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Biodegradation of Diesel Oil using Yeast *Rhodosporidium toruloides*

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ABSTRACT

Yeast *Rhodosporidium toruloides* was isolated from soil contaminated with petroleum hydrocarbons from Vellore, India. Yeast strain showed 99% sequence similarity with *Rhodosporidium toruloides*. *In vitro* study to check the efficiency of indigenous yeast to degrade hydrocarbon present in diesel oil was done in shake flask. Isolated yeast was enriched in mineral salt media containing diesel oil as sole source of carbon. It utilized hydrocarbons present in diesel oil efficiently. Degradation was preliminarily determined by change in physical appearance of medium viz., colour and viscosity. Degradation of hydrocarbons present in diesel oil was further confirmed by gravimetric analysis and gas chromatography. Residual oil after 15 days of degradation period was quantified gravimetrically and subjected to gas chromatography with flame ionization detector analysis. Chromatogram obtained after GC analysis clearly showed degradation of hydrocarbons present in diesel oil.

Key words: Indigenous yeast, biodegradation, diesel oil, gas chromatography

INTRODUCTION

Petroleum hydrocarbons are important source of energy and are extensively used everyday world wide. Petroleum hydrocarbon in the form of LPG, gasoline, diesel oil etc., is used as fuel for internal combustion engines. It can be used to manufacture fuels, plastics, detergents, lubricant, solvents, elastomers and fibers such as nylons and polysters. Large amounts of hydrocarbons (HCs) are extracted, produced, refined and handled every year and despite improvements in careful handling, transportation and containment, there is still the possibility that some may contaminate the soil and water environment.

In addition, oil pollution, accidents are nowadays become a common phenomenon and have caused ecological and social catastrophes (Burns *et al*., 1993). Accidental release of oil in the environment has been shown to cause serious damage to natural ecosystems in both prevalence and quantity (Rahman *et al*., 2002a; Chayneau *et al*., 2005). Crude oil is a complex mixture of hydrocarbons and other organic compounds. Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants and are of great concern due to their hazardous or potential toxicity, mutagenicity and carcinogenicity (Bogan and Sullivan, 2003; Hadibarata *et al*., 2009; Kim *et al*., 2001; Zhang *et al*., 2010; Kasai *et al*., 2006; Wolicka and Suszek, 2008).

Traditional methods of cleaning oil pollution are confined to physical and chemical processes. These processes to decontaminate the oil polluted areas have been limited in their application. Physical cleaning method includes booms, skimmers, incineration, brick making, adsorbents etc. It cannot remove more than 10-15% of the spilled oil. Whereas, use of chemical surfactants as remediating agent is not favourable due to their toxic effects on the existing biota in the polluted area (Sood and Lal, 2009; Thavasi *et al*., 2011). Moreover, mechanical method to reduce hydrocarbon pollution is expensive, time consuming and less effective. Therefore, despite decades of research, successful bioremediation of oil contaminated environment still remains a challenge (Perfumo *et al*., 2010; Thavasi *et al*., 2011; Sood and Lal, 2009; Vasudevan and Rajaram, 2001).

Biological methods can have an edge over the physico-chemical treatment processes in removing spills. Bioremediation is the conversion of chemical compounds into energy, cell mass and biological waste products using living organisms, especially microorganisms. (Rahman *et al*., 2002a). Bioremediation convert contaminants to harmless end products and the process is economical and efficient (Rahman *et al*., 2002b; Hasanshahian and Emtiazi, 2008; Das and Mukherjee, 2007; Ferrari *et al*., 1996).

The objective of the study was to isolate indigenous yeast from oil contaminated soil with potential to degrade hydrocarbons present in diesel oil. Degradation studies carried out can help to find out the degradation potential of the yeast and its effectiveness in bioremediation of oil contaminated soil in future.

MATERIALS AND METHODS

Sample collection: Four soil samples were collected from Vellore district by random sampling method. Soil, twigs and gravel present on the surface was removed and soil was collected at a depth of 3 cm using a sterilized knife. Samples were collected in separate sterile plastic bags and duly labelled samples were stored in refrigerator at -4°C before analysis.

