td>57.1 ml</td>
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<tr>
<td>EDTA [0.5M] pH: 8.00</td>
<td>100 ml</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>1000 ml</td>
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</table>

Tris EDTA buffer

<table>
<thead>
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<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris chloride</td>
<td>10 mM</td>
</tr>
<tr>
<td>EDTA [pH 8]</td>
<td>1 mM</td>
</tr>
</tbody>
</table>
Annexure

*Ethidium Bromide*

- **Stock**: 10 mg/ml
- **Working concentration**: 0.6 μg/ml

*Gel loading dye*

- **Bromophenol blue**: 0.25 g
- **Xylene cyanol**: 0.25 g
- **Ficoll**: 15 g
- **Distilled water**: 100 ml
Patents and Publications

Patents

- Bioremediation of acidic sludge


Publications


Publications

**Oral/Poster Presentations**

- **Nitu Sood et al.** 2003. Bioremediation of acidic tar sludge at Digboi refinery. Poster presentation at **Petrotech 2003**.

- **Nitu Sood et al.** 2005. Selection of microbes for removal of paraffins from oil wells. Poster presentation at **Petrotech 2005**.


- Banwari Lal, **Nitu Sood et al.** 2009. Prevention of paraffin deposition in oil well tubing by using theromophilic paraffin degrading strain *Geobacillus kaustophilus*. Accepted at **Petrotech 2009**.

- **Nitu Sood.** 2004. Biodegradation of acidic oily sludge at Digboi refinery: A case study. Presentation at Institute of High Technology (IHT), Delhi in collaboration with IOCL, Faridabad, India.

- **Nitu Sood** and Banwari Lal. 2004. Biodegradation of acidic tar sludge at Digboi refinery. ISFL, Delhi, India.

Isolation of a novel yeast strain Candida digboiensis TERI ASN6 capable of degrading petroleum hydrocarbons in acidic conditions

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A B S T R A C T
A novel yeast species Candida digboiensis TERI ASN6 was isolated from soil samples contaminated with acidic oily sludge (pH 1–3) from the Digboi refinery (Northeast India). The strain TERI ASN6 could degrade 73% of the total petroleum hydrocarbons present in the medium at pH 3 in a week. This strain presents a dimorphic behaviour and showed mycelia morphology when grown under stressed conditions such as low pH and in a medium containing petroleum hydrocarbons. The C. digboiensis strain could efficiently degrade the aliphatic and aromatic fractions of the acidic oily sludge at pH 3 as confirmed by gas chromatography. During the growth of TERI ASN6 in dibenzothiophene (DBT), DBT-sulfone and biphenyl-2-ol were detected. An active cytochrome P450 system, implicated in hydrocarbon oxidation, was also detected in this yeast using degenerated primers based on its conserved regions. This yeast is a potential candidate for petroleum bioremediation treatment of hydrocarbon contaminated acidic soils. Its physiological behaviour allows the strain to work efficiently where other hydrocarbon-degrading bacteria may not survive.

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1. Introduction

India’s estimated oil reserves are 5 billion barrels with exploration still on. Through its 18 refineries, India processes approximately 128 million metric tonnes of crude oil per annum (TEDDY, 2006). These refineries generate enormous amounts of oily sludge every year as a result of crude oil processing (0.002–0.1% of the weight of crude processed). This oily sludge contains alkanes, aromatic hydrocarbons, NSO (nitrogen, sulphur and oxygen) compounds and asphaltenes (Bhattacharya et al., 2003). Safe disposal of this oily sludge is a major problem for refineries since it threatens the environment through soil and ground water pollution. Oily sludge constituents are also known to be potent carcinogens and immunotoxicants (Mishra et al., 2001). The Digboi refinery in Assam state of north-eastern India, however, is faced with a rather unique problem. It has nearly 50,000 tonnes of an acidic oily sludge (pH 1.5–3) lying inside the refinery premises. The acidity of this sludge is owing to sulphuric acid that was used in old wax refining methods in the refinery. While the use of sulphuric acid is now obsolete, the existing inventory is in itself a huge bioremediation challenge. When oily sludge disposal strategies like incineration, brick making etc. were met with limited success, microbial bioremediation was attempted as an environmentally benign and economically feasible alternative. As bioremediation candidates, both bacteria and fungi are relatively plentiful in soil and both the groups contribute substantially to the biodegradation of hydrocarbons (Bartha and Bossert, 1984). The low pH at Digboi, however, prevented the growth of known hydrocarbon-degrading bacteria. It was therefore required to isolate microorganisms from this acidic sludge contaminated environment that would be better adapted to degrade the contaminating hydrocarbons at low pH.

The present study details the isolation of a novel yeast strain from the Digboi refinery that is capable of utilizing hydrocarbons at pH 3. The ability of this strain to utilize alkanes and transform aromatic hydrocarbons to their oxidative forms has also been presented. The strain was identified as a novel yeast strain Candida digboiensis and has been described by Prasad et al. (2005). The effect of stressful growth conditions on the morphology of C. digboiensis TERI ASN6 and the possible advantage of the dimorphic behaviour to this yeast strain when applied to the bioremediation field is also discussed.

2. Methods

The oily sludge contained 45% aromatic fraction and around 25% each of alkanes and NSO (nitrogen, sulphur and oxygen) containing compounds and asphaltenes with a pH ranging from 1 to 3.
2.1. Enrichment and isolation of acidic oily sludge degrading microbes

The microbial strains were isolated by enrichment culture technique from soil samples contaminated with acidic oily sludge from Digboi refinery, Assam, north-eastern India. The composition of the acidic oily sludge was determined following the protocol described by Misra et al. (2001). For enrichment, 5 g of soil samples were inoculated into 100 mL of minimal salts medium (MSM) (0.1% K₂HPO₄, 0.1% KH₂PO₄, 0.05% MgSO₄.7H₂O, 0.05% NH₄Cl, 0.05% KNO₃ (w/v) and 0.1% (v/v) trace metals solution in MiliQ water), pH 3 (adjusted with H₂SO₄). The trace metals solution contained 0.15% nitrolitriacetic acid, 0.5% MnSO₄.2H₂O, 0.001% FeSO₄·7H₂O, 0.01% CaCl₂, 0.01% ZnSO₄, 0.001% CuSO₄.5H₂O, 0.01% CoCl₂, 0.001% Al(III)₂O₃, 0.001% H₂BO₃ and 0.001% Na₂MoO₄. The medium was supplemented with steam sterilized oily sludge (1% w/v) as carbon source and incubated at 30 °C on a rotary shaker (180 rpm) for 7 days. After ten cycles of enrichment, 1 mL of the culture was diluted 10⁷ fold, and 100 μL were plated on MSM agar plates with oily sludge (0.1% w/v) as the carbon source. The colonies obtained were further purified on the MSM (pH 3) agar plates with petroleum hydrocarbons of the oily sludge. The isolates were routinely subcultured in MSM containing the hydrocarbons of the oily sludge as the carbon source and stored in 25% glycerol at −70 °C.

2.2. Degradation of petroleum hydrocarbons under acidic conditions by the selected isolates

Degradation of the total petroleum hydrocarbons of the acidic oily sludge by the isolates was monitored in 250 mL Erlenmeyer flasks (in triplicate) containing 50 mL MSM (pH 3) with 0.1% (w/v) of the petroleum hydrocarbons of the oily sludge as the sole carbon source and flasks were incubated on a rotary shaker (180 rpm) at 30 °C for 7 d. The isolates were grown overnight in Luria Bertani broth (LB, HiMedia) (pH 3) to a cell density of 10⁷ CFU mL⁻¹ and 5% (v/v) inoculum was used to inoculate MSM with oily sludge as the sole carbon source. Uninoculated controls were maintained to monitor abiotic loss of the oily sludge. The residual degraded oily sludge hydrocarbons were extracted thrice with equal volumes of hexane and chloroform.

The profile of the hydrocarbon fraction, extracted from inoculated flasks, was compared with that obtained from uninoculated control flask. The isolate with the maximum degradation was selected for further studies.

2.3. Characterization of the efficient acidic petroleum hydrocarbon-degrading strain

2.3.1. Hydrocarbon utilization profile

The ability of the selected strain to utilize hydrocarbons as the sole carbon source was tested by growing it in minimal salts medium (MSM) with different hydrocarbons in separate flasks for 72 h. Alkanes with varying carbon chain lengths ranging from octane (C₈) to tricloanthac (C₃₀) and aromatic hydrocarbons (fluoranthene, pyrene, phenanthrene and dibenzothiophene) were tested. The medium was inoculated with 5 ml of 24 h-old culture of the selected isolate, grown in LB broth at pH 3 and incubated at 30 °C under shaking conditions. The culture growth was monitored by absorbance measured at 600 nm (UV–vis Hitachi spectrophotometer) and confirmed by measuring the total cell protein content. Protein was measured using Biuret’s method (Layne, 1957). The ability of the strain to grow in MSM with hydrocarbons as the sole carbon source was indicative of its ability to utilize the hydrocarbons.

2.3.2. Morphological characterization

The morphological peculiarities of the selected strain were studied by growing the strain in MSM containing petroleum hydrocarbons as the carbon source and media without hydrocarbons. Non-hydrocarbon media were GYP (1% (w/v) each of glucose, yeast extract and peptone) and Luria Bertani broth. Petroleum hydrocarbon containing MSM was prepared by adding 0.1% (w/v) of the following hydrocarbon substrates: the straight chain alkane, eicosane, the polycyclic aromatic hydrocarbon (PAH), fluoranthene, crude oil and the hydrocarbons of the acidic oily sludge as the sole carbon source.

Solid medium was prepared by adding agar (HiMedia) separately to the broth after autoclaving. All the solid and liquid media were prepared at pH 3 and 7.

The morphology of the colonies on the solid media was monitored at 4× magnification (Olympus microscope BH2, USA) and the cells were observed at 40× magnification.

The selected strain was cultivated in LB and GYP broth. The overnight grown culture was centrifuged at 5000 rpm for 1 min. The cells were then resuspended in phosphate buffer and centrifuged again. After three such washes, the cells were fixed overnight in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The cells were again washed with the buffer following which sputter coating (SCD 020) of the sample was done. Scanning electron microscopy (SEM) was performed using the LEO 435 VP (30 kV voltage) electron microscope at All India Institute of Medical Sciences (AIIMS), New Delhi, India.

2.4. Degradation of the alkane and aromatic fractions of the acidic oily sludge

In order to determine the degradation of the alkane and aromatic fractions of the oily sludge by the selected isolate, the total petroleum hydrocarbon (TPH) was obtained following the method described by Misra et al. (2001).

The alkane and aromatic fractions obtained using this method were then used as the carbon source (0.1% w/v) in MSM at pH 3 to determine the extent of degradation by the selected isolate. To study the degradation, flasks were inoculated with 5% (v/v) of a 24 h-old-culture of the selected isolate (10⁷ CFU/ml) in LB (pH 3) and incubated at 30 °C under shaking conditions (180 rpm). Uninoculated control flasks were maintained to monitor abiotic losses. After 7 days of incubation, the degraded alkane hydrocarbon residue was extracted thrice with equal volumes of hexane and the aromatic residue with toluene. The degradation was quantified gravimetrically after the evaporation of solvents. One microlitre of the alkane fraction (dissolved in 10 mL hexane) was analyzed by gas chromatographic analysis (GC Hewlett Packard 5890 series II) fitted with Flame Ionization Detectors (FID) and DB 2887 methyl silicon 10 m long column (0.53 mm × 3 μm film thickness). Similarly, the aromatic fraction was dissolved in 5 mL acetone and 1 μl was analyzed by GC–FID using a 30 m long DB 5625 (0.25 mm × 0.25 μm film thickness) column. During analysis the injector and detector of GC were maintained at 300 °C and the oven temperature was programmed to rise from 80 °C to 240 °C with an increase of 5 °C per minute and then held at 240 °C for 30 min. Individual compounds present in the alkane and aromatic fractions were determined by matching the retention times with authentic standards (The n-alkanes: C₈, C₁₀, C₁₂, C₁₄, C₁₆, C₁₈, C₂₀, C₂₂, C₂₄, C₂₆, and C₂₈ and the aromatic hydrocarbon standards fluoranthene, pyrene, phenanthrene, dibenzothiophene were obtained from Sigma–Aldrich, USA).
3. Results and discussion

3.1. Isolation and screening of microorganisms capable of degrading the hydrocarbons of the acidic oily sludge

The enrichment procedure for obtaining acidic oily sludge hydrocarbon-degrading microbes was performed in minimal salts medium in multiple cycles to ensure that the microbes that were obtained at the end of the enrichment cycle were capable of utilizing the acidic oily sludge rather than just tolerating it. The enrichment protocol from acidic oily sludge contaminated soil yielded eight isolates. These isolates showed a varying degradation profile for the total petroleum hydrocarbon (TPH) of the oily sludge. It varied from 27.7% to 77% of the 100 mg TPH used as the sole carbon source in a week. The isolate with the best degradation potential was selected for further studies.

3.2. Characterization of the selected strain

3.2.1. Hydrocarbon utilization profile

The selected isolate studied in this work had previously been identified by Prasad et al. (2005) as C. digboiensis TERI ASN6. The isolate TERI ASN6 was capable of growing on the medium containing petroleum hydrocarbons, from octane (C8) to triacontane (C30), as the sole carbon source. This includes the straight chain petroleum hydrocarbons found in the oily sludge at Digboi refinery. However, maximum growth was observed on octadecane (C18) and heptadecane (C17). The hydrocarbon utilization pattern of TERI ASN6 indicated that the growth was less on short chain alkanes like octane, decane, undecane, dodecane and tridecane but it started increasing from tetradecane with a maximum growth being observed on heptadecane, octadecane and nonadecane after which the growth was again comparatively reduced with tricosane, tetracosane, octacosane and triacontane (Fig. 1). The hydrocarbon utilization pattern by TERI ASN6 was the same as determined by total cell protein and absorbance. TERI ASN6 is capable of utilizing a wide range of hydrocarbons under acidic conditions, with a preference for alkanes with intermediate carbon chain lengths. The decreased growth pattern in short chain could be attributed to the toxicity of these alkanes to cell membranes (Teh, 1975) while growth on long chain alkanes could be limited by decreased bioavailability of the substrate (Lal and Khanna, 1996). While TERI ASN6 was capable of efficient utilization of alkanes as the carbon source, it was unable to completely degrade aromatic hydrocarbons.
as the sole carbon source. However, oxidation of the aromatic hydrocarbons was observed.

3.2.2. Morphological characterization

The composition of the medium directly influenced the growth and the phenotype of TERI ASN6. It was observed that TERI ASN6 exhibited yeast morphology in GYP medium at pH 7 (Fig. 2a, e). However, it produced pseudohyphae when grown on the same medium at pH 3 (Fig. 2f). When grown on LB agar pH 3, the cells of TERI ASN6 showed pseudohyphal morphology (Fig. 2h, d) while on LA pH 7, the cells were initially in the yeast form (Fig. 2g) but after 24 h of growth the cells switched to the pseudohyphal form.
When grown on MSM with hydrocarbons as the carbon source, the cells of TERI ASN6 showed only mycelial morphology. The cells were not observed in the yeast form in this medium with any hydrocarbon source. The growth of TERI ASN6 on MSM containing eicosane, fluoranthene or crude oil as the carbon source showed a variation in the morphological pattern. The pattern of mycelial emergence from the edges of the colonies was uniform when they grew on crude oil (Fig. 3c) unlike the mycelial pattern of TERI ASN6 on eicosane (Fig. 3a) and fluoranthene (Fig. 3b) carbon sources. The growth of this yeast strain on acidic oily sludge however, showed distorted colony structure (Fig. 3d). The morphological behaviour of TERI ASN6 remained unchanged at incubation temperatures of 30°C and 37°C if the same medium was used.

Fungi, like all living organisms, must be able to respond to changes in environmental conditions and hence develop a response which enables their adaptation to the new physiological situation (Alonso-Monge et al., 1999). In C. digboiensis TERI ASN6, like many other yeasts, this response comprises formation of filaments. In this study, C. digboiensis TERI ASN6 was challenged with conditions similar to those found in open-field such as hydrocarbon substrates, pH and temperature fluctuations. The isolate TERI ASN6 always produced pseudohyphae or hyphae in addition to the yeast form at low pH irrespective of medium components or growth temperatures. This is in contrast to the dimorphic behaviour exhibited by Candida albicans and Mucor rouxii which occur as yeast cells in acidic conditions and as filaments near neutrality (Bartnicki-Garcia, 1962). The hydrocarbon utilizing yeast Yarrowia lipolytica also shows maximum mycelial formation at pH 7 and almost no mycelia at pH 3 (Ruiz-Herrera and Sentandreu, 2002). The haploid cells of the plant pathogen Ustilago maydis, on the other hand, show filamentation in acidic conditions like C. digboiensis TERI ASN6 (Sánchez-Martínez and Pérez-Martín, 2001).

The dimorphic behaviour of the TERI ASN6, like Y. lipolytica (Perez-Campo and Dominguez, 2001), does not seem to be affected by temperature variation. For environmental applications like bioremediation, the tolerance of slight temperature fluctuations augurs well for the performance of the isolate in the field. C. digboiensis TERI ASN6 was however, very strongly affected by the carbon source provided in the growth medium. At neutral pH, when GYP provided glucose to this strain, luxurious growth was observed in the yeast form, however, when grown on LB agar (pH 7) in the absence of glucose, it was observed to form pseudohyphae to forage nutrients. Because of the limited bioavailability of hydrophobic hydrocarbons, C. digboiensis TERI ASN6 produced hyphae and pseudohyphae, to reach the substrate, as a response to carbon stress. The earlier onset of filaments on the solid medium compared with those in liquid medium also indicates the mycelial emergence is due to nutritional stress, since the point of colony growth would be locally starved of nutrients whereas in an agitated liquid culture there would be a uniform mixing of nutrients.

Morphological differentiation plays a vital role in the pathogenesis of fungal infection (Ferretti de Lima et al., 2004). The invasive filaments help penetrating the substrate, thereby allowing better access to nutrients in the surrounding growth environment. We believe that, just like pathogenic fungi, the environmental isolate TERI ASN6 has adapted both nutrient and stress response systems to trigger morphological changes that allow it to survive in harsh conditions and to adapt better in unfavourable environments.

3.3. Degradation of the alkane and aromatic fractions of the acidic oily sludge by C. digboiensis

The strain TERI ASN6 was capable of utilizing 73.64% of the total petroleum hydrocarbon of the oily sludge in a week as determined by gravimetric weight loss technique. The strain TERI ASN6 was grown in MSM containing variable concentrations of the acidic oily sludge (0.05% (w/v) to 1% (w/v)). Maximum degradation efficiency was observed at 0.1% (w/v) concentration. Hence this concentration
was selected for the degradation study. The degradation of the alkane and aromatic hydrocarbons by TERI ASN6 at pH 3 was analyzed by gas chromatography. The strain TERI ASN6 depicted efficient utilization of the alkane fraction compared to the untreated control (Fig. 4). The strain was capable of degrading the entire range of \( n \)-alkanes from tetradecane to dotriacontane. The strain also showed considerable utilization of components from aromatic fraction as shown in Fig. 5. However, the oxidation of alkanes was considerably more profound than that for the aromatic hydrocarbons. This finding is in agreement with other reports where hydrocarbon-degrading microbes have shown preferential degradation of alkanes to aromatics and NSO-asphaltene fractions (Lal and Khanna, 1996; Mishra et al., 2001).

3.4. Biotransformation of phenanthrene and dibenzothiophene by C. digboiensis

Identification of the oxidation products showed a peak at 27 min in GC EIMS with a molecular ion at \( m/z \) 194 (Fig. 6a). This mass fragmentation pattern is indicative of the introduction of an oxygen...
atom to the phenanthrene molecule. Trace amounts of the phenanthrene conjugate phenanthrene quinone were also detected.

The strain *C. digboiensis* TERI ASN6 was unable to utilize dibenzothiophene (DBT) as the sole carbon source for its growth as evidenced by the limited growth observed when it was grown in MSM using DBT as the sole carbon and energy source. However, during the growth of this strain in the presence of DBT, two oxidative metabolites of the DBT biodesulfurization pathway were detected.
detected. The two metabolites, DBT-sulfone and biphenyl-2-ol were detected by GC–MS and identified by comparing the mass spectrum of these metabolites with authentic standards. The [M⁺] at m/z 216 and the fragment ion at m/z 187 were identical to those of the standard DBT-sulfone (Fig. 6b) and biphenyl-2-ol with the parent ion at m/z of 170 was also detected (Fig. 6c). The DBT desulfurization pathway is known to occur among bacteria being most notable among Rhodococcus IGTS8 (Kilbane and Jackowski, 1992) and Rhodococcus erythropolis strain D-1 (Bressler and Fedorak, 2000). Besides these, Beijerinckia has also been reported to oxidize DBT (Laborde and Gibson, 1977) and Corynebacterium SY1 (now Rhodococcus) utilizes DBT as the sole sulphur source (Omori et al., 1998). Among eukaryotes some fungi including lignolytic fungi have been studied for PAH degradation and oxidative transformation of DBT. The lignolytic enzymes that lead to the formation of quinones as well as fungal cytochrome P450 monoxygenases are responsible for the oxidation of a wide variety of aromatic hydrocarbons (Prenafeta-Boldú et al., 2006). Cytochrome P450 mediated benzo(a)pyrene hydroxylation has been studied in Phanerachete chrysosporium (Masaphy et al., 1996). The cytochrome P450 detected in Pleurotus ostreatus have been implicated in oxidation of fluorene and DBT similar to the nonlignolytic fungus Cunninghamella elegans (Bezalel et al., 1996). There are, however, fewer reports of desulfurizing yeasts. Baldi et al. (2003) studied

![Fig. 6.](image)

![Fig. 7.](image)
3.5. Detection of the cytochrome P450 gene in C. digboiensis

Alkaline assimilating yeasts have P450s that can be induced by alkanes. The genes encoding P450 which participate in n-alkane metabolism belong to the CYP 52 family and have been cloned from Candida maltosa, Candida tropicalis and Candida apicola (Iida et al., 1998). The degenerate primers Helix 1 and HR2 corresponding to the conserved Helix and heme binding regions were used. After the RT-PCR reaction the PCR product of 395 bp was purified and sequenced (GenBank accession no. DQ486834). The deduced amino acid sequence of the PCR product was similar to the cytochrome P450 alkane hydroxylases of Y. lipolytica and Candida strains (40–60% identity). This sequence shows 62.25% and 63.79% identity with cytochrome P450 alkane hydroxylase 5 (ALK5) and ALK 7 of Y. lipolytica. The identities with Lodderomyces elongisporus and C. tropicalis were 54% and 55.66% respectively. A dendrogram showing the position of this sequence with respect to similar sequences in other yeasts is shown (Fig. 7). Different fungi involve different enzymatic systems, generally, extracellular lignin peroxidase, manganese peroxidase and laccase for white rot fungi and intracellular cytochrome P450 for most non-lignolytic fungi. We believe that C. digboiensis follows the non-lignolytic pathway as evident from PCR based detection of an active cytochrome P450 system. Hydrocarbon oxidation in yeasts is generally known to be evident from PCR based detection of an active cytochrome P450 system. A number of encoding genes of CYP 52 family have been cloned from the yeasts, C. maltosa, C. tropicalis, C. apicola and Y. lipolytica (Eschenfeldt et al., 2003; Iida et al., 1998).

The degradation of the total petroleum hydrocarbons of acidic oily sludge by the novel yeast strain C. digboiensis TERI ASN6 is now being tested under field conditions.

4. Conclusions

The enrichment of the contaminated soil from Digboi refinery yielded a novel yeast isolate, identified as C. digboiensis TERI ASN6, that had developed adaptation mechanisms to survive in the harsh environments of that refinery. This yeast strain was capable of utilizing the hydrocarbons found in the oily sludge at pH 3. An active cytochrome system that gives this novel yeast strain, C. digboiensis its ability to utilize alkane hydrocarbons and oxidize PAHs, was also detected.

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The authors would like to thank the Centre for Mycorrhizal Research, TERI for their help with the optical microscope and the Electron Microscopy Division of All India Institute of Medical Sciences for SEM photography. The authors are grateful to the Indian Oil Corporation Limited, R & D Centre, Faridabad, India and the Department of Biotechnology, India for financial support. The authors would also like to thank Dr. Parag Sadhale of the Indian Institute of Science, Bangalore for his guidance, Ms. Sonali, Mr. Dileep Kumar, Ms. Jyoti, Mr. Akhil and Ms. Gunet for their kind help and Mr. Vinod Kumar and Rambaran for their technical assistance.

References


Mishra, S., Jyot, J., Kuhad, R.C., Lal, B., 2001. In situ bioremediation potential of an Alkane assimilating yeasts have P450s that can be induced by alkanes. The genes encoding P450 which participate in n-alkane metabolism belong to the CYP 52 family and have been cloned from Candida maltosa, Candida tropicalis and Candida apicola (Iida et al., 1998). The degenerate primers Helix 1 and HR2 corresponding to the conserved Helix and heme binding regions were used. After the RT-PCR reaction the PCR product of 395 bp was purified and sequenced (GenBank accession no. DQ486834). The deduced amino acid sequence of the PCR product was similar to the cytochrome P450 alkane hydroxylases of Y. lipolytica and Candida strains (40–60% identity). This sequence shows 62.25% and 63.79% identity with cytochrome P450 alkane hydroxylase 5 (ALK5) and ALK 7 of Y. lipolytica. The identities with Lodderomyces elongisporus and C. tropicalis were 54% and 55.66% respectively. A dendrogram showing the position of this sequence with respect to similar sequences in other yeasts is shown (Fig. 7). Different fungi involve different enzymatic systems, generally, extracellular lignin peroxidase, manganese peroxidase and laccase for white rot fungi and intracellular cytochrome P450 for most non-lignolytic fungi. We believe that C. digboiensis follows the non-lignolytic pathway as evident from PCR based detection of an active cytochrome P450 system. Hydrocarbon oxidation in yeasts is generally known to be evident from PCR based detection of an active cytochrome P450 system. A number of encoding genes of CYP 52 family have been cloned from the yeasts, C. maltosa, C. tropicalis, C. apicola and Y. lipolytica (Eschenfeldt et al., 2003; Iida et al., 1998).

The degradation of the total petroleum hydrocarbons of acidic oily sludge by the novel yeast strain C. digboiensis TERI ASN6 is now being tested under field conditions.

The enrichment of the contaminated soil from Digboi refinery yielded a novel yeast isolate, identified as C. digboiensis TERI ASN6, that had developed adaptation mechanisms to survive in the harsh environments of that refinery. This yeast strain was capable of utilizing the hydrocarbons found in the oily sludge at pH 3. An active cytochrome system that gives this novel yeast strain, C. digboiensis its ability to utilize alkane hydrocarbons and oxidize PAHs, was also detected.

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The authors would like to thank the Centre for Mycorrhizal Research, TERI for their help with the optical microscope and the Electron Microscopy Division of All India Institute of Medical Sciences for SEM photography. The authors are grateful to the Indian Oil Corporation Limited, R & D Centre, Faridabad, India and the Department of Biotechnology, India for financial support. The authors would also like to thank Dr. Parag Sadhale of the Indian Institute of Science, Bangalore for his guidance, Ms. Sonali, Mr. Dileep Kumar, Ms. Jyoti, Mr. Akhil and Ms. Gunet for their kind help and Mr. Vinod Kumar and Rambaran for their technical assistance.
Isolation and characterization of a potential paraffin-wax degrading thermophilic bacterial strain *Geobacillus kaustophilus* TERI NSM for application in oil wells with paraffin deposition problems

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Abstract

Paraffin deposition problems, that have plagued the oil industry, are currently remediated by mechanical and chemical means. However, since these methods are problematic, a microbiological approach has been considered. The bacteria, required for the mitigation of paraffin deposition problems, should be able to survive the high temperatures of oil wells and degrade the paraffins under low oxygen and nutrient conditions while sparing the low carbon chain paraffins. In this study, a thermophilic paraffinic wax degrading bacterial strain was isolated from a soil sample contaminated with paraffinic crude oil. The selected strain, *Geobacillus TERI NSM*, could degrade 600 mg of paraffinic wax as the sole carbon source in 1000 ml minimal salts medium in 7 d at 55 °C. This strain was identified as *Geobacillus kaustophilus* by fatty acid methyl esters analysis and 16S rRNA full gene sequencing. *G. kaustophilus TERI NSM* showed 97% degradation of eicosane, 85% degradation of pentacosane and 77% degradation of triacontane in 10 d when used as the carbon source. The strain TERI NSM could also degrade the paraffins of crude oil collected from oil wells that had a history of paraffin deposition problems.

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Keywords: Wax deposition; Wax degradation; Paraffinic crude oil; Long chain alkane degradation

1. Introduction

Paraffins are high molecular weight alkane hydrocarbons. Paraffinic wax is generally considered to be composed of alkanes of carbon chain length C₁₆ (hexadecane) to C₃₈ (octadecane) and above (Addison, 1984; Kotlar et al., 2007). In reservoirs, under high temperature–pressure conditions, paraffins remain in equilibrium. However, as the crude oil is pumped out from the reservoir to the surface, temperature and pressure are reduced. Below the cloud point, the paraffinic hydrocarbons (wax) precipitate out of the crude oil and form deposits on the oil production systems. This paraffinic wax deposition causes the oil companies millions of dollar per year as removal costs and the cost of lost production (Barker et al., 2001). Paraffin problems are usually mitigated by thermal fluid treatments, pigging (scraping) or using chemicals and solvents. While most of these remedies are in use, they have certain disadvantages. Mechanical scraping causes losses due to production downtime. Chemicals and solvents, besides being hazardous, only partially dissolve or disperse paraffins and they are deposited elsewhere in the tubing. Moreover, these remedial options are expensive.

A microbial intervention to prevent paraffin deposition in oil well tubing, promises to be a non hazardous and economically viable approach.

Alkanes, that comprise paraffins, have long been known to be degraded by microbes. *Pseudomonas putida GPo1* (commonly known as *Pseudomonas oleovorans GPo1*) is
among the most extensively studied alkane degrading bacteria. While this strain is most known for efficient utilization of medium chain alkanes (van Beilen et al., 2001) and carbon chain length alkanes from C_{12} to C_{22} (van Beilen et al., 2005), the Pseudomonas aeruginosa strain WatG could degrade paraffinic alkanes hexatriacontane (C_{36}) and tetracontane (C_{40}) in a mineral salts medium containing crude oil (Hasanuzzaman et al., 2007). Several Acinetobacter strains are also known to degrade paraffinic hydrocarbons with the strain M-1 being known for its ability to degrade paraffins with carbon chain lengths of C_{20} to C_{44} (Tani et al., 2001). Koma et al. (2001), on the other hand, have studied an Acinetobacter strain that can degrade long chain n-paraffins from waste oil of car engines at 30 °C. Kotlar et al. (2007) have isolated an Acinetobacter sp. 6A2 strain capable of utilizing paraffins with a melting point of 52–54 °C. While, several bacteria are known to degrade alkanes at mesophilic temperatures of 30–37 °C, reports of thermophilic bacteria that are capable of degrading wax as the carbon source are fewer in number. Marchant et al. (2002) have found Geobacillus strains that can grow at temperatures of 40–80 °C and utilize alkanes.

This investigation was aimed at finding a bacterial strain/consortium that could survive at high temperature conditions encountered in oil well tubing while being able to effectively degrade paraffin wax that causes depositions in oil wells.

2. Materials and methods

2.1. Chemicals and media

All the standards for hydrocarbons used in this study were procured from Sigma–Aldrich (St. Louis, Mo, USA). The media for bacterial cultivation was from HiMedia (Mumbai, India) and the paraffinic wax (Melting point 58–60 °C) used in the study was from Merck India Ltd. (Mumbai, India).

2.2. Enrichment and isolation of paraffin wax degrading bacteria

The bacterial strains were isolated by enrichment culture technique from soil samples contaminated with paraffinic crude oil procured from an oil field in Mehsana of Gujarat state of western India. For enrichment, 5 g soil samples were inoculated into 100 ml of minimal salts medium (MSM) (Lal and Khanna, 1996) containing steam sterilized paraffinic wax (1% w/v) as carbon source and incubated at 70 °C on a rotary shaker (180 rpm) for 7 d. After ten cycles of enrichment, 1 ml of culture was diluted 10^{4} fold, and 100 μl was plated on MSM agar plates with paraffinic wax (0.1% w/v) as the carbon source. The bacterial colonies thus obtained were further purified on the MSM agar plates with paraffinic wax. The isolates were routinely subcultured and frozen stock cultures were stored in 25% glycerol at −70 °C.