Isolation and enrichment of isolated yeast: Oil-contaminated soil samples were homogeneously mixed, air dried and sorted to remove stones. Soil was homogenized and ground and passed through 2 mm sieve to remove gravel. Mixed soil samples were used for isolation of yeast. Mineral salts medium (Opasols and Adewoye, 2010) with 2% v/v diesel oil as substrate was used for enrichment and isolation of yeast.

The procedure for isolation of potential strain is same as followed by Zhang *et al*. (2010) five grams of soil were inoculated in 100 mL MSM containing 2% (v/v) diesel oil as sole source of carbon and energy and incubated with shaking at 100 rpm for 7 days. After three cycles of enrichment, 1 mL of the culture broth was serially diluted and plated on Sabouraud's dextrose agar (SDA). Orange, pin head colonies obtained on SDA was selected for degradation studies. Colonies were subcultured and purified on SDA.

Characterization of isolated yeast: Microscopic examination of colonies was done. Colonies showing yeast morphology was selected. Sequence analysis was done using ITS1 and ITS4 primers. The sequences were matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm at NCBI. Sequences of closely related taxa were retrieved and aligned using the Clustalx. A phylogenetic tree was constructed by the Neighbour-joining method (Fig. 1).

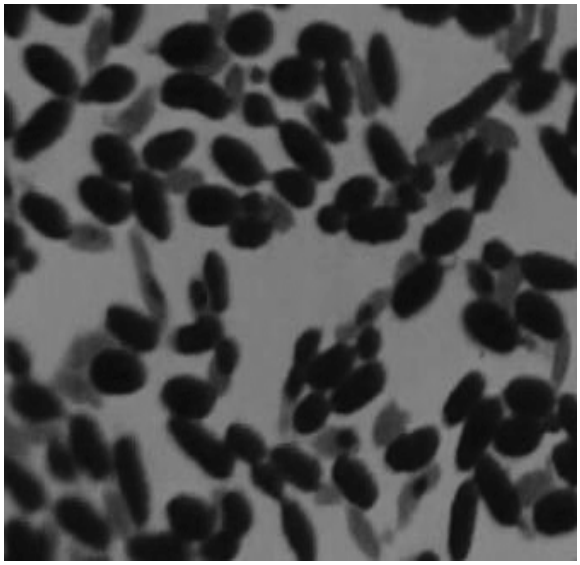

Fig. 1: Budding yeast cells of *Rhodosporidium toruloides* (microscopic view)