2.3. Degradation of paraffinic wax by selected bacterial strains

Degradation of paraffin-wax by the selected isolates was monitored in 250 ml Erlenmeyer flasks (in triplicate) containing 100 ml MSM with 0.1% (w/v) of paraffinic wax as the sole carbon source and samples were incubated on a rotary shaker (180 rpm) at 55 °C for 7 d. The isolates were grown overnight in Luria Bertani broth to a cell density of 10^{8} CFU ml^{-1} and 5% (v/v) inoculum was used to inoculate MSM with paraffin wax as the sole carbon source. Uninoculated controls were maintained to monitor abiotic loss of paraffin-wax. The residual undegraded paraffin-wax was extracted thrice with equal volumes of hexane. For quantitative analysis, 1 μl of the paraffinic wax (dissolved in 10 ml hexane) was analysed by GC (Hewlett Packard 5890 Series II fitted with flame ionization detectors (FID)) on a DB 2887 column using the method described by Mishra et al. (2001). The profile of the paraffinic fraction, extracted from inoculated flasks, was compared with that obtained from uninoculated control flask.

2.4. Characterization of the efficient paraffin-wax degrading strain

The efficient paraffin-wax degrading bacterial strain was characterized by morphology studies and substrate utilization ability. Morphological studies of the most efficient paraffin wax degrading bacterial strain was done using light microscopy at 100x magnification (Olympus microscope BH2, USA). The detailed morphology of the selected strain grown on Luria Bertani agar at 55 °C was studied using SEM (LEO 435 VP, 30 kV voltage).

To study the ultra structure of this bacterial strain, it was grown following the method described by Ishige et al. (2002). The cells were then studied by TEM (FEI Philips Morgagni 268D).

The fatty acid analysis of the selected strain was done at MIDI Labs (Newark, DE, USA).

The ability of this strain to degrade alkanes was tested by growing it in MSM containing each of the alkanes from octane (C_{8}) to triacontane (C_{30}) as the sole carbon source at 55 °C under shake flask conditions. Growth measured in terms of absorbance (600 nm) and total cell protein was indicative of the ability of the strain to utilize alkanes.

2.5. Identification of the most efficient paraffin-wax degrading strain by 16S rRNA gene sequencing

The 16S rRNA gene sequence was determined using the Microseq 16S rRNA full gene kit (PE Applied Biosystems). The amplification and sequencing was performed following the manufacturer’s protocol. The cycle sequenced product was analyzed with the ABI Prism 310 genetic analyzer (PE Applied Biosystems). The sequence was analyzed using the NCBI (National Center for Biotechnology Information) databank. The sequence was deposited in the NCBI.
database and was assigned the accession number EF199739.

2.6. Degradation of alkanes representing the paraffinic-wax

A mixture of 50 mg each of eicosane (C₂₀), pentacosane (C₂₅) and triacontane (C₃₀) was added as carbon source to 10 Erlenmeyer flasks of 250 ml capacity, each containing 50 ml of MSM. Each of these flasks was inoculated with 5% (v/v) of overnight grown TERI NSM culture in Luria Bertani broth (LB) to a cell density of 10⁸ CFU ml⁻¹ and kept on a rotary shaker at 55 °C for 10 d. Every 24 h one flask was removed and the residual undegraded alkane hydrocarbons were extracted with twice the volume of hexane. This experiment was repeated twice. The hexane was evaporated at room temperature in a fume hood and the residual alkanes analysed by GC as described in Section 2.3.

2.7. Degradation of paraffinic crude oil by the selected strain

The ability of the selected strain to degrade paraffinic crude oil was tested by preparing 50 ml MSM in 250 ml flasks containing 0.01% Tween 80 (Sigma chemicals, USA) and taking 1% (w/v) of paraffinic crude oil collected from problematic wells of Limbodara oil wells of Gujarat state, western India. The medium was inoculated with an overnight grown culture of selected paraffin wax degrading strain in LB (10⁸ CFU ml⁻¹) and incubated in shaking conditions at 55 °C for 7 d. The experiment was done in triplicates. At the end of the incubation period, the residual paraffin crude oil was extracted thrice with equal volumes of hexane, eluted with hexane through a silica gel column to obtain the paraffinic fraction and then analyzed by GC-FID as described in Section 2.3.

3. Results and discussion

Indian crude oils are highly paraffinic in nature (Agrawal and Joshi, 1984) and most of the oil wells that have a history of paraffin deposition related problems in oil well tubings, have crude oil containing paraffins of carbon chain length of C₁₆ (hexadecane) and higher alkanes (Lazar et al., 1999). We, therefore, obtained paraffinic wax with a carbon chain length of C₁₈ (octadecane) to C₃₀ as being representative of most problematic paraffinic crude oils of India.

3.1. Isolation and screening of paraffin wax degrading bacteria

In order to selectively enrich thermophilic bacterial strains that could degrade paraffin wax, the growth medium was supplied with wax as the sole carbon source and incubated at 70 °C. Enrichment at higher temperature would increase the probability of obtaining thermophilic bacteria with heat stable enzymes and reduced oxygen requirements (Mohamed et al., 2006) which are desirable in reservoir conditions. After repeated sub culturing, a stabilized wax-degrading consortium was obtained. This consortium when plated on MSM agar plates containing wax yielded 10 morphologically distinct looking bacterial colonies. These bacterial isolates when tested for their paraffin wax degrading ability, revealed a varying paraffin wax degradation of 10–50% in 7 d. Of the ten isolates that we had obtained, we checked their ability to degrade wax and paraffinic crude oil and selected the strain TERI NSM for further studies.

3.2. Characterization of the selected bacterial isolate

The selected strain TERI NSM was a catalase positive, Gram positive rod. It could grow at temperatures from 50 to 85 °C and could utilize glucose, mannose, raffinose, trehalose, cellobiose, galactose, starch, arabinose, ribose, xylose, sucrose, maltose, lactose and mannitol. However, it could not utilize Tween 80 as the sole carbon source. It could utilize n-alkanes of carbon chain length above C₁₅ as the sole carbon source while it could not utilize lower carbon chain alkanes (≤C₁₅) which are valuable and desirable for the prevention of the deposition of paraffins in oil well tubing. The inability of this strain to utilize lower alkanes is good for the prevention of paraffin deposition in oil wells, since the shorter alkanes are also valuable components and should not be degraded. Cell morphology studies of this isolate revealed a cell size of approximately 2–3 μm width, however, in addition to normal rod shaped morphology, the cells of strain TERI NSM showed the presence of long flexuous rods of variable length with some going up to 100 μm at 70 °C. The light microscopy photographs and scanning electron micrographs of these cells are shown in Fig. 1. The occurrence of these flexuous thread shaped morphology was higher when the bacterial strains were cultured at 70 °C than at 55 °C. It was also noted that, this morphology was apparent in LB medium as well as MSM medium containing wax at 70 °C but not so in MSM containing wax at 55 °C. While the length of the rods can extend easily up to 80 μm upon repeated subculturing at 70 °C (Fig. 1b), the length of the rods on Luria Agar at 55 °C has been recorded around 20 μm (Fig. 1d). However, at all times the bacterial shapes are a mixture of shorter rods (Fig. 1a and d) and the longer flexuous rods. Marchant et al. (2002) had also observed similar morphology in their Geobacillus strains. They have also observed a greater presence of the longer rods at 70 °C compared to 40–50 °C temperatures similar to our observations with Geobacillus TERI NSM.

The ultra structure of Geobacillus TERI NSM is shown in Fig. 2. The TEM micrographs show the presence of terminal endospores (Fig. 2a and b) that allow bacterial strains to withstand environmentally harsh conditions. Ishige et al. (2002) had observed disc shaped intracellular inclusions in an alkane utilizing Acinetobacter strain M-1 concomitant with wax ester accumulation, however, following the same protocol, no such inclusions were observed.
Fig. 1. (a) and (b) Light micrograph (100x magnification) of Gram stained cells of TERI NSM grown in minimal salts medium with wax as the carbon source at 70 °C. (c) and (d) Scanning electron micrograph of the strain TERI NSM showing mixed short cells and long flexuous rods when grown on Luria Agar at 55 °C.

Fig. 2. Ultra structure photographs of the strain TERI NSM using Transmission electron microscopy.
in the ultra structure micrographs of our strain TERI NSM (Fig. 2c and d).

The cell wall fatty acid profile of Geobacillus TERI NSM grown on LB at 55 °C revealed that it was largely composed of iso-C15:0 and iso-C17:0 fatty acids. The iso-C15:0 fraction formed 47.8% and the iso-C17:0 formed 23.9% of the fatty acid composition. Other fatty acids detected were iso-C16:0 at 6.9%, iso-C14:0 at 0.3%, 1.8% of anteiso C15:0 and 2.3% of iso-C17:1 w5c. Besides these, C17:0 anteiso at 5.1% also formed a sizable fatty acid fraction. On the basis of the dendrogram cluster analysis technique, that produces unweighted pair matching based on fatty acid compositions to show the relatedness between organisms, TERI NSM showed similarity with Geobacillus kaustophilus and Geobacillus thermocatenulatus (graphically depicted in Fig. 3). Nazina et al. (2004) have also shown several Geobacilli including the novel thermophile Geobacillus gargensis to be largely composed of iso-C15:0, iso-C16:0 and iso-C17:0 fatty acids. Other Geobacilli with similar fatty acid profiles include Geobacillus thermodenitrificans, Bacillus thermoleovorans and Bacillus thermocatenulatus (Nazina et al., 2001). Poli et al. (2006), however, have found a Geobacillus toebii subsp decananicus strain that differs from the aforementioned Geobacilli in that it lacked in iso-C15 fatty acid, while iso-C16 and iso-C17 were predominant.

3.3. Identification of the selected strain Geobacillus TERI NSM using 16S rRNA gene sequencing

Using universal primers that bind at the 5' and 3' ends of 16S rDNA of Eubacteria, the entire 16S rRNA gene sequence was done to ascertain TERI NSM's species identity. The sequence, when analysed by comparison with 16S rDNA nucleotide sequences of GenBank, revealed a 100% homology to the 16S sequence of the thermophilic Geobacillus strain N60 isolated from a volcanic island. The strain also showed 99% homology to several thermophilic strains of the Geobacillus gp. It showed single nucleotide difference with Bacillus caldotenax, G. kaustophilus, a Bacillus sp. WPD616, Geobacillus sp. STB2 and 2 nucleotide difference (99% homology) with G. kaustophilus strain BGSC 90A1, Geobacillus stearothermophilus BGSC9A19 and B. thermoleovorans and 4 nucleotide difference with Bacilli strains Bacillus vulcani and Bacillus caldolyticus. The strain TERI NSM was assigned to the G. kaustophilus sp. considering the results of the 16S sequence and the supporting evidence provided by fatty acid methyl ester analysis. The relatedness of TERI NSM was to members of the family Bacillaceae.

The occurrence of Geobacillus strains in hydrocarbon contaminated environments is not uncommon. Others (Nazina et al., 2001, 2005) have obtained hydrocarbon oxidizing Geobacilli strains from formation waters of oil fields. Mohamed et al. (2006) have also found hydrocarbon degrading members of the Bacillaceae family to dominate the oil contaminated soils of Kuwait. Recently, Wang et al. (2006) isolated a thermophilic bacterial strain G. thermodenitrificans that shows selective degradation of long chain alkanes, similar to the degradation pattern of TERI NSM. However, in the NCBI search of 16S rRNA gene sequence, TERI NSM does not show a match with this strain. The 16S gene sequence alignment of the two strains by Clustalw (EMBL) revealed a 98% similarity.

Fig. 4. Degradation of the paraffinic alkanes: eicosane, pentacosane and triacontane by Geobacillus kaustophilus TERI NSM at 55 °C.

Fig. 3. The dendrogram cluster analysis based on fatty acid compositions showing relatedness of TERI NSM to closely related bacterial strains (provided by MIDI Labs).
3.4. Degradation of the alkanes of paraffin-wax by G. kaustophilus TERI NSM

The degradation pattern, of a mixture of paraffinic alkanes by G. kaustophilus TERI NSM, is shown in Fig. 4. The degradation of paraffins was studied at 55 °C since it is the cloud point of the paraffinic crude oil that was collected from oil wells with a history of paraffin deposition related problems. While more than 60% of all the three alkanes, C20, C25 and C30, were utilized in a week, there was a difference in the rate of utilization of the three alkanes. On the 10th day of incubation, 77% of triacontane and 85% of pentacosane were degraded as compared to 97% utilization of eicosane. Thus, G. kaustophilus TERI NSM also shows a preferential degradation of eicosane to pentacosane and triacontane.

3.5. Degradation of paraffinic crude oil by the selected strain G. kaustophilus TERI NSM

The paraffinic crude oil from an oil well, in the Limbodara region of Gujarat, India, showed a variable amount of paraffins (approximately 50%) that contained alkanes with a carbon chain length from C14 to C32. The growth studies of TERI NSM indicated an efficient utilization of the paraffinic crude oil that was supplied as the sole carbon source. The residual undegraded paraffinic crude oil was extracted when the growth of TERI NSM declined. The thermophilic strain G. kaustophilus TERI NSM could effectively degrade all the carbon fractions of this crude oil. It could degrade 60% of this paraffinic fraction in 7 d. Gas chromatographic analysis revealed that this strain could utilize paraffins efficiently (Fig. 5). Lazar et al. (1999) had prepared a mixed consortium of 15 bacterial isolates and 3 bacterial consortia to get 95.3% petroleum hydrocarbon utilization from Romanian paraffinic crude oil at 28 °C in 10–12 d. Etoumi (2006) also found a reduction in wax appearance temperature and heavy hydrocarbon fractions by biodegradation of paraffinic hydrocarbons using Pseudomonas and Actinomyces species.

The strain TERI NSM revealed that it could degrade paraffins at high temperatures and under limited nutrient and oxygen conditions that are known to be associated with thermophilic environments. This bacterial strain could not produce H2S, as tested by Sulfide determination kit (Merck, India), that causes well fouling. These indicate that the strain TERI NSM is suitable for application in oil wells having a history of paraffin deposition problems and will be tested in actual oil wells shortly.

4. Conclusions

A thermophilic wax degrading bacterial strain (TERI NSM) was obtained by enrichment technique and identified as G. kaustophilus. The ability of this strain, to efficiently degrade paraffin wax in low nutrient conditions as the sole carbon source, grow at high temperatures and selectively degrade long carbon chain alkanes (leaving the short carbon chain alkanes intact) make it a good candidate for application to oil wells with paraffin deposition problems.

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References


Candida digboiensis sp. nov., a novel anamorphic yeast species from an acidic tar sludge-contaminated oilfield

G. S. Prasad, S. Mayilraj, Nitu Sood, Vijeyta Singh, Kakoli Biswas and Banwari Lal

Two strains (TERI-6T and TERI-7) of a novel yeast species were isolated from acidic tar sludge-contaminated soil samples collected from Digboi Refinery, Assam, India. These two yeast strains were morphologically, physiologically and phylogenetically identical to each other. No sexual reproduction was observed on corn meal agar (HiMedia), malt agar, Gorodkowa agar (Yarrow, 1998), YM agar (HiMedia) or V8 agar (Difco). Physiologically, the novel isolates were most closely related to Candida blankii, but differed in eight physiological tests. The prominent differences were the ability of the isolates to assimilate melibiose and inulin and their inability to assimilate D-glucuronate, succinate and citrate. Phylogenetic analysis using the D1/D2 variable domain showed that the closest relative of these strains is Candida blankii (2-8 % divergence). Other related species are Zygoascus hellenicus and Candida bituminiphila. The isolates differed from C. blankii by 11 base substitutions in the 18S rRNA gene sequence and by 58 base substitutions in the internal transcribed spacer sequences. The physiological, biochemical and molecular data support the contention that strains TERI-6T and TERI-7 represent a novel species, for which the name Candida digboiensis sp. nov. is proposed. The type strain is TERI-6T (=MTCC 4371T =CBS 9800T =JCM 12300T).

During a research programme isolating bacteria from acidic tar sludge-contaminated soils, two yeast strains (TERI-6T and TERI-7) belonging to the genus Candida were isolated from such a soil (pH 2-0) collected from the Digboi oil refinery in Assam, India, a state in the north-eastern part of India (27°33'N 95°40'E). These two isolates were obtained on the nutrient agar plates used for isolating bacteria from the sludge-contaminated soil of an oilfield. Small colonies appeared on that medium and were sent for characterization to the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. There are very few reports on the isolation of yeast species from this habitat: yeast strains have been isolated from oilfields and oil brines in Japan (Iizuka & Goto, 1965; Iizuka & Komagata, 1965). Recently, Candida bituminiphila from tar was reported (Robert et al., 2001). On the basis of conventional morphological and physiological tests, the strains were shown to be related to Candida blankii and Zygoascus hellenicus, though they differed from them in several physiological tests. No sexual reproduction was observed on corn meal agar (HiMedia), malt agar, Gorodkowa agar (Yarrow, 1998), YM agar (HiMedia) or V8 agar (Difco). Sequence analysis of rRNA genes also showed that the strains are closely related to C. blankii. All these data supported the assignment of strain TERI-6T to a novel species, for which we propose the name Candida digboiensis.

The yeast strains examined in this study are listed in Table 1 and are available from the MTCC, the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, and the Japan Collection of Microorganisms (JCM), Wako-shi, Japan. All the strains were grown on YM agar at 25 °C. Phenotypic characteristics were examined using standard methods for yeast taxonomy (Yarrow, 1998).
The yeast strains were grown in YM broth (HiMedia) for 24 h at 25 °C, harvested by centrifugation, resuspended in sterile 1 M sorbitol and transferred to 1-5 ml microfuge tubes. The cell pellet was used for DNA isolation with the MasterPure Yeast DNA purification kit (Epicentre Technologies) according to the manufacturer’s instructions.

Each PCR was performed in a final reaction mixture (50 µl) containing 50 ng genomic DNA, 25 pmol each primer, 200 mM each of dATP, dTTP, dGTP and dCTP (Promega), 2-5 mM MgCl2, 2-0 U Taq polymerase (Promega) and 5 µl 10× reaction buffer (Promega). Primers ITS1 and ITS4 were used to amplify the internal transcribed spacer (ITS) region, while NS1 and NS8 were used for amplifying the small-subunit (SSU) rRNA gene (White et al., 1990). The primers were obtained from Integrated DNA Technologies. Amplification reactions were performed in a PTC 150 Mini Cycler (MJ Research) with the following cycling parameters: initial denaturation for 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C and 1-0 min at 72°C (for ITS and D1/D2 regions) or 2-0 min (for SSU rRNA gene), with a final extension for 10 min at 72°C, and cooled to 4°C. The amplified products were separated on 1-2 % agarose (Sisco Research Laboratories) gel by electrophoresis and visualized by staining with ethidium bromide (0-5 µg ml⁻¹).

The amplicons were purified using the Qiagen gel extraction kit. Direct sequencing of gel-purified PCR products was performed with the ABI BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems). Both strands of the PCR product were sequenced. The SSU rRNA gene was sequenced with primers NS1–NS8, the ITS region with primers ITS1 and ITS4 (White et al., 1990) and the D1/D2 domain with primers NL1 and NL4 (Kurtzman & Robnett, 1998). Sequencing reactions were purified by ethanol and sodium acetate precipitation. The pellet was washed twice with 70 % ethanol, which considerably improved the removal of dye terminators from the reaction. Processing of the samples for loading onto an ABI 310 model sequencer was performed according to the instructions of the manufacturer (Applied Biosystems).

A sequence-similarity search was done using GenBank BLASTN (Altschul et al., 1997). Sequences of closely related taxa were retrieved and aligned using the CLUSTAL X program (Thompson et al., 1997). For the neighbour-joining analysis (Saitou & Nei, 1987), distances between the sequences were calculated using Kimura’s two-parameter model (Kimura, 1980). Bootstrap analysis was performed to assess the confidence limits of the branching (Felsenstein, 1985).

### Latin diagnosis of *Candida digboiensis* G. S. Prasad, Mayilraj, Sood & Lal sp. nov.

*Coloniae in agaro multi humiles convexae, integrae vel fimbriatae, albae vel cremeae, butyrosae. In medio liquido cum dextroso et peptono et extracto levedinis et extracto malti post multiis 3 dies ad 25 °C cellulae sunt ellipsoideae ad cylindricalae pro maxima partes irregularès (2-0–3-5 × 3-0–9-0 µm), singulae vel binae. Reproductio vegetativa per holoblastice gemmationem. In lamina Dalmau post 7 dies pseudohyphae formantur dense ramosae. Hyphae verae nonnumquam praesentes. Fermentatio nulla. Sucrosum, galactosum, L-sorbosum, D-ribosum, D-xilosum, L-arabinosum, D-arabinosum, L-rhamnosum, maltosum, trehalosum, methyl D-D-glucosidum, cellobiosum, salicinum, melibiosum, lactosum, raffinosum (exigue), melezitosum, inulinum, erythritolum, ribotilum, xylitolum (lente), D-arabinotilum, L-arabinotilum, glucotilum, mannitolum, galactitol, myo-inositolum, gluconolactonum (lente), acidum gluconicum (exigue), ethanolum, arbutinum, amyllum solubile et glycerolum (lente) assimilantur, neque glucosaminum, acidum glucuronicum, acidum succinicum, acidum citricum, aut methanolum. Ethylaminum, lysinum et cadaverinum velut substrata nigrigeni utuntur, neque sodii nitratum aut nitritum. Vitaminis vel acidi aminosis absentibus haud crescit. 42 °C crescit. In agaro calci carbonato addito acidum non formatur. In liquido 50 % glucosii addito non crescit. 0-01 % cycloheximidum non crescit. Amyllum non formatum, urea non finditur, diazolium coeruleum B probatio negativa. Holotypus TERI-6T (=MTCC 4371T) lyophilis, isolatus e terra inquinata ‘acid tar sludge’, circa Digboi oil refinery, Digboi, Assam, India.

### Description of *Candida digboiensis* G. S. Prasad, Mayilraj, Sood & Lal sp. nov.

*Candida digboiensis* [dig.boi.en’sis. N.L. nom. fem. adj, digboiensis referring to Digboi (27-33 ° N 95-40 ° E), a town

### Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>Location</th>
<th>ITS accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. digboiensis sp. nov. TERI-6T (=MTCC 4371T) =CBS 9800T =JCM 12300T</td>
<td>Acid tar sludge-contaminated soil</td>
<td>Digboi, Assam, India</td>
<td>AJ697745</td>
</tr>
<tr>
<td>C. digboiensis sp. nov. TERI-7 (=MTCC 4372) =CBS 9801 =JCM 12301</td>
<td>Acid tar sludge-contaminated soil</td>
<td>Digboi, Assam, India</td>
<td>AJ697746</td>
</tr>
<tr>
<td>C. blankii MTCC 1442T (=CBS 1898T)</td>
<td>Blood of mink (<em>Putorius vison</em>) Canada</td>
<td></td>
<td>AJ697747</td>
</tr>
<tr>
<td>C. blankii MTCC 624 (=CBS 6734)*</td>
<td>Soil Japan</td>
<td></td>
<td>AJ697748</td>
</tr>
</tbody>
</table>

*Type strain of *C. hydrocarbofumarica.*
in Assam State, north-eastern India, where the type strain was isolated.

Colonies on malt agar are low-convex, entire or fringed, white to cream and butyrous. After 3 days in YM broth at 25 °C, cells are ellipsoidal to short cylindrical (2-0–3.5 × 3.0–9.0 μm) and occur singly, in pairs or in short chains (Fig. 1). Some irregular shapes and a few elongated (up to 15 μm) cells are also present. Sympodial holoblastic conidiogenesis results in ovoid to obclavate conidia (2-4 μm) that arise from pronounced protuberances. After 1 week in Dalmau plate culture, pseudohyphae consisting of branched chains of elongated cells are visible. True hyphae may be present. Fermentation absent. Sucrose, galactose, sorbose, ribose, xylose, D-arabinose, L-arabinose, L-rhamnose, maltose, trehalose, methyl α-D-glucoside, cellobiose, salicin, melibiose, lactose, raffinose, melezitose, inulin, erythritol, ribitol, xylitol, D-arabitol, L-arabitol, D-glucitol, D-mannitol, galactitol (weak), myo-inositol, D-glucono-1,5-lactone (weak), D-glucuronate, ethanol, arbutin, starch (slow) and glycerol (weak) are assimilated. D-Gluconate, D-glucosamine, succinate, citrate and methanol are not assimilated. Ethylamine, lysine and cadaverine are utilized as sole nitrogen sources; sodium nitrate and nitrite are not. Does not grow in the absence of vitamins. Grows in the absence of amino acids. Grows at 42 °C. Acid is not produced on chalk agar. Does not grow in the presence of 50 % (w/w) glucose, 0 % and 0.1 % (w/v) cycloheximide. It differs from Candida auringiensis and Candida salmanticensis in its ability utilize melibiose and inulin and its inability to grow in the presence of 0.01 % and 0.1 % (w/v) cycloheximide. C. digboiensis differs from Candida auringiensis and Candida salmanticensis in its ability utilize melibiose and inulin and its inability to grow in the presence of 0.01 % and 0.1 % (w/v) cycloheximide. It differs from Z. hellenicus in its ability to assimilate melibiose, inulin, erythritol, arabitol and its inability to assimilate D-glucosamine. Z. hellenicus cannot grow at 42 °C, whereas strains TERI-6T and TERI-7 show growth at this temperature. Selected phenotypic differences between C. digboiensis and related species are shown in Table 2.

The variable D1/D2 domain of the large-subunit rRNA gene has been sequenced for all currently recognized ascomycetous yeasts (Kurtzman & Robnett, 1998). These studies have shown that strains belonging to separate species generally exhibit greater than 1% sequence divergence. Strains TERI-6T and TERI-7 show 2-8% divergence (16 base substitutions out of 556 nt) from C. blankii, indicating that C. digboiensis could be a novel species. However, in a recent study with the yeast species Clavispora lusitaniae, it was found that the sequence variation in the D1/D2 region among mating strains of that species could exceed 6-0% (Lachance et al., 2003). At present, it is not clear whether this constitutes a rare example of polymorphism in the D1/D2 region or whether some other species are also polymorphic. For further confirmation of the novelty of the strains under study, we also sequenced the SSU rRNA gene and the ITS region (comprising ITS1, 5.8S rRNA gene and ITS2 regions) of strains TERI-6T and TERI-7.

A BLAST search (Altschul et al., 1997) using the C. digboiensis SSU rRNA gene sequence showed that C. blankii is the closest relative, although most other sequences retrieved in this search were those of filamentous fungal species. A discontiguous Mega BLAST (http://www.ncbi.nlm.nih.gov/blast/) search retrieved the yeast sequences. In the SSU rRNA gene sequence, C. digboiensis differs from C. blankii by 11 base substitutions and two deletions. C. salmanticensis and C. auringiensis are also related but show sequence divergence of 2-3 and 2-7%, respectively, from C. digboiensis. Most species of the Stephanoascus clade (Stephanoascus smithiae, Stephanoascus farinosus, Arxula adeninovorans, Z. hellenicus, C. bituminiphila etc.) show more than 5%
divergence, in the SSU rRNA gene sequence, from C. digboiensis, which suggests that they may be distantly related to C. digboiensis. Further confirmation of the novelty of C. digboiensis came from sequencing of the ITS region. As the ITS region of C. blankii was not available in the nucleic acid databases, we sequenced the ITS regions of two strains of C. blankii (MTCC 1442T and MTCC 624). In this region, C. digboiensis differs from C. blankii by 58 base substitutions; in addition, there are 33 base deletions in C. blankii and nine deletions in C. digboiensis. This strongly suggests the separation of C. digboiensis from C. blankii.

A BLAST search with the ITS sequence of C. digboiensis retrieved the partial ITS sequence of C. blankii (deposited as part of this study) and only the 5.8S rRNA gene sequences of other species, the first being C. bituminiphila, followed by Z. hellenicus and different varieties of Zygoascus species. This again shows that the strains isolated from acidic tar sludge-contaminated soil represent a novel species.

In the phylogenetic tree constructed using the D1/D2 variable domain of the large-subunit rRNA gene (Fig. 2), C. digboiensis along with C. blankii were placed within a broad cluster comprising Stephanoascus/Arxula/Blastobotrys/Zygoascus (the Stephanoascus clade) and some species of Candida supported by high bootstrap values (90 %). However, C. digboiensis and C. blankii appear to be more

![Fig. 2. Phylogenetic placement of C. digboiensis sp. nov. based on the D1/D2 variable domain sequence of the large-subunit rRNA gene. The tree was generated by NJPlot (Perrière & Gouy, 1996). Bootstrap values (each expressed as a percentage of 1000 replications) greater than 70 % are given at nodes. The scale shows 5 % sequence divergence.](image-url)
closely related to C. auringiensis, C. salmanticensis and Candida tartarivorans than to the other species of the Stephanoascus clade. Kurtzman & Robnett (1998) showed that C. blankii is phylogenetically distantly related to the Stephanoascus clade. Similarly, C. auringiensis, C. salmanticensis and C. tartarivorans appear to be only distantly related to the Stephanoascus clade (Fonseca et al., 2000). Middelhoven & Kurtzman (2003) examined the ability of several yeast species to assimilate glycine, uric acid, n-hexadecane, putrescine and branched-chain aliphatic compounds such as isobutanol, leucine and isoleucine. Among the Saccharomycetales, most of the species belonging to the Stephanoascus clade utilized most, or all, of these compounds. C. blankii, which was considered as distantly related to the above clade, utilized n-hexadecane and five other compounds, indicating that it is phylogenetically more similar to members of the Stephanoascus clade. It would be interesting to examine the ability of C. digboiensis strains to utilize the above compounds.

Lachance & Starmer (1998) commented that ‘unfortunately too many times in the past, workers engaged in yeast isolation have not gone beyond the mere nomenclatural description of new species, failing to typify also their community, habitat, and possible interactions’. As C. digboiensis strains were isolated from an acid tar sludge-contaminated soil, we have investigated which other species have been isolated from hydrocarbon habitats. Although very few reports are available on the isolation of yeast species from oil-contaminated soil, yeast strains have been isolated from oilfields and oil brines in Japan (Iizuka & Goto, 1965; Iizuka & Komagata, 1965), and some yeast species have been reported to utilize petroleum hydrocarbons (Ismailov, 1985a, b; Palittapongarnpim et al., 1998; Radwan et al., 2001). We have examined the habitats of the closest relatives of C. digboiensis. Candida hydrocarbofumarica MTCC 624 (=CBS 6734), a synonym of C. blankii, was isolated from soil; it has been reduced to synonymy with C. blankii because of the high degree (>90%) of nuclear DNA reassociation between the type strains of two species (Meyer et al., 1998). We have determined the sequence of the D1/D2 variable domain of the type strain of C. hydrocarbofumarica and found it to be identical to the sequence of the type strain of C. blankii, confirming their conspecificity. The SSU rRNA gene and ITS sequences of C. blankii and C. hydrocarbofumarica were also identical. C. blankii strains utilize hydrocarbons (Furukawa et al., 1970, 1978; Yamada et al., 1970). Strains of C. digboiensis could utilize alkane as well as aromatic fractions of acidic tar sludge (data not shown). On the basis of D1/D2 sequence analysis, C. auringiensis, C. salmanticensis and C. tartarivorans are the other relatives of C. digboiensis. Both C. auringiensis and C. salmanticensis were isolated from ‘alpechin’, the waste produced by olive oil extraction (Meyer et al., 1998). C. bituminiphila and Z. hellenicus are the other relatives of this cluster. Interestingly, C. bituminiphila was isolated from tar, which is a by-product of the crude oil industry (Robert et al., 2001). Three strains of Z. hellenicus (CBS 4028, CBS 4075 and CBS 5839) have the ability to split fat (CBS web site: http://www.cbs.knaw.nl/databases/index.htm). Yeasts are relatively rarely isolated from crude oil or hydrocarbon sources. However, phylogenetic analysis of C. digboiensis and related species suggests that several closely related yeast species may exist in nature. Physiological and biochemical aspects of acidic tar sludge degradation by C. digboiensis strains are under investigation and will be reported separately.

Acknowledgements

This study was supported by the Department of Biotechnology and the Council of Scientific and Industrial Research, Government of India (G. S. P.), and by the Indian Oil Corporation Ltd and the Department of Biotechnology, Government of India (B. L.). G. S. P. is grateful to Dr Tapan Chakrabarti for encouragement, to Mr Paramjit for his excellent technical assistance and to Dr K. W. Gams (CBS, Utrecht, The Netherlands) for help with the Latin description. This is IMTECH Communication number 24/2004.