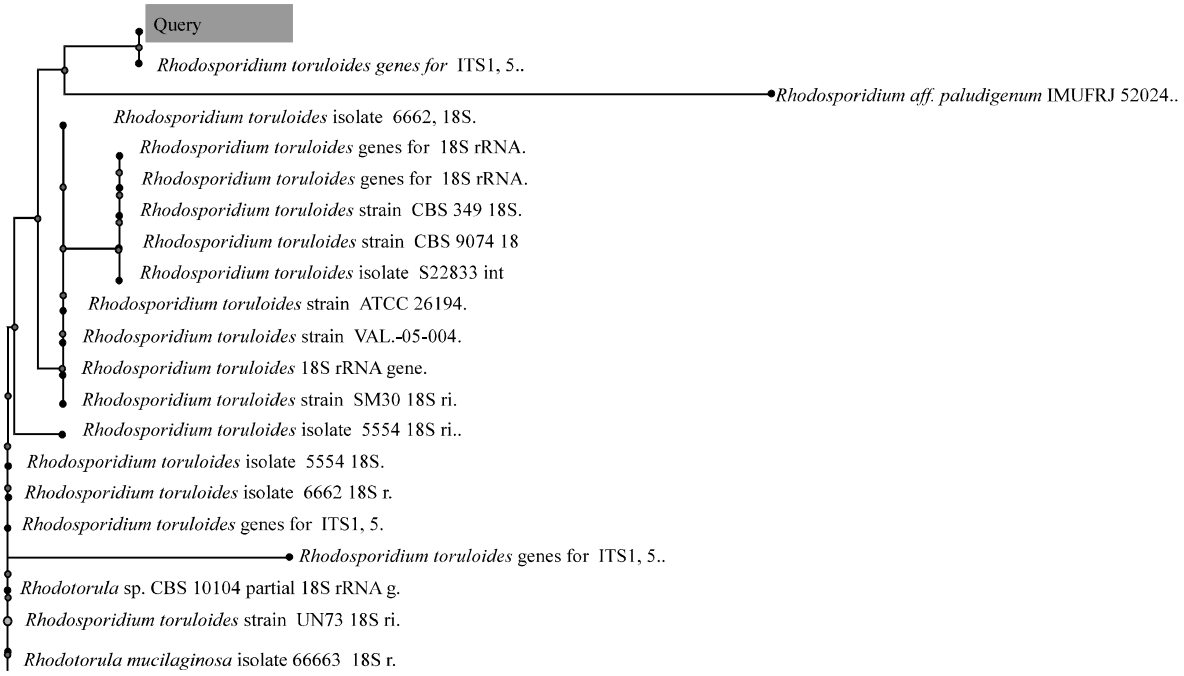

Fig. 2: Phylogenetic tree of the isolated yeast strain

Biodegradation of diesel oil: Loop full of culture was taken from slant in 100 mL MSM with 2% v/v diesel oil in 250 mL Erlenmeyer flask. Control flask with oil and without inoculum was also maintained. Flasks were then incubated in rotary shaker (100 rpm, 25°C), for 15 days (Das and Mukherjee, 2007). Inoculated and uninoculated flasks were harvested on day 15.

Culture broth was centrifuged at 5000 rpm for 5 min. Pellets were discarded and supernatant was taken. It was then extracted using hexane (1:1) in separatory funnel. Supernatant and hexane was shaken vigorously for 5 min and kept stationary for 10 min (Fig. 2). Top layer of hexane with diesel oil and lower aqueous layer was taken out in separate clean beaker. Anhydrous sodium sulphate was used to remove the remaining moisture. Solvent hexane was then evaporated in fume hood at room temperature. The residual oil left was estimated gravimetrically and degradation of hydrocarbon was analysed using gas chromatography.

Gas chromatographic analysis of residual degraded crude oil: Analysis was done in SAIF, IIT Chennai. One micro litre of the alkane fraction of diesel oil was made up to 10 mL using n-hexane and was analyzed by gas chromatography an (GC Hewlett Packard 5890) fitted with Flame ionization detector (FID) using 30 m long column. During analysis the injector and detector of GC were maintained at 300°C and the oven temperature was programmed to rise from 80 to 240°C with an increase of 5°C per min and then held at 240°C for 30 min (Sood and Lal, 2009).

RESULTS

Isolation of yeast: Among 10 isolated organisms, orange pin head colonies were selected for the biodegradation studies based on its distinct colony morphology. Microscopic examination after Lactophenol Cotton Blue (LCB) staining showed budding yeast cells (Fig. 3). Morphological and biochemical tests were done for identification of yeast and are shown in Table 1. Growth of these microorganisms in diesel oil containing media was assessed by viable cell count method. Yeast isolate showed increase in cell count indicating its growth in media.

Molecular characterization of yeast: 28S rDNA sequencing was done using ITS1 and ITS4 primers. The sequences were matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm at NCBI and the Acession number is BankIt1485676 JN966991. Isolate shows 99% similarity with Rhodosporidium toruloides.

Biodegradation studies: After 15 days of incubation, degradation of oil was assessed by visual observation of the medium. Flask inoculated with *Rhodosporidium toruloides* were found with tiny oil droplets dispersed in the medium resulting in browning of the medium (Fig. 4). Increase in viscosity of the medium was also observed. Gravimetric analysis of the residual oil was done. Amount of diesel oil recovered after 15 days was extremely low (Table 2). Loss in volume of oil in control is regarded as abiotic loss. Extracted oil in sample flask was compared with that of the