References


Title: BIOREMEDIATION OF ACIDIC SLUDGE

Abstract: The present invention relates to a process of remediation of acidic sludge, which comprises: mixing the sludge with soil, adding microbial consortia capable of degrading hydrocarbon, to soil-sludge mixture, in which the microorganisms is supported on a carrier, and providing nutrient for the microorganisms to the soil-sludge mixture.
BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
5 TITLE OF THE INVENTION

BIOREMEDIATION OF ACIDIC SLUDGE.

FIELD OF THE INVENTION

The present invention relates to a method of disposal of sludge and more particularly to a method of disposal of acidic sludge utilizing microorganisms capable of degrading of hydrocarbon i.e., bioremediation at acidic pH.

BACKGROUND OF THE INVENTION

Over the past several decades worldwide production, processing, storage, transportation and utilization of synthetic and naturally occurring chemical substances has led to the introduction of significant quantities of hazardous materials into the environment. Unintentional spillage of petroleum distillates, industrial solvents and other chemical substances has been caused, for example, by weathering, chemical corrosion and accidental damage to pipes, storage vessels, processing equipment, transportation vehicles, etc. Deliberate acts and carelessness have also contributed to the release of hazardous substances into the environment. The spillage of such materials has resulted in large numbers of polluted sites and enormous volumetric quantities of soil and groundwater, which have been contaminated with hazardous substances. Soil contamination can cause extensive damage to the local ecosystem by accumulating in the tissue of animals and plants and by causing death thereto and/or mutation to the progeny thereof. Such contamination can also present a serious health threat to humans, and, in extreme cases, can render the contaminated area unsuitable for human habitation. In many cases, contaminated sites can pose a danger to adjacent property, such as by entrainment of hazardous substances by local groundwater flow, and local laws frequently mandate remediation prior to the sale or lease of property wherein the soil has been contaminated with hazardous materials.
Various methods have been utilized for the treatment, remediation or disposal of petroleum sludge. These methods generally include permanent removal of the contaminated soil to a secure landfill, incineration, indirect thermal treatment, aeration, venting, air sparging and bioremediation. Removal of contaminated soil to landfills is no longer an attractive alternative on account of the high excavation, transportation and disposal costs, and because of the potential for residual liability. Incineration and indirect thermal treatment can be achieved either on-site or off-site, but in either case involves excavation, handling and treatment of substantially all of the contaminated soil as well as significant amounts of soil adjacent to the contaminated soil. The soil must then either be transported to the treatment facility or else the treatment apparatus must be installed on-site. In either case, these methods generally involve enormous transportation and handling costs, and require large amounts of energy to combust or volatilize the contaminants. Other elaborate and expensive techniques, which have been utilized, involve excavation and treatment of the contaminated soil using multistep unit operations for separating and recovering the soil from the contaminants. Removal, incineration, indirect thermal treatment and other methods of handling contaminated soil, which involve complete excavation of the contaminated soil, have the advantage that they can be accomplished in a relatively short amount of time. These methods are particularly attractive in those situations where there is a substantial risk that the contamination will rapidly spread to adjacent property. However, in most cases such methods are prohibitively expensive.

Old refineries have long lasting problem of safe disposal of spent acid tar (acidic sludge), which is accumulated over several decades as a result of acid treatment of wax and lube base oils. However, the acid treatment of lube oil and wax has been discontinued but there is huge
inventory of this acid tar in refineries. For example Digboi refinery in India have an inventory of 20,000-50,000 KL that is stored in the open sludge ponds. In past several attempts to dispose the accumulated spent tar has been made but met with only a limited success. Conventional practices generally do not provide a practical, affordable technology for remediating acidic sludge in ecofriendly and intensive manner.

It is known to exploit microorganisms to detoxify or degrade hydrocarbon contaminants. This treatment method is known as bioremediation. Bioremediation may be affected under aerobic and anaerobic conditions. Major requirements for effective bioremediation are:

- a biodegradable organic substrate, an appropriate and active microbial community (consortium), bioavailability of the polluting substrate to the microorganisms, and the creation of optimal conditions for microbial metabolism. Sometimes bioremediation requires further biostimulation with nutrients or some specific analogue substrate; it may also require bioaugmentation of the microbial community if the site does not have an appropriate indigenous biodegrading population. Bioremediation is a cost effective, less energy intensive and ecofriendly method for disposal of sludge. However, biodegradation of acidic sludge is a difficult task because at low pH the normal sludge degrading microorganisms are unable to grow.

U.S. Pat. No. 3,152,983 discloses the microbial disposal of oily wastes. This method is designed for large industrial waste separation and disposal, beginning with an oil water separator.

U.S. Pat. No. 3,462,275 discloses a process for treating biodegradable organic waste material using a thermophilic aerobic microorganism culture capable of digesting cellulose and produce cellular proteinaceous material.

U. S. Pat. No. 3,769,164 teaches a process for the microbial degradation of spilled petroleum by treating it with specially mutated
species of microorganisms *Candida parapsilosis* (ATCC 20246), *Aspergillus* sp. (ATCC 20253), *Nocardia corallina* (ATCC 21504), etc.

U.S. Pat. No. 3,838,198 is directed at conditioning raw waste input for digestion by thermophilic aerobic microorganisms. The process is geared towards animal waste.

U.S. Pat. No. 3,871,956 features a method for cleaning accidental oil spills on water or in soil. The method of this patent does not utilize temperature or oxygen controls and is not a self-perpetuating process.

U.S. Pat. No. 3,871,957 teaches methods of applying certain microorganisms for rapid dispersal of oil spills. The microorganisms so employed include a wide variety of bacteria yeasts, actinomycoses and filamentous fungi. This reference also teaches use of certain clays such as kaolin or zeolites as carriers for such microorganisms.

The US patent 4,415,662 discloses the use of a particular fungus to degrade petroleum crude oil and products. The method comprises the application to the crude petroleum and petroleum products in an environment of an effective amount of the fungus *Actinomucor elegans* (Ediam) Benj. and Hasselt., Strain No. TC-405, its enzymatic active principle compound, a broth comprising it, or a carrier medium comprising the fungus or its enzymatic active principal compound.

U.S. Pat. No. 4,668,388 discloses a high rate reactor for the treatment of biosludge, wherein the reaction is conducted in an enclosure.

U.S. Pat. No. 4,913,586 describes a process and apparatus for safely handling and detoxifying contaminated soil substantially saturated with contaminants such as fuel and petroleum hydrocarbons. The contaminated soil is treated with an additive consisting of low grade humic acid and lime that is mixed with the soil in the approximate ratio of nine parts soil to one part of additive. The treatment involves breaking down the contaminated soil particles to a fine silt or sand, mixing the additive into the soil in a
tumbler and, finally, discharging the thoroughly additive-covered and encapsulated toxic soil particles into a previously dug trench.

U.S. Pat. No. 5,035,537, discloses a method for treatment of soil, porous rock and similar material contaminated by petroleum, hydrocarbon and volatile organic compounds and includes the steps of gathering the contaminated soil, disbursing it uniformly on an impervious horizontal surface to a depth of four to six inches, treating it with an emulsifying agent and allowing the emulsifying agent to seep through the soil and volatilize the hydrocarbon and organic compounds in the soil.

U.S. Pat. No. 5,039,415, relates to a method of treating hydrocarbon contaminated soil by excavating the soil, forming the soil into a flowing particulate stream; forming an aqueous liquid mixture of water and a microbe-containing solution that reacts with hydrocarbon to form CO$_2$ and water; dispersing the liquid mixture into the particulate soil stream to wet the particulate; and allowing the substance to react with the wetted soil particulate to thereby form CO$_2$ and water.

U.S. Pat. No. 5,055,196 discloses a process for treating soil or sludge to remove contaminants in contact with the soil or sludge. More particularly, this method relates to a process in which inorganic contaminants, such as metal or metal salts, or organic contaminants, such as PCBs, are removed from water-wet soil and sludge.

United States Patent 5,059,252 discloses a method for on-site bioremediation of soils contaminated with petroleum derived hazardous wastes. This method for enhancing bioremediation includes the step of applying a cationic ion exchange resin to the contaminated soil in an amount sufficient to promote growth of organisms capable of degrading the hazardous waste.

United States Patent 5,415,777 discloses a method of decontaminating soil contaminated by petroleum products on the site of the contamination, which is accomplished by chemical breakdown,
enzymatic action and biological microbial degradation. Significant byproducts of this degradation of the hydrocarbon material are: water, sodium salts, ammonium salts, carbon dioxide, free amino acids and heat. United States Patent 5,427,944 teaches a process for biodegrading polycyclic aromatic hydrocarbon contaminants using a mixed bacteria culture of Achromobacter sp. and Mycobacterium sp and nutrient. The mixed bacteria culture was utilized for in situ or ex situ bioremediation of contaminated soil, or in any of various conventional bioreactors to treat contaminated liquids such as landfill leachates, groundwater or industrial effluents.

U.S. Pat. No. 5,453,133 relates to a process for removing contaminants, such as hydrocarbons, from soil. The process involves contacting the contaminated soil with a suitable solvent for the contaminant, in the presence of a bridging liquid, which is immiscible with the solvent, while agitating. The amount of the bridging liquid and the degree of agitation are balanced to control the particle size of the substantially contaminant- and solvent-free soil agglomerates so formed.

U.S. Pat. No. 5,494,580 relates to a method for decontamination of a hydrocarbon-polluted environment by the use of certain bacterial compositions.

United States Patent 5,609,667 discloses a method of treating hydrocarbon-contaminated soils. A powdered cellulose, containing essentially 3-8% of ammonium sulfate forms a biologically active media which preferentially adsorbs hydrocarbons in the presence of water and supports the growth of naturally occurring hydrocarbon reducing bacterial forms resulting in rapid decomposition of the adsorbed hydrocarbons into water, carbon dioxide and other benign waste products.

United States Patent 5,624,843 discloses method for improving the bioremediation of hydrocarbon contaminated water with indigenous microorganisms comprising adding to the hydrocarbon contaminated water
a hydrocarbon solution of an additive selected from the group consisting of
(a) a mixture of a sorbitan ester of a C\textsubscript{7} to a C\textsubscript{22} monocarboxylic acid and a
diacyloxyalkylene adduct of a sorbitan monoester of a C\textsubscript{7} to C\textsubscript{22}
monocarboxylic acid; the adduct having from 6 to 50 polyyoxyalkylene units,
(b) an alkylglycoside wherein the alkyl group has from about 8 to 18 carbon
atoms and the glycoside is a mono or a diglycoside, and (c) a mixture of (a)
and (b), the solution being added in amounts sufficient to promote the
growth of indigenous micro organisms.

United States Patent 5,807,724 teaches a method and means for
enabling the remediation and removal of spilled oils or fats (lipids) from
land, moving or standing fluids, slurries, or semi-solid, processed
manufactures, or from food substances using three particular species of the
yeasts (Candida albicans; Candida guillermondii; and Candida yarrowii) which
possess the capability to produce an enzyme, lipase, which is capable of
breaking down hydrocarbon-based substances, e.g., crude oil and other
petroleum distillates, either paraffin-based or possessing other peculiar
chemical bases.

US patent 5811290 describes a method for enhancing
bioremediation of hydrocarbon-contaminated soils, water and/or sludge
using urea-surfactant clathrate and U.S. Pat. No. 5,834,540 relates to a
composition and process for the remediation of contaminated materials,
and in particular for soil remediation. The reference relates to compositions
and processes that cause the breakdown of unwanted contaminates, such as
hydrocarbon wastes. The reference relates to the further use of radiation,
preferably microwave radiation, to effect the degradation of contaminates.
The reference is equally effective in treating materials that have been
exposed to harmful or infectious biological contaminates.

United States Patent 6,057,147 discloses an apparatus and method
for enhanced bioremediation of hydrocarbons removed from a
contaminated object. The device and method promote continuous
microbial bioremediation of hydrocarbon contaminants in a self-
propagating manner while cleaning the solution and filtering sediment
without generating an environmentally dangerous waste trail. The cleaning
solution comprises microorganisms of the genus *Achromobacter, Actinobacter,
Alcaligenes, Arthrobacter, Bacillus, Nocardia, Flavobacterium, Pseudomonas* and
mixtures thereof.

United States Patent 6,503,746 pertains to isolated strains
*Paenibacillus validus* that degrade polyaromatic hydrocarbons, a method of
isolating the strains of the invention and a method of using the strains of
the invention for bioremediation.

The United States Patent 20020187545 application discloses a
process for Bioremediation of hydrocarbon-contaminated waste using corn
material. The hydrocarbonaceous contaminant is contacted with the corn
material in the presence of nutrients and bacteria effective for
bioremediation.

The 20030100098 US patent application discloses the application of
bacterial strains to bioremediate polyaromatic hydrocarbon contaminated
soils and groundwater.

**OBJECTS AND SUMMARY OF THE INVENTION**

The object of the present invention is to develop a process that will
rapidly decontaminate acidic hydrocarbon sludge in an efficient and
environmentally acceptable manner. Accordingly, the object of the present
invention is to provide a method for bioremediation of acidic sludge by
acid tolerant hydrocarbon assimilating microbes.

The object is achieved by the present invention, which describes a
method and means for enabling the remediation and removal of acidic oily
sludge, spilled oils, and slurries.

The method of the present invention relates to isolation of two
particular species of the yeast which possess the capability of breaking
down hydrocarbon substances, e.g., crude oil and other petroleum
5 distillates, either paraffin-based or possessing other peculiar chemical bases in acidic as well as in neutral pH.

These isolates have been used to develop a process for remediation of acidic sludge.

The present invention relates to a process of remediation of acidic sludge, which comprises: mixing the sludge with soil, adding microbial consortia capable of degrading hydrocarbon to soil sludge mixture, in which the microorganism is supported on a carrier; and providing nutrient for the microorganism to the soil sludge mixture.

DETAILED DESCRIPTION OF THE INVENTION

In past several attempts to dispose the accumulated spent tar has been made but met with only a limited success. These methods generally include secure landfill, incineration, indirect thermal treatment, aeration, venting, air sparging and bioremediation. Sludge stored in storage pits was source for contamination the ground water. Conventional practices generally do not provide a practical, affordable technology for remediating acidic sludge in ecofriendly and intensive manner. One of the methods was neutralization with lime followed by drying and then use of the material in brick kilns as fuel. This method was not also successful because of combustion-related problems and emission issues.

However, as mentioned bioremediation of acidic sludge is a difficult task because at low pH the normal sludge degrading microorganisms are unable to grow. However, the specialized sludge-degrading microorganisms, which have special adaptations to survive in acidic environment, can degrade the sludge. Such microbes can be developed from indigenous microbial population of acidic sludge by the selective enrichment technique.

Major requirements for effective bioremediation are a biodegradable organic substrate, an appropriate and active microbial community (consortium), bioavailability of the polluting substrate to the
microorganisms, and the creation of optimal conditions for microbial
growth. Sometimes bioremediation requires further biostimulation with
nutrients, surfactants, or some specific analogue substrate; it may also
require bioaugmentation of the microbial community if the site does not
have an appropriate indigenous biodegrading population. In the
bioaugmentation the naturally occurring but selected specific microbes are
cultivated in laboratory condition and then supplemented in the field.

The process of development of bioremediation techniques includes
steps namely, Isolation and characterization naturally occurring
microorganisms with bioremediation potential by selective enrichment
technique, optimization of large-scale cultivation of these microbes and its
absorption on suitable carrier. Next step is evaluation of its ability to
degradate petroleum sludge and its different fraction separately in liquid
media as well as in lab scale soil experiments and optimization of
conditions like nutrient, moisture condition, aeration for enhanced
biodegradation in experimental conditions. Then the process is tested and
optimized in field.

The hydrocarbon-degrading microbe of present invention was
isolated by selective enrichment culture technique from indigenous
microbial population of acidic sludge taking acidic sludge as carbon source
and inoculum.

Enrichment culture technique is based on basic principle of
selection and allows growth of organism of interest, and as unfavorable as
possible for competing organisms. In this technique the growth parameters,
including cultural nutrient medium, conditions are controlled to favor the
development of a specific organism or group of organisms.

The first cycle of enrichment was carried out by adding 5 gms of the
soil in 100 ml of medium and flask were incubated at 30°C on shaking
condition. An enrichment cycle was repeated four times using above
mentioned minimal salt media and acid tar as sole carbon source.
A suitable nutrient medium for use during the enrichment process should, at minimum include a nitrogen source, such as an ammonium salt, and a phosphorus source, such as an alkali metal phosphate compound. The nutrient system used during the process of acclimating the mixed bacteria culture to hydrocarbon compounds desirably includes a magnesium source, such as a magnesium salt, and can optionally include other nutrients such as sodium, calcium and iron salts. A suitable nutrient system which can be effectively utilized during the enrichment process includes an ammonium salt and a phosphate compound, along with minor amounts of other conventional nutrients, wherein the molar ratio of elemental nitrogen to phosphorus is from about 5:1 to about 15:1, and more preferably from about 8:1 to about 12:1. A particularly preferred nutrient system for use during the enrichment process includes ammonium chloride, from about 5 to about 20 parts by weight of hydrated magnesium sulfate (M.sub.a SO.sub.4H.sub.2 0) per 100 parts by weight of ammonium chloride, from about 5 to about 20 parts by weight of sodium chloride per 100 parts by weight of ammonium chloride, and from about 15 to about 50, and more preferably from about 20 to about 30 parts by weight of monobasic potassium phosphate (KH.sub.2 PO.sub.4) per 100 parts by weight of ammonium chloride and traces of vitamins and trace elements. The pH of the medium was kept acidic preferably 3. The foregoing nutrients are dissolved in a suitable amount of water to dissolve the nutrients and combined with appropriate quantities of a suitable initial primary food source and the mixed bacteria culture. A suitable initial primary food source is generally total acidic sludge or its total petroleum hydrocarbon or its saturates, aromatic, asphaltene fractions. In particular, it is recommended that the acclimation process be carried out by adding samples of the soil, which is to be remediated to the mixed microbial culture.
Isolation of the pure desired isolates was done by streaking on minimal salt agar medium containing 0.05-5% acidic sludge as carbon source and pH of medium was maintained 3.0. These plates were incubated at 30°C. Eight microbial strains were obtained at the end of the enrichment cycle from the acidic tar sludge.

The two acidic sludge degrading isolates showing highest growth and degradation potential have been identified as *Candida digboigenesis* (dig, boi.en'sis) L. nom.Fem. Adj.digboigenesis refers to place Digboi in Assam state of India. These cultures are deposited in MTCC (Microbial Type Culture Collection and Gene Bank) at Institute of Microbial Technology, Chandigarh, under number MTCC 4371 and 4372 respectively. The type strain of *C. digboigenesis* is strain MTCC 4371 and also deposited in collection of Yeast Division of the Central bureau voor Schimmelcultures, Utrecht, The Netherlands. These two microbes were combined to make the microbial blend for bioremediation and were used in present invention.

The isolates were evaluated separately and in combination for their potential to degrade acid tar and its fractions at acidic pH. For that the individual isolate or mixture was inoculated in 5 ml of mineral salt medium. After 48 hr growth this 5 ml culture was transferred in 20 ml fresh medium. After 48 hrs growth 20 ml culture was transferred to minimal salt 80 ml, which contained acidic sludge or its toluene or hexane soluble fraction (1%) as carbon source. All experiments were carried out at pH 3 and 30°C temperature in shaking condition. After incubation of 10 days the residual substrate was measured. In addition, the ability of pure strains to grow separately on various fraction of acidic sludge at pH 3 was also examined.

After successful isolation and efficacy test in laboratory scale the microbial consortium/blend was grown in bioreactor. An inoculum for bioreactor, of the mixed culture of the present invention was prepared by first growing the individual microbe on separate agar plates in a conventional manner. After sufficient growth of the individual microbe is
achieved, both microbes can be transferred to a fresh agar plate for simultaneous growth of both organisms together in a mixed culture. After the mixed culture exhibits successful growth, it can be transferred to a suitable vessel containing a nutrient solution. The vessel preferably should contain control devices for temperature, pH, agitation, aeration and stirring.

Particularly preferred nutrient system for growth includes (g per liter) KH₂PO₄ 0.5-1.0, K₂HPO₄ 0.5-1.0, Mg SO₄ 0.5-1.0, (NH₄)₂SO₄ 0.25-0.75, KNO₃ 0.25-0.75, Trace element 5 ml to 20 ml of solution and Multi vitamin solution 0.5-5 ml, fermentable sugars as carbon source 1–5%.

The composition of Trace element solution (gram per liter) is Nitrilotriacetic acid 1-1.5, FeSO₄ .7H₂O 0.05-0.15, MnCl₂.4H₂O 0.005-0.015, CoCl₂.6H₂O 0.15-0.2, CaCl₂.2H₂O 0.05-0.15, ZnCl₂ 0.05-0.15, CuCl₂.2H₂O 0.01-0.03, H₃BO₃ 0.01-0.02, Na₂MoO₄ 0.01-0.02, Na₂SeO₃ 0.015-0.02, NiSO₄ 0.01-0.03, SnCl₂ 0.01-0.03. The composition of Trace element solution Multivitamin solution (g/l) is Biotin 0.001-0.003, Folic acid 0.001-0.003, Pyridoxine HCl 0.05-0.02, Thiamine HCl 0.002-0.008, Riboflavin 0.001-0.01, Nicotinic acid 0.002-0.01, Ca-Pentothionate 0.002-0.01, Lipoic acid 0.0025-0.0075, Vitamin B₁₂ 0.0005-0.0015, PABA 0.0025-0.0075.

The appropriately grown mixed microbial culture of the invention was adsorbed on suitable carrier. In the invention, microorganisms capable of degrading hydrocarbon contaminants are dispersed in soil while being supported on, i.e., fixed in a carrier.

As the carrier for supporting microorganisms there may be used any known material so far as it can be applied to contaminated soil with microorganisms supported thereon. From the standpoint of effective supporting of useful microorganisms, carrier materials, which can firmly adsorb microorganisms to the surface thereof, be helpful in transport and dispersal of final bioremediation agent.

The carrier made of the materials that can retain microorganisms
thereon relatively mildly and thus allows easy release of microorganisms thus proliferated. The carrier is inexpensive and can act as a nutrient source for the microorganisms thus applied, particularly a nutrient source, which can be gradually released to advantage.

Further, the formation of a carrier by a biodegradable material is advantageous in that any problems arising from secondary contamination by residual carrier or the effect of applied microorganisms on the soil ecological system can be avoided. As such a biodegradable carrier there is preferably used a material, which gradually decomposes and disappears after the remediation of soil by applied microorganisms. When such a carrier is used, applied microorganisms which have been released into soil after the disappearance of the carrier are then put in environments which are severe to growth such as competition with predominant native-born microorganisms in soil and predation by protozoan. The microorganisms are then driven out of soil and gradually decrease in number to extinction.

As a result, the ecological system in soil can be restored to the original state.

Preferred examples of the biodegradable carrier material employable herein include corn husk, sugar industry waste or any agricultural waste.

The water content of the carrier is from 1% to 99% by weight, preferably from 5% to 90% by weight, more preferably from 10% to 85% by weight. When the water content of the carrier is too low, microorganisms find difficulty in survival. On the contrary, when the water content of the carrier is too high, the resulting carrier exhibits a deteriorated physical strength that makes itself difficult to handle.

The carrier adsorbed microbial blend was tested for its efficacy in liquid medium as well in lab scale soil experiments. After successful lab scale validation the process was optimized in field. The parameters of field studies are volume of soil bed, sludge dosing and mixing, microbial blend dosing, nutrient composition and dosing and tilling and moisture content. A plot was brick lined and cemented and impermeable plastic sheet was put
in bottom. This area was demarcated with cemented boundary preferably of 45 cm height. In this pit a uniform soil layer preferably of 15 cm was prepared.

On the plot, acidic sludge was spread uniformly around 5 –40%, preferably 10% (w/w) and thoroughly mixed with soil bed.

After mixing bed was inoculated with microbial blends around 0.1-5% w/w based on sludge concentration. Inoculum size was preferably 0.2%.

Nutrients were also applied at the rate of 10 mg to 100 mg /100 kg of sludge after making its 5-25% solution in water.

As nutrients there are preferably used materials containing carbon, nitrogen and phosphorus. A culture solution suitable for the growth of microorganisms may be used. As such a culture solution there is widely used a material having a meat juice, a yeast extract, a malt extract, bactopeptone, glucose, inorganic salts, mineral, etc. in admixture at a proper ratio is widely used. These components may be mixed at a proper ratio depending on the kind of microorganisms. As the nutrients to be used in the invention there may be used any nutrients containing proper organic and inorganic nutrients besides the aforementioned culture solution. Preferable nutrient is mixture of yeast extract and potassium nitrate in 1:1.

The site was again tilled to mix the content i.e. acid tar and microbial blend along with nutrient, to a depth of around 7-10 cm. Tilling and watering of plot was carried out to enhance growth of applied microbes. The moisture content of the soil-sludge mix should be more preferably from 30% to 80% of the water retention capability of the soil - sludge mix. When the water content is too low, microorganisms find difficulty in survival. On the contrary, when the water content is too high, it stops aeration.

An important aspect of the remediation treatment is that mechanical mixing and tilling of soil sludge mix at starting of treatment and
in between of treatment. This helps in the aeration, which is essentially required of microbe of present invention for growth and survival. Preferably the tiling should be carried out in 15 days interval and it preferably up to depth of plastic sheet put in the bottom. The advantages of mechanical agitation to promote mixing are several. Tilling of the soil creates better contact between organic compound contaminants, and microbes to promote faster reaction rates and increase the likelihood that the degradative reactions will proceed to completion. Secondly, the thorough mixing enabled by mechanical agitation aids in controlling and dissipating then heat generated by the microbial growth.

The temperature at which soil remediation is effected needs to be suitable for the action of microorganisms, i.e., from 3°C to 50°C, preferably from 10°C to 45°C, more preferably from 18°C to 40°C. In order to keep the microorganisms within this temperature range, heating such as spraying and injection of hot water may be affected depending on the situation. In cold districts, a heat conductor may be inserted into soil so that heat from a heat source can be transferred to soil. Alternatively, the heat conductor inserted in soil may be electrically energized to heat soil. As such a heat conductor there may be used any material, which can transmit heat such as metal and ceramics.

The present invention therefore provides a safe, effective and inexpensive means for eliminating acidic oily sludge from environment.

To further illustrate the present invention, reference is made to the following examples-

Example 1

Isolation and Characterization of acidic sludge-degrading microbe

The nutrient media (mineral salt media, MSM) used for enrichment process includes 1.0% KH₂PO₄, 1.0 % K₂HPO₄, 0.5% Mg SO₄ 0.75%, NH₄Cl 2.0%, NaCl 0.2 % (V/V) of trace element solution and 0.01%
(V/V) vitamin solution. The trace element contained 0.15% nitrolotriacetic acid, 0.001% FeSO₄·7H₂O, 0.005% MnCl₂·4H₂O, 0.01 %CaCl₂·2H₂O, 0.05-% 0.05% ZnCl₂, 0.001%CuCl₂·H₂O. The composition of Multivitamin 0.01%Biotin, 0.03% Folic acid, 0.02%, Thiamine HCl, 0.01% Riboflavin, 0.02% Nicotinic acid, and 0.005%Vitamin B₁₂. The foregoing nutrients are dissolved in a suitable amount of water to dissolve the nutrients. In this medium 5% acidic sludge was used as inoculum and total carbon source. The pH of media kept acidic preferably 3. The first cycle of enrichment was carried out by adding 5 gms of the soil in 100 ml of medium and flask were incubated at 30°C on shaking condition. The cultures were monitored for the presence of microorganisms by gram staining and microscopy and subcultured into fresh MSM medium when growth was detected. After 3 to 4 subcultures, the bacteria were plated onto MSM agar plates containing the same substrate as the enrichment.

The mixed culture obtained by enrichment was evaluated for its potential to degrade acid tar. For that the mixed culture was inoculated in 5 ml of MSM medium. After 48 hr growth this 5 ml culture was transferred in 20 ml medium. After 48 hrs growth 20 ml culture was transferred to minimal salt 80 ml, which contained toluene and hexane extract of acidic sludge (1%) as carbon source. All experiments were carried out at pH3.

After incubation of 10 days the residual substrate was measured. Pure desired isolates from the mixed culture was isolated by streaking on minimal salt (MSM) agar medium containing .05-5% acidic sludge as carbon source and pH of medium was maintained 3.0. These plates were incubated at 30°C. Eight microbial strains were obtained at the end of the enrichment cycle from the acidic tar sludge.

In addition, the ability of pure strains to grow separately on various fraction of acidic sludge at pH 3 was also examined. The two acidic sludge degrading isolates showing highest growth and degradation have been
identified by MTCC (Microbial Type Culture Collection and Gene Bank) at Institute of Microbial Technology, Chandigarh. These were identified as *Candida digboigenesis* (dig, boi.en'sis) L. nom.Fem.Adj.digboigenesis refers to place Digboi in Assam state of India. These cultures are deposited in MTCC under number MTCC 4371 and 4372 respectively. The type strain of *C. digboigenesis* is strain MTCC 4371 and also deposited in collection of Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

**Example 2**

Large-scale cultivation and mixing with carrier

An inoculum of the mixed culture of the present invention was prepared by first growing the individual microbe on separate Luria agar plates in a conventional manner. After sufficient growth of the individual microbe is achieved, both microbes can be transferred to a fresh agar plate for simultaneous growth of both organisms together in a mixed culture.

After the mixed culture exhibits successful growth, it can be transferred to a suitable vessel containing a nutrient solution. The vessel preferably should contain control devices for temperature, pH, agitation, aeration and stirring. The temperature was kept 30°C, ph 3, stirring 200 2pm and air 5 L/min. Used nutrient system for growth includes (g per liter) KH₂PO₄ 0.5-1.0, K₂HPO₄ 0.5-1.0, MgSO₄ 0.5-1.0, (NH₄)₂SO₄ 0.25-0.75, KNO₃ 0.25-0.75, Trace element 5 ml to 20 ml of solution and Multi vitamin solution 0.5-5 ml, 10% sucrose as carbon source. The composition of Trace element solution (gram per liter) is Nitrilotriacetic acid 1-1.5, FeSO₄·7H₂O 0.05-0.15, MnCl₂·4H₂O 0.005-0.015, CoCl₂·6H₂O 0.15-0.2, CaCl₂·2H₂O 0.05-0.15, ZnCl₂ 0.05-0.15, CuCl₂·H₂O 0.01-0.03, H₂BO₃ 0.01-0.02, Na₂MoO₄·0.01-0.02, Na₂SeO₃·0.015-0.02, NiSO₄·0.01-0.03, SnCl₂·0.01-0.03. The composition of Trace element solution Multivitamin solution (g/l) is Biotin 0.001-0.003, Folic acid 0.001-0.003, Pyridoxine HCl 0.05-0.02, Thiamine HCl 0.002-0.008, Riboflavin
0.001-0.01, Nicotinic acid 0.002-0.01, Ca-Pantotheconate 0.002-0.01, Lipoic acid 0.0025-0.0075, Vitamin B12 0.0005-0.0015, PABA 0.0025 -0.0075.

The appropriately grown mixed microbial culture of the invention was adsorbed on previously UV sterilized corn floor in ratio 1:6(v/w). This material was packed in UV sterilized plastic bag keeping 80% space for air and it was sealed.

Example 3

Biodegradation in liquid media

The media used for biodegradation consisted of MSM supplemented with 0.25% yeast extract. Runs to determine the biodegradation of sludge with respect to incubation time were carried out in 250-ml Erlenmeyer flasks containing 50 ml of media and 5 g of sludge. The flasks were inoculated with 3 g of microbial blend adsorbed on carrier material; described above the flasks were incubated at 30 °C and at 220 rpm.

Residual total petroleum hydrocarbons (TPH) content was determined by extraction with 1:1 mixture of hexane and toluene. The solvent layer was separated in separating funnel and transferred to pre-weighted vial. The solvent was evaporated in fume hood and residual oil was weighed to determine total petroleum hydrocarbons.

The results are as follows-

Table 1: Biodegradation of acidic sludge in soil in liquid media

<table>
<thead>
<tr>
<th>Incubation time (days)</th>
<th>TPH degradation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>22</td>
</tr>
<tr>
<td>14</td>
<td>41</td>
</tr>
<tr>
<td>21</td>
<td>63</td>
</tr>
<tr>
<td>28</td>
<td>78</td>
</tr>
</tbody>
</table>
It was found that over a period of 56 days, about 92% degradation of TPH occurred.