Table 1: Tests done for identification of yeast isolate

Test	Results
Morphology on SDA	Orange, round, pin head, elevated, mucoid colonies with entire margin
LFB staining	Oval shaped, budding yeast cells
Nitrate reduction	Negative
Urease	Negative
Dextrose	A^+G^-
Mannose	A^+G^-
Galactose	A^-G^-
Lactose	A^-G^-

where, A is Acid production and G is gas production

Table 2: Volume (mL) of extracted diesel oil after 15 days of incubation

Sample	Initial volume	Residual oil	Degraded oil
Control	2	1.8	0.2
Yeast	2	0.5	1.5

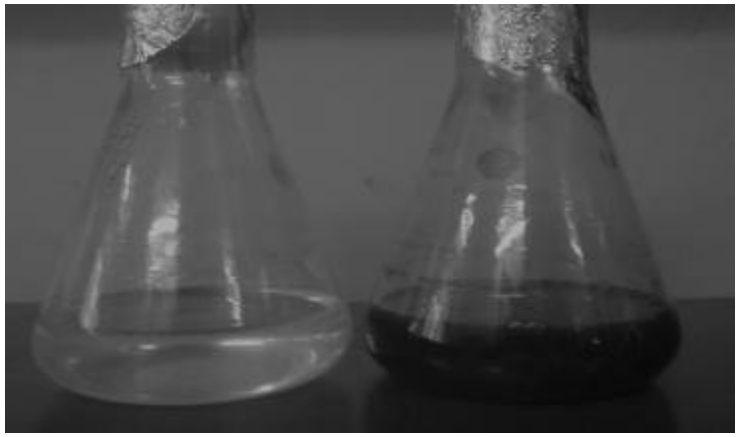

Fig. 3: Diesel oil degradation by selected yeast isolate Bd1 *Rhodosporidium toruloides*

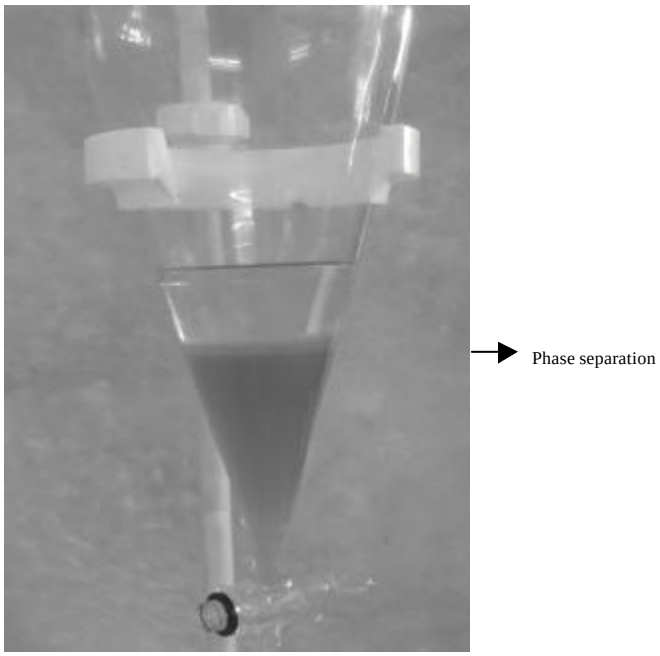

Fig. 4: Extraction of diesel oil in separatory funnel

control. Isolated yeast strain degraded 1.5 mL of the oil showing that the organism has degraded oil to a large extent. Extract obtained had an upper layer of degraded products and lower layer was that of the oil as shown in Fig. 5.

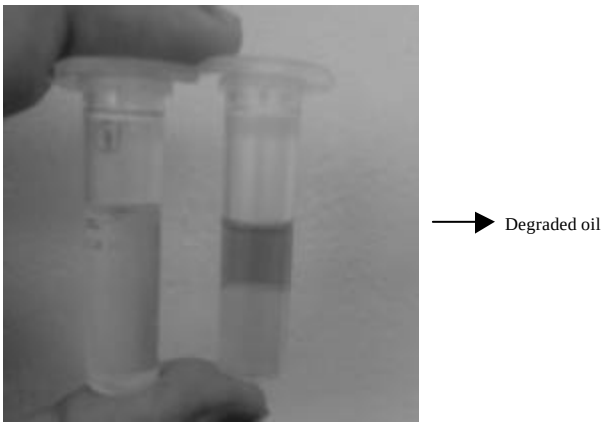

Fig. 5: Decrease in volume of the extracted oil in sample after 15 days of incubation