Example 4

Degradation of Sludge in soil

A plot of 200 square meters was marked and lined with cemented brick and impermeable plastic sheet was put in bottom over the bricklayer. This area was demarcated with cemented boundary of 45 cm height. In this pit a uniform soil layer preferably of 15 cm was prepared. On the plot around 5 MT of acidic sludge was spread uniformly. This was thoroughly mixed with soil bed.

After mixing bed was inoculated with 80 kg microbial blends along with 3 kg of nutrient (KNO3 and yeast extract in 1:1 (w/w) dissolved in 100 lit of tap water. The site was again tilled to mix the content. Mechanical tilling of site was carried out on every 15 days. Watering was carried out to maintain appropriate moisture level.

Residual total petroleum hydrocarbons (TPH) content was determined as follows: At timed intervals, 10 g of representative soil-sludge mix was refluxed with 50 ml of hexane. The residual material was then refluxed with 50 ml of toluene. The solvent layers was separated in separating funnel and pooled and transferred to preweighted vial. The solvent was evaporated in fume hoods and residual oil was weighted to determine total petroleum hydrocarbons.

The results are as follows -

Table 2: Biodegradation of acidic sludge in soil

<table>
<thead>
<tr>
<th>Days</th>
<th>TPH degradation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The pH of the samples was determined by taking 25 g of representative and homogenized sample of sludge/soil mix in a beaker containing 25 ml ultra pure water and allowed to mix in rotatory shaker at 180 rpm for four hours. The content were allowed to settle and filtered. The pH of the supernatant was measured by pH meter on zero days and after every 30 day of bioremediation.

The results are as follows-

Table 3. pH profile of soil mixed with acidic sludge under treatment

<table>
<thead>
<tr>
<th>Days</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.9</td>
</tr>
<tr>
<td>60</td>
<td>3.6</td>
</tr>
<tr>
<td>90</td>
<td>4.3</td>
</tr>
<tr>
<td>120</td>
<td>5.2</td>
</tr>
<tr>
<td>150</td>
<td>5.7</td>
</tr>
</tbody>
</table>
We claim:

1. A process of remediation of acidic sludge, which comprises: mixing the sludge with soil, adding microbial consortia capable of degrading hydrocarbon, to soil-sludge mixture, in which the microorganisms is supported on a carrier; and providing nutrient for the microorganisms to the soil-sludge mixture.

2. The method of remediation of acidic sludge as claimed in claim 1, wherein the microorganisms capable of degrading acidic sludge include microorganisms capable to degrade aromatic, aliphatic, naphthenic and other fraction of sludge at acidic pH.

3. The process of remediation of acidic sludge as claimed in claim 1, wherein the microorganism is yeast.

4. The process of remediation of acidic sludge as claimed in claim 3, wherein the microbial consortia includes Candida dighoigensis MTCC 4371 and Candida dighoigensis MTCC 4372.

5. The method of remediation of acidic sludge as claimed in claim 1, wherein the carrier for support of microorganisms is biodegradable.

6. The method of remediation of acidic sludge as claimed in claim 1, wherein the carrier is made of materials that can retain microorganisms thereon relatively mildly and thus allows easy release of microorganisms thus proliferated.
7. The method of remediation of acidic sludge as claimed in claim 1, wherein the microorganisms are supported on the natural carrier material like cornhusk, sugar industry waste or any agricultural waste.

8. The method of remediation of acidic sludge as claimed in claim 1, wherein the water content of the carrier is from 1% to 99% by weight.

9. The method of remediation of acidic sludge as claimed in claim 1, wherein the water content of the carrier is 10% to 85% by weight.

10. The method of remediation of acidic sludge as claimed in claim 1, wherein the nutrients are compounds that can be used as nitrogen source and phosphorus source by microorganisms for growth.

11. The method of remediation of acidic sludge as claimed in claim 10, wherein the nutrient system further includes a magnesium source.

12. The method of remediation of acidic sludge as claimed in claim 10, wherein the nutrient system optionally includes sodium, calcium and iron salts.

13. The method of remediation of acidic sludge as claimed in claim 10, wherein the nitrogen source is an ammonium salts.

14. The method of remediation of acidic sludge as claimed in claim 10, wherein the phosphorus source is an alkali metal phosphate compound.

15. The method of remediation of acidic sludge as claimed in claim 10, wherein the molar ratio of elemental nitrogen to phosphorus is 5:1-15:1.

16. The method of remediation of acidic sludge as claimed in claim 10, wherein the preferable molar ratio of elemental nitrogen to phosphorus is 8:1-12:1.

17. The method of remediation of acidic sludge as claimed in claim 1, wherein the nutrient system has the following composition ammonium chloride, from about 5 to about 20 parts by weight of hydrated magnesium sulfate (M.sub.a SO.sub.4.H.sub.2O) per 100 parts by
weight of ammonium chloride, from about 5 to about 20 parts by weight of sodium chloride per 100 parts by weight of ammonium chloride, and from about 15 to about 50 and more preferably from about 20 to about 30 parts by weight of monobasic potassium phosphate (KH.sub.2 PO.sub.4) per 100 parts by weight of ammonium chloride and traces of vitamins and trace elements.

18. The method for remediation of acidic sludge, comprising the steps of: Mixing the sludge with soil on to an impermeable sheet of material and introducing into the soil sludge mix an inoculum comprised of mixed yeast culture, which have been acclimated to degradation of sludge at acidic pH along with a nutrient system containing a nitrogen source and a phosphorus source.

19. The method of claim 8, further comprising the step of:
   periodic tilling the soil-sludge mixes to loosen and to increase aeration for bacterial growth;
   maintaining the moisture content, which is at least 80% of the water retention capability of the soil –sludge mix; and
   testing the soil-sludge mix periodically to monitor reduction in amount of hydrocarbon contaminants contained in the soil.
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   U.S. :

   Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

   Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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</thead>
</table>

Further documents are listed in the continuation of Box C. See patent family annex.

Date of the actual completion of the international search

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Date of mailing of the international search report
DEC 2005

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Form PCT/ISA/210 (second sheet) (July 1998)
Isolation of a novel yeast strain Candida digboiensis TERI ASN6 capable of degrading petroleum hydrocarbons in acidic conditions

Nitu Sood, Banwari Lal

The Energy and Resources Institute (TERI), Darbari Seth Block, India Habitat Centre, Lodhi Road, New Delhi 110 003, India

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A B S T R A C T

A novel yeast species Candida digboiensis TERI ASN6 was isolated from soil samples contaminated with acidic oily sludge (pH 1–3) from the Digboi refinery (Northeast India). The strain TERI ASN6 could degrade 73% of the total petroleum hydrocarbons present in the medium at pH 3 in a week. This strain presents a dimorphic behaviour and showed mycelia morphology when grown under stressed conditions such as low pH and in a medium containing petroleum hydrocarbons. The C. digboiensis strain could efficiently degrade the aliphatic and aromatic fractions of the acidic oily sludge at pH 3 as confirmed by gas chromatography. During the growth of TERI ASN6 in dibenzothiophene (DBT), DBT-sulfone and biphenyl-2-ol were detected. An active cytochrome P450 system, implicated in hydrocarbon oxidation, was also detected in this yeast using degenerated primers based on its conserved regions. This yeast is a potential candidate for petroleum bioremediation treatment of hydrocarbon contaminated acidic soils. Its physiological behaviour allows the strain to work efficiently where other hydrocarbon-degrading bacteria may not survive.

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1. Introduction

India’s estimated oil reserves are 5 billion barrels with exploitation still on. Through its 18 refineries, India processes approximately 128 million metric tonnes of crude oil per annum (TEDDY, 2006). These refineries generate enormous amounts of oily sludge every year as a result of crude oil processing (0.002–0.1% of the weight of crude processed). This oily sludge contains alkanes, aromatic hydrocarbons, NSO (nitrogen, sulphur and oxygen) compounds and asphaltenes (Bhattacharya et al., 2003). Safe disposal of this oily sludge is a major problem for refineries since it threatens the environment through soil and ground water pollution. Oily sludge constituents are also known to be potent carcinogens and immunotoxicants (Mishra et al., 2001). The Digboi refinery in Assam state of north-eastern India, however, is faced with a rather unique problem. It has nearly 50,000 tonnes of an acidic oily sludge (pH 1.5–3) lying inside the refinery premises. The acidity of this sludge is owing to sulphuric acid that was used in old wax refining methods in the refinery. While the use of sulphuric acid is now obsolete, the existing inventory is in itself a huge bioremediation challenge. When oily sludge disposal strategies like incineration, brick making etc. were met with limited success microbial bioremediation was attempted as an environmentally benign and economically feasible alternative. As bioremediation candidates, both bacteria and fungi are relatively plentiful in soil and both the groups contribute substantially to the biodegradation of hydrocarbons (Bartha and Bossert, 1984). The low pH at Digboi, however, prevented the growth of known hydrocarbon-degrading microorganisms to survive in this sludge. It was therefore required to isolate microorganisms from this acidic sludge contaminated environment that would be better adapted to degrade the contaminating hydrocarbons at low pH.

The present study details the isolation of a novel yeast strain from the Digboi refinery that is capable of utilizing hydrocarbons at pH 3. The ability of this strain to utilize alkanes and transform aromatic hydrocarbons to their oxidative forms has also been presented. The strain was identified as a novel yeast strain Candida digboiensis and has been described by Prasad et al. (2005). The effect of stressful growth conditions on the morphology of C. digboiensis TERI ASN6 and the possible advantage of the dimorphic behaviour to this yeast strain when applied to the bioremediation field is also discussed.

2. Methods

The oily sludge contained 45% aromatic fraction and around 25% each of alkanes and NSO (nitrogen, sulphur and oxygen) containing compounds and asphaltenes with a pH ranging from 1 to 3.

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2.1. Enrichment and isolation of acidic oily sludge degrading microbes

The microbial strains were isolated by enrichment culture technique from soil samples contaminated with acidic oily sludge from Digboi refinery, Assam, north-eastern India. The composition of the acidic oily sludge was determined following the protocol described by Mishra et al. (2001). For enrichment, 5 g of soil samples were inoculated into 100 mL of minimal salts medium (MSM) (0.1% K2HPO4, 0.1% KH2PO4, 0.05% MgSO4 7H2O, 0.05% NH4Cl, 0.05% KNO3 (w/v) and 0.1% (v/v) trace metals solution in MiliQ water), pH 3 (adjusted with H2SO4). The trace metals solution contained 0.15% nitrolotrriaic acid, 0.5% MnSO4.2H2O, 0.001% FeSO4, 0.01% CaCl2.2H2O, 0.01% ZnSO4, 0.001% CuSO4.5H2O, 0.01% CoCl2, 0.001% AlK(SO4)2, 0.001% H3BO3 and 0.001% Na2MoO4. The medium was supplemented with steam sterilized oil sludge (1% w/v) as carbon source and incubated at 30 °C on a rotary shaker (180 rpm) for 7 days. After ten cycles of enrichment, 1 mL of the culture was diluted 103 fold, and 100 μL were plated on MSM agar plates with oily sludge (0.1% w/v) as the carbon source. The colonies obtained were further purified on the MSM (pH 3) agar plates with oily sludge (0.1% w/v) as the carbon source. The isolates were routinely subcultured in MSM containing the hydrocarbons of the oily sludge as the carbon source and stored in 25% glycerol at −70 °C.

2.2. Degradation of petroleum hydrocarbons under acidic conditions by the selected isolates

Degradation of the total petroleum hydrocarbons of the acidic oily sludge by the isolates was monitored in 250 mL Erlenmeyer flasks (in triplicate) containing 50 mL MSM (pH 3) with 0.1% (w/v) of the petroleum hydrocarbons of the oily sludge as the sole carbon source and flasks were incubated on a rotary shaker (180 rpm) at 30 °C for 7 d. The isolates were grown overnight in Luria Bertani broth (LB, HiMedia) (pH 3) to a cell density of 107 CFU mL−1 and 5% (v/v) inoculum was used to inoculate MSM with oily sludge as the sole carbon source. Un inoculated controls were maintained to monitor abiotic loss of the oily sludge. The residual underestimated oil sludge hydrocarbons were extracted thrice with equal volumes of hexane and chloroform.

The profile of the hydrocarbon fraction, extracted from inoculated flasks, was compared with that obtained from un inoculated control flask. The isolate with the maximum degradation was selected for further studies.

2.3. Characterization of the efficient acidic petroleum hydrocarbon-degrading strain

2.3.1. Hydrocarbon utilization profile

The ability of the selected strain to utilize hydrocarbons as the sole carbon source was tested by growing it in minimal salts medium (MSM) with different hydrocarbons in separate flasks for 72 h. Alkanes with varying carbon chain lengths ranging from octane (C8) to triacontane (C30) and aromatic hydrocarbons (fluoranthene, pyrene, phenanthrene and dibenzo[ghi]perylene) were tested. The medium was inoculated with 5 mL of a 24 h-old-culture of the selected isolate, grown in LB broth at pH 3 and incubated at 30 °C under shaking conditions. The culture growth was monitored by absorbance measured at 600 nm (UV–vis Hitachi spectrophotometer) and confirmed by measuring the total cell protein content. Protein was measured using Biuret’s method (Layne, 1957). The ability of the strain to grow in MSM with hydrocarbons as the sole carbon source was indicative of its ability to utilize the hydrocarbons.

2.3.2. Morphological characterization

The morphological peculiarities of the selected strain were studied by growing the strain in MSM containing petroleum hydrocarbons as the carbon source and media without hydrocarbons. Non-hydrocarbon media were GYP (1% (w/v) each of glucose, yeast extract and peptone) and Luria Bertani broth. Petroleum hydrocarbon containing MSM was prepared by adding 0.1% (w/v) of the following hydrocarbon substrates: the straight chain alkane, eicosane, the polycyclic aromatic hydrocarbon (PAH), fluoranthene, crude oil and the hydrocarbons of the acidic oily sludge as the sole carbon source.

Solid medium was prepared by adding agar (HiMedia) separately to the broth after autoclaving. All the solid and liquid media were prepared at pH 3 and 7.

The morphology of the colonies on the solid media was monitored at 4× magnification (Olympus microscope BH2, USA) and the cells were observed at 40× magnification.

The selected strain was cultivated in LB and GYP broth. The overnight grown culture was centrifuged at 5000 rpm for 1 min. The cells were then resuspended in phosphate buffer and centrifuged again. After three such washes, the cells were fixed overnight in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The cells were again washed with the buffer following which sputter coating (SCD 020) of the sample was done. Scanning electron microscopy (SEM) was performed using the LEO 435 VP (30 kV voltage) electron microscope at All India Institute of Medical Sciences (AIIMS), New Delhi, India.