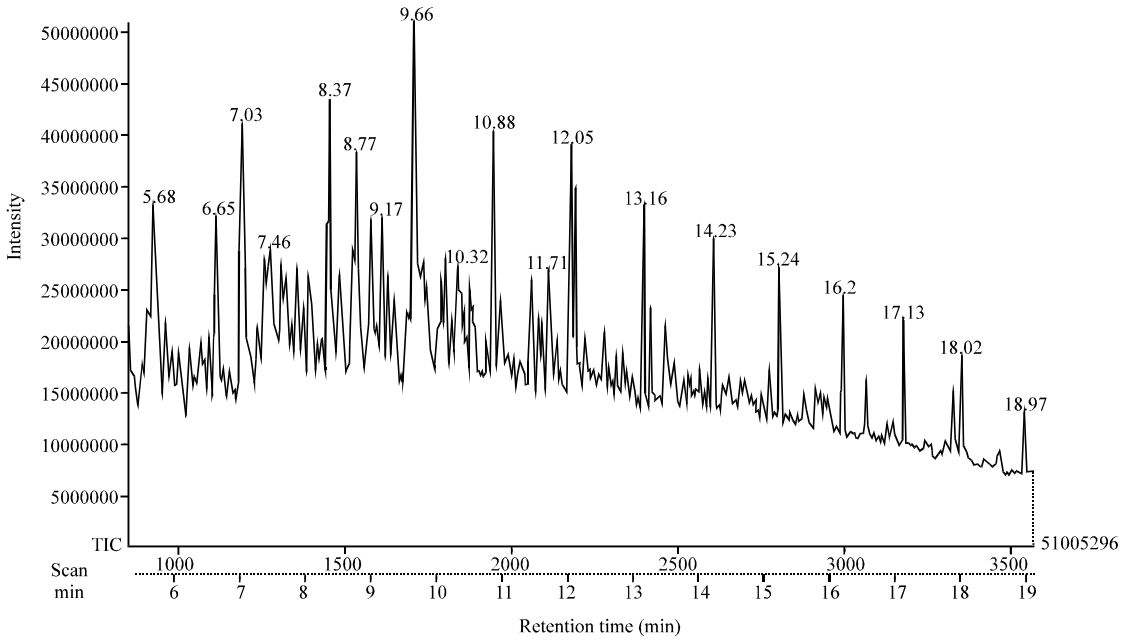

Fig. 6: Gas chromatogram of control diesel oil after 15 days incubation

GC-FID analysis of the alkane fraction: GC chromatogram showed reduction in the intensity of the hydrocarbon peaks of the sample with *Rhodosporidium toruloides* when compared with control diesel oil, which was taken as 100% (Fig. 6). Intensity or relative abundance was taken on Y-aixs. Retention time was taken on X-axis. Many peaks decreased tremendously and few decreased only to some extent. Relative abundance of hydrocarbons reduced from 50 ppm in control to 27 ppm in sample oil with *Rhodosporidium toruloides*. This is shown in Fig. 7. Result of GC confirms the degradation of hydrocarbon by the isolated yeast *Rhodosporidium toruloides*.