2.4. Degradation of the alkane and aromatic fractions of the acidic oily sludge

In order to determine the degradation of the alkane and aromatic fractions of the oily sludge by the selected isolate, the total petroleum hydrocarbon (TPH) was obtained following the method described by Mishra et al. (2001). The alkane and aromatic fractions obtained using this method were then used as the carbon source (0.1% w/v) in MSM at pH 3 to determine the extent of degradation by the selected isolate. To study the degradation, flasks were inoculated with 5% (v/v) of a 24 h-old-culture of the selected isolate (107 CFU/ml) in LB (pH 3) and incubated at 30 °C under shaking conditions (180 rpm). Un inoculated control flasks were maintained to monitor abiotic losses. After 7 days of incubation, the degraded alkane hydrocarbon residue was extracted thrice with equal volumes of hexane and the aromatic residue with toluene. The degradation was quantified gravimetrically after the evaporation of solvents. One microtitre of the alkane fraction (dissolved in 10 mL hexane) was analyzed by gas chromatographic analysis (GC Hewlett Packard 5890 series II) fitted with Flame Ionization Detectors (FID) and DB 2887 methyl silicon 10 m long column (0.53 mm × 3 μm film thickness). Similarly, the aromatic fraction was dissolved in 5 mL acetone and 1 μl was analyzed by GC–FID using a 30 m long DB 5.625 (0.25 mm × 0.25 μm film thickness) column. During analysis the injector and detector of GC were maintained at 300 °C and the oven temperature was programmed to rise from 80 °C to 240 °C with an increase of 5 °C per minute and then held at 240 °C for 30 min. Individual compounds present in the alkane and aromatic fractions were determined by matching the retention times with authentic standards (The n-alkanes: C6, C10, C11, C12, C13, C14, C17, C18, C19, C20, C21, C22, C23, C25, C28, and C30 and the aromatic hydrocarbon standards fluoranthene, pyrene, phenanthrene, dibenzo[ghi]perylene were obtained from Sigma-Aldrich, USA).
2.5. Biotransformation of aromatic hydrocarbons phenanthrene and dibenzothiophene by the selected strain

The intermediate metabolites detected during the growth of the selected strain on dibenzothiophene and phenanthrene were analyzed by GC–Mass Spectrometry (GC–MS, Perkin-Elmer, USA). The isolate was grown for 24 h in LB (pH 3) (10^7 cells/ml) and 5% (v/v) inoculum was added to the 2 L Erlenmeyer flasks containing 500 ml of minimal salts medium (supplemented with 0.5% (w/v) yeast extract) with 0.01% (w/v) each of dibenzothiophene and phenanthrene separately. Cells were harvested by centrifugation at 7500 × g on the 5th day of growth and the entire supernatant was acidified to pH 2 with 2 N HCl. The intermediate metabolites were extracted three times with equal volumes of diethyl ether. The extracts were concentrated to 1 ml and derivatized following the protocol of Kaushik and Agnihotri (1997). After cooling, the derivatized sample (1 µl) of the extract was analyzed by GC–MS. A DB5 MS column was used for analysis with Helium (1 ml/min) as the carrier gas. The injector was maintained at 300 °C for 3 min and then held for 30 min at this temperature. The MS was programmed to rise from 80 °C to 300 °C with an increment of 5 °C/min and then held for 30 min at this temperature. The MS was programmed with a two-minute solvent delay and a mass scan range of 50–450 with an EI of 70 eV. Various intermediate metabolites were identified by matching the retention times with authentic standards and by comparing the mass spectrum of the intermediates with those of the standard compounds of the mass spectral libraries (Turbomass Gold software- NIST-Mass Spectral Library).

2.6. Detection of the cytochrome P450 gene in the selected strain

The total RNA from the cells induced by octadecane was prepared using the Qiagen RNeasy kit following the manufacturer’s protocol (Qiagen, USA). RT-PCR was carried out by using the degenerated primers Helix I and HR2 designed by Iida et al. (1998) based on the sequences of the conserved helix and heme binding regions of cytochrome P450. The PCR reaction was 35 cycles of denaturation for 20 s at 94 °C, annealing for 20 s at 45 °C, and extension for 60 s at 72 °C. The resulting PCR product was applied on agarose gel electrophoresis and DNA fragment was recovered, purified using the Microcon column (Millipore Corporation, USA) and sequenced using the ABI Prism 310 Genetic Analyzer according to the supplier’s instructions (Applied Biosystems). The cytochrome P450 partial sequence was submitted to GenBank.

3. Results and discussion

3.1. Isolation and screening of microorganisms capable of degrading the hydrocarbons of the acidic oily sludge

The enrichment procedure for obtaining acidic oily sludge hydrocarbon-degrading microbes was performed in minimal salts medium in multiple cycles to ensure that the microbes that were obtained at the end of the enrichment cycle were capable of utilizing the acidic oily sludge rather than just tolerating it. The enrichment protocol from acidic oily sludge contaminated soil yielded eight isolates. These isolates showed a varying degradation profile for the total petroleum hydrocarbon (TPH) of the oily sludge. It varied from 27.7% to 77% of the 100 mg TPH used as the sole carbon source in a week. The isolate with the best degradation potential was selected for further studies.

3.2. Characterization of the selected strain

3.2.1. Hydrocarbon utilization profile

The selected isolate studied in this work had previously been identified by Prasad et al. (2005) as C. digboiensis TERI ASN6. The isolate TERI ASN6 was capable of growing on the medium containing petroleum hydrocarbons, from octane (C8) to triacontane (C30), as the sole carbon source. This includes the straight chain petroleum hydrocarbons found in the oily sludge at Digboi refinery. However, maximum growth was observed on octadecane (C18) and heptadecane (C17). The hydrocarbon utilization pattern of TERI ASN6 indicated that the growth was less on short chain alkanes like octane, decane, undecane, dodecane and tridecane but it started increasing from tetradecane with a maximum growth being observed on heptadecane, octadecane and nonadecane after which the growth was again comparatively reduced with tricosane, tetradecane, octacosane, and triacontane (Fig. 1). The hydrocarbon utilization pattern by TERI ASN6 was the same as determined by total cell protein and absorbance. TERI ASN6 is capable of utilizing a wide range of hydrocarbons under acidic conditions, with a preference for alkanes with intermediate carbon chain lengths. The decreased growth pattern in short chain could be attributed to the toxicity of these alkanes to cell membranes (Teh, 1975) while growth on long chain alkanes could be limited by decreased bioavailability of the substrate (Lal and Khanna, 1996). While TERI ASN6 was capable of efficient utilization of alkanes as the carbon source, it was unable to completely degrade aromatic hydrocarbons...

![Fig. 1. Hydrocarbon utilization ability of TERI ASN6 measured in terms of growth in 72 h on alkanes (0.1% w/v) as the sole carbon source with respect to uninoculated controls.](image-url)
as the sole carbon source. However, oxidation of the aromatic hydrocarbons was observed.

3.2.2. Morphological characterization

The composition of the medium directly influenced the growth and the phenotype of TERI ASN6. It was observed that TERI ASN6 exhibited yeast morphology in GYP medium at pH 7 (Fig. 2a, e). However, it produced pseudohyphae when grown on the same medium at pH 3 (Fig. 2b, f). When grown on LB agar pH 3, the cells of TERI ASN6 showed pseudohyphal morphology (Fig. 2h, d) while on LA pH 7, the cells were initially in the yeast form (Fig. 2g) but after 24 h of growth the cells switched to the pseudohyphal form (Fig. 2c).

Fig. 2. Cellular morphology of TERI ASN6 growing on non-hydrocarbon liquid and solid media by scanning electron microscopy (a, b, c, and d) and light microscopy (e, f, g, and h). (a, e) yeast morphology on Glucose yeast extract peptone medium at pH 7; (b, f) pseudohyphal formation in Glucose yeast extract peptone medium at pH 3; (c) pseudohyphae and (g) yeast morphologies in Luria Bertani Agar medium at pH 7; (d, h) pseudohyphal formation in Luria Bertani Agar medium at pH 3.
When grown on MSM with hydrocarbons as the carbon source, the cells of TERI ASN6 showed only mycelial morphology. The cells were not observed in the yeast form in this medium with any hydrocarbon source. The growth of TERI ASN6 on MSM containing eicosane, fluoranthene or crude oil as the carbon source showed a variation in the morphological pattern. The pattern of mycelial emergence from the edges of the colonies was uniform when they grew on crude oil (Fig. 3c) unlike the mycelial pattern of TERI ASN6 on eicosane (Fig. 3a) and fluoranthene (Fig. 3b) carbon sources. The growth of this yeast strain on acidic oily sludge however, showed distorted colony structure (Fig. 3d). The morphological behaviour of TERI ASN6 remained unchanged at incubation temperatures of 30°C and 37°C if the same medium was used.

Fungi, like all living organisms, must be able to respond to changes in environmental conditions and hence develop a response which enables their adaptation to the new physiological situation (Alonso-Monge et al., 1999). In C. digboiensis TERI ASN6, like many other yeasts, this response comprises formation of filaments. In this study, C. digboiensis TERI ASN6 was challenged with conditions similar to those found in open-field such as hydrocarbon substrates, pH and temperature fluctuations. The isolate TERI ASN6 always produced pseudohyphae or hyphae in addition to the yeast form at low pH irrespective of medium components or growth temperatures. This is in contrast to the dimorphic behaviour exhibited by Candida albicans and Mucor rouxii which occur as yeast cells in acidic conditions and as filaments near neutrality (Bartnicki-Garcia, 1962). The hydrocarbon utilizing yeast Yarrowia lipolytica also shows maximum mycelial formation at pH 7 and almost no mycelia at pH 3 (Ruiz-Herrera and Sentandreu, 2002). The haploid cells of the plant pathogen Ustilago maydis, on the other hand, show filamentation in acidic conditions like C. digboiensis TERI ASN6 (Sánchez-Martínez and Pérez-Martín, 2001).

The dimorphic behaviour of the TERI ASN6, like Y. lipolytica (Perez-Campo and Dominguez, 2001), does not seem to be affected by temperature variation. For environmental applications like bioremediation, the tolerance of slight temperature fluctuations augurs well for the performance of the isolate in the field. C. digboiensis TERI ASN6 was however, very strongly affected by the carbon source provided in the growth medium. At neutral pH, when GYP provided glucose to this strain, luxurious growth was observed in the yeast form, however, when grown on LB agar (pH 7) in the absence of glucose, it was observed to form pseudohyphae to forage nutrients. Because of the limited bioavailability of hydrophobic hydrocarbons, C. digboiensis TERI ASN6 produced hyphae and pseudohyphae, to reach the substrate, as a response to carbon stress. The earlier onset of filaments on the solid medium compared with those in liquid medium also indicates the mycelial emergence is due to nutritional stress, since the point of colony growth would be locally starved of nutrients whereas in an agitated liquid culture there would be a uniform mixing of nutrients.

Morphological differentiation plays a vital role in the pathogenesis of fungal infection (Ferretti de Lima et al., 2004). The invasive filaments help penetrating the substrate, thereby allowing better access to nutrients in the surrounding growth environment. We believe that, just like pathogenic fungi, the environmental isolate TERI ASN6 has adapted both nutrient and stress response systems to trigger morphological changes that allow it to survive in harsh conditions and to adapt better in unfavourable environments.

3.3. Degradation of the alkane and aromatic fractions of the acidic oily sludge by C. digboiensis

The strain TERI ASN6 was capable of utilizing 73.64% of the total petroleum hydrocarbon of the oily sludge in a week as determined by gravimetric weight loss technique. The strain TERI ASN6 was grown in MSM containing variable concentrations of the acidic oily sludge (0.05% (w/v) to 1% (w/v)). Maximum degradation efficiency was observed at 0.1% (w/v) concentration. Hence this concentration
was selected for the degradation study. The degradation of the
alkane and aromatic hydrocarbons by TERI ASN6 at pH 3 was
analyzed by gas chromatography. The strain TERI ASN6 depicted
efficient utilization of the alkane fraction compared to the
untreated control (Fig. 4). The strain was capable of degrading
the entire range of n-alkanes from tetradecane to dotriacontane.
The strain also showed considerable utilization of components
from aromatic fraction as shown in Fig. 5. However, the oxidation
of alkanes was considerably more profound than that for the aromatic
hydrocarbons. This finding is in agreement with other reports
where hydrocarbon-degrading microbes have shown preferential
degradation of alkanes to aromatics and NSO-asphaltene fractions
(Lal and Khanna, 1996; Mishra et al., 2001).

3.4. Biotransformation of phenanthrene and dibenzothiophene by
C. digboiensis

Identification of the oxidation products showed a peak at 27 min
in GC EIMS with a molecular ion at m/z 194 (Fig. 6a). This mass
fragmentation pattern is indicative of the introduction of an oxygen

Fig. 4. Gas chromatogram showing the biodegradation of the alkane fraction (0.1% w/v) of acidic oily sludge from Digboi refinery by Candida digboiensis TERI ASN6 at pH 3 compared to the control.

Fig. 5. Gas chromatogram showing the biotransformation of the aromatic fraction (0.1% w/v) of acidic oily sludge from Digboi refinery by Candida digboiensis TERI ASN6 at pH 3 compared to the control.
atom to the phenanthrene molecule. Trace amounts of the phenanthrene conjugate phenanthrene quinone were also detected.

The strain C. digboiensis TERI ASN6 was unable to utilize dibenzothiophene (DBT) as the sole carbon source for its growth as evidenced by the limited growth observed when it was grown in MSM using DBT as the sole carbon and energy source. However, during the growth of this strain in the presence of DBT, two oxidative metabolites of the DBT biodesulfurization pathway were
detected. The two metabolites, DBT-sulfone and biphenyl-2-ol were detected by GC–MS and identified by comparing the mass spectrum of these metabolites with authentic standards. The [M+H]+ at m/z 216 and the fragment ion at m/z 187 were identical to those of the standard DBT-sulfone (Fig. 6b) and biphenyl-2-ol with the parent ion at m/z of 170 was also detected (Fig. 6c). The DBT desulfurization pathway is known to occur among bacteria being most notable among Rhodococcus IGTS8 (Kilbane and Jackowski, 1992) and Rhodococcus erythropolis strain D-1 (Bressler and Fedorak, 2000). Besides these, Beijerinckia has also been reported to oxidize DBT (Laborde and Gibson, 1977) and Corynebacterium SY1 (now Rhodococcus) utilizes DBT as the sole sulphur source (Omori et al., 1998). Among eukaryotes some fungi including lignolytic fungi have been studied for PAH degradation and oxidative transformation of DBT. The lignolytic enzymes that lead to the formation of quinones as well as fungal cytochrome P450 monooxygenases are responsible for the oxidation of a wide variety of aromatic hydrocarbons (Prenafeta-Boldú et al., 2006). Cytochrome P450 mediated benzo(a)pyrene hydroxylation has been studied in Phaneracheta chrysosporium (Masaphy et al., 1996). The cytochrome P450 detected in Pleurotus ostreatus have been implicated in oxidation of fluorene and DBT similar to the nonlignolytic fungus Cunninghamamella elegans (Bezalel et al., 1996). There are, however, fewer reports of desulfurizing yeasts. Baldi et al. (2003) studied

Fig. 6. (continued).

Fig. 7. Phylogenetic tree based on the partial Cytochrome P450 sequence analysis with the sequences of closely related genes of other species. Bootstrap values are shown at branch points.
a basidiomycete yeast strain Rhodosporidium toruloides for its ability to utilized DBT as a sulphur source. An active cytochrome P450 system has been detected in our strain Candida digboiensis TERI ASN6, which we believe might be involved in the desulfurization of DBT. To the best of our knowledge, this is the first report of biodesulfurization of dibenzothiophene by a Candida sp.

3.5. Detection of the cytochrome P450 gene in C. digboiensis

Alkane assimilating yeasts have P450s that can be induced by alkanes. The genes encoding P450 which participate in n-alkane metabolism belong to the CYP 52 family and have been cloned from Candida maltosa, Candida tropicalis and Candida apicola (Iida et al., 1998). The degenerate primers Helix 1 and HR2 corresponding to the conserved Helix and heme binding regions were used. After the RT-PCR reaction the PCR product of 395 bp was purified and sequenced (GenBank accession no. DQ846834). The deduced amino acid sequence of the PCR product was similar to the cytochrome P450 alkane hydroxylases of Y. lipolytica and Candida strains (40–60% identity). This sequence shows 62.2% and 63.7% identity with cytochrome P450 alkane hydroxylase 5 (ALK5) and ALK 7 of Y. lipolytica. The identities with Lodderomyces elongisporus and C. tropicalis were 54% and 55.66% respectively. A dendrogram showing the position of this sequence with respect to similar sequences in other yeasts is shown (Fig. 7). Different fungi involve different enzymatic systems, generally, extracellular lignin peroxidase, manganese peroxidase and laccase for white rot fungi and intracellular cytochrome P450 for most non-lignolytic fungi. We believe that C. digboiensis follows the non-lignolytic pathway as evident from PCR based detection of an active cytochrome P450 system. Hydrocarbon oxidation in yeasts is generally known to be through the Cytochrome P450 oxidation system. A number of encoding genes of CYP 52 family have been cloned from the yeasts, C. maltosa, C. tropicalis, C. apicola and Y. lipolytica (Eschenfeldt et al., 2003; Iida et al., 1998).

The degradation of the total petroleum hydrocarbons of acidic oil sludge by the novel yeast strain C. digboiensis TERI ASN6 is now being tested under field conditions.

4. Conclusions

The enrichment of the contaminated soil from Digboi refinery yielded a novel yeast isolate, identified as C. digboiensis TERI ASN6, that had developed adaptation mechanisms to survive in the harsh environments of that refinery. This yeast strain was capable of utilizing the hydrocarbons found in the oil sludge at pH 3. An active cytochrome system that gives this novel yeast strain, C. digboiensis its ability to utilize alkane hydrocarbons and oxidize PAHs, was also detected.

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References