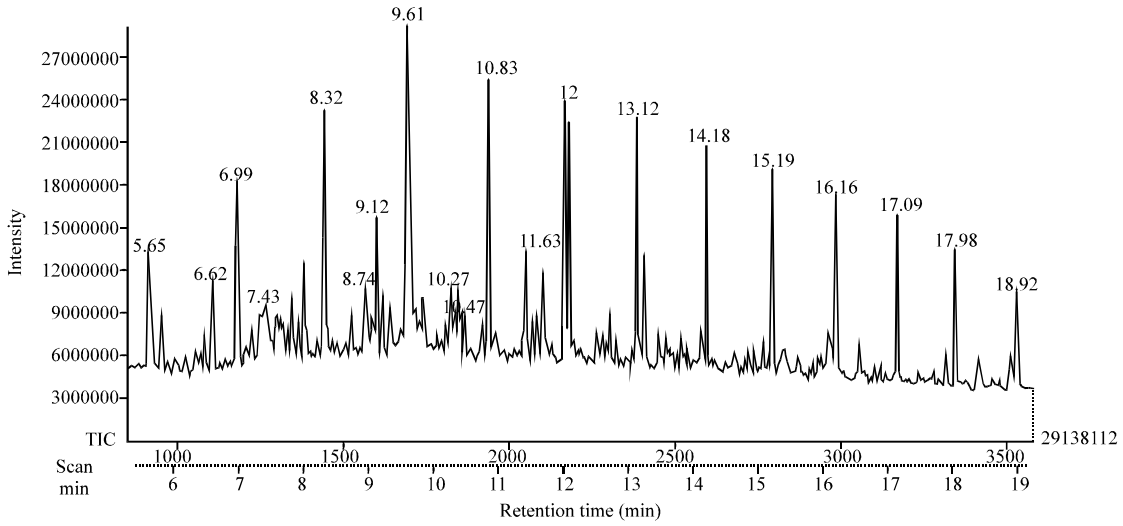

Fig. 7: Gas chromatogram of *Rhodosporidium toruloides* inoculated diesel oil after 15 days incubation

DISCUSSION

The yeast isolated and employed for the biodegradtion studies was found to be *Rhodosporidium toruloides* which was grown MSM and characterised. According to Sood and Lal (2009) the ability of the strain to grow in MSM with hydrocarbons as the sole carbon source is indicative of its ability to utilize the hydrocarbons. Thus, increase in plate count Yeast isolate *Rhodosporidium toruloides* during a study period of 15 days, shows that these isolates have potential to degrade hydrocarbons. Growth of the isolate in MSM with diesel oil as sole source of carbon and energy indicates its ability to degrade diesel oil. Increase in viable cell count showed adaptation of isolate in diesel oil containing medium. There was prominent change in colour of the medium after 15 d of incubation which was due to the dispersion of oil in the medium as well as break down of hydrocarbons. Increase in viscosity also indicates degradation of diesel oil as density of oil increases with the degradation. Gravimetric analysis of residual oil was done which showed decrease in content of oil when compared with the control. Finally, degradation was confirmed by GC-FID. Chromatogram clearly showed reduction in intensity of hydrocarbons after 15 days of incubation in flask with *Rhodosporidium toruloides*. In a similar study conducted by Sood and Lal (2009) isolated a novel yeast species *Candida digboiensis* TERI ASN6 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. The *C. digboiensis* strain could efficiently degrade the aliphatic and aromatic fractions of the acidic oily sludge at pH 3 and this was confirmed by gas chromatography. Canet *et al*. (2001) demonstrated the potential of four white-rot fungi *Phanerochaete chrysosporium* IMI 232175, *Pleurotus ostreatus, Coriolus versicolor* IMI 210866 and Wye isolate #7 to degrade PAH. These microorganisms naturally decompose lignin to obtain the cellulose inside wood fibre using a nonspecific enzymatic complex, which also enables them to degrade a wide range of contaminants if they are introduced to soils. In soil, the fungi release these enzymes to the extracellular medium, allowing the fungi to degrade large molecules that they would otherwise be unable to incorporate across cell walls.

Such metabolism enables fungi to avoid the uptake of potentially toxic substances. Furthermore, induction of the extracellular enzyme system is independent of the presence of contaminants; therefore, the fungi can degrade contaminants at extremely low concentrations.

CONCLUSION

GC chromatogram results were in accordance with that of the viable cell count and gravimetric analysis. Results of GC analysis showed that many peaks decreased and some even disappeared when compared with peaks of the control oil. Thus the indigenous yeast isolate *Rhodosporidium toruloides* used in the study have potential to degrade petroleum hydrocarbon. The growth and oil degrading potential of the isolates need to be optimized further. These isolates may have a potential application in bioremediation.

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