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Biodegradation of fungi isolated from Marine Ecosystem

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Abstract

The present study was confined to the marine ecosystem in and around kanyakumari, Tamil Nadu comprising of Sanguthurai (S1), Chothavilai (S2), Vivekananda Rock (S3), VattakottaiFort (S4), Chinna muttom (S5) and Water, sediment, and natural substrates of marine organisms were collected to isolate the fungi. All the collected samples were plated, incubated and the fungal colonies were identified. The baits samples were regularly observed under aseptic conditions using stereoscopic dissection microscope. Totally, 19 species of fungi were isolated from marine samples and they are as follows: *Aspergillus,Penicillium, Rhizopus, Cladosporium, Neurospora, Mucor and Fusarium.* In diesel degradation study, *P.janthinellum* had a maximum growth peak on the 5th day at an optical density of 2.05 while *R .nigricans* had the least growth peak on the 5th day at an optical density of 0.88. *P.janthinellum* had maximum growth peaks on the 5th day at an optical density of 0.34 with *A. carbonarius* and *A. flavus*earlier having this minimum growth peak (0.08) on the 5th day in petrol degradation. The growth pattern of fungi in Crude oil and minimal salt broth showed that *R. stolonifer* had a maximum growth peak at optical density 0.48 on the 5th day and in kerosene and minimal salt broth observed that *P.janthinellum* had a maximum growth peak at optical density 0.56 on the 4th day while *R.oryzae* had the lowest optical density at 0.03 on the 2th day.

Keywords: Marine ecosystem, Fungi, Biodegradation studies.

Introduction

The petroleum industry is responsible for the generation of high amounts of organic residues, as well as for the pollution of soils, rivers and seas. The potentiality of the microorganisms, pointed out in literature as agents of degradation of several compounds, indicates biological treatments as the most promising alternative to reduce the environmental impact caused by oil spills. It is known that the main microorganisms consuming petroleum hydrocarbons are bacterias and fungi. However, the filamentous fungi possess some attributes that enable them as good potential agents of degradation, once those microorganisms ramifies quickly on the substratum, digesting it through the secretion of extracellular enzymes. Besides, the fungi are capable to grow under environmental conditions of stress, for example: environment with low pH values or poor in nutrients and with low water activity.

Several authors have made lists containing bacteria and fungi genera that are able to degrade a wide spectrum of pollutants, proceeding from marine atmosphere as well as the soil (Boucher *et al*., 1996; Yateem *et al*., 1998; Juhasz and Naidu, 2000). In accordance with several scientific publications, can be pointed out that, amongst the filamentous fungi

Trichoderma and *Mortierella* spp are the most common ones isolated from the soil. *Aspergillus* and *Penicillium* spp have frequently been isolated from marine and terrestrial environments. In this way, microbiology of hydrocarbons degradation constitutes a field of research under development, once microbiological procedures may be used in the decontamination processes (Bonaventura and Johnson,1997).

Petroleum hydrocarbon-degrading fungi were isolated from *Detarium senegalense* seeds. An assessment of the relative ability of each fungus to degrade petroleum crude oil, diesel, unspent and spent engine oils, kerosene and *Detarium senegalense* oil extract, on minimal salt broth, was done measuring change in optical density read on a spectrophotometer. Five fungi were isolated from diseased namely: *Aspergillus flavus, A.niger, Mucor, Rhizopus*, and *Talaromyces*. The fungi isolated were used in the experiment and it was evident that all the fungi were capable of biodegrading the petroleum oil, though at different rates. *Aspergillus niger* had the highest ability to degrade unspent engine oil and *Detarium senegalense* oil extract while *Rhizopus* had the highest ability to degrade kerosene and diesel, and Talaromyces had the highest ability to degrade spent engine oil. There was fluctuation in the growth pattern of the fungi in the petroleum oil medium.

The white rot fungus *Polyporus* sp. S133 collected from petroleum contaminated soil was tested for its ability to grow and degrade crude oil, obtained from petroleum industry. The ability of *Polyporus* sp. S133 pre-grown on wood meal to degrade crude oil was measured. Maximal degradation (93%) was obtained when *Polyporus* sp. S133 was incubated in 1000 ppm of crude oil for 60 days, as compared to 19% degradation rate in 15000 ppm. Increased concentration of crude oil decreased the degradation rate.

Annual worldwide consumption of petroleum hydrocarbons was estimated to be of the order of 1012 US gallon (Prince, 1993). Much of it travels by water and at some instances some amount of oil inevitably spills from tankers and pipelines. Some of spilled crude oil or the washings of storage tanks pollute the beaches by the formation of tar balls and render them unusable. Oil refineries generate huge volume of oily sludge during the refining of crude oil. Oily sludge is carcinogenic and a potent immunotoxicant (Propst *et al*., 1999). Improper disposal and handling of oily sludge contaminates soil and may pose a serious threat to groundwater. Bioremediation offers a promising means to reclaim such contaminated soil (Bartha, 1986; Bragg *et al*., 1994). Bioremediation employs microorganisms capable of degrading toxic contaminants (Bossert and Bartha, 1984; Eriksson *et al*., 1995). Augmenting the contaminated site with an appropriate inoculum of microorganisms is a promising technique to enhance the biodegradation of hydrocarbons.

Moreover, using an indigenous microorganism consortium ensure that the organisms have a higher tolerance to the toxicity of hydrocarbons and are materials, mostly agricultural byproducts, are used to transfer the microorganism consortium to the fields effectively. The carrier materials provides nutrients, moisture, and physical support for the increased aeration needed by the microorganisms, and also assist in extending the survival of the microorganisms until they are applied in the field. Extended survival of the microorganisms under field conditions is essential for efficient degradation of the toxic hydrocarbons, especially of the multi-ringed aromatic and the recalcitrant hydrocarbons (Lal and Kanna, 1996).

Based on the necessary basic information obtained on marine fungi and marine ecosystem of Biodegradation potentials of fungi, the present study has been undertaken in the proposed study area in marine ecosystem , a coastal deltaic habitat along the East coast of Tamil Nadu, Kanyakumari.

Materials and Methods

Study area

Totally five (5) sampling stations were selected. The five sampling stations are:

1. Sanguthurai (S1)
2. Chothavilai (S2)
3. Vivekananda Rock (S3)
4. Vattakottai Fort (S4)
5. Chinna Muttom (S5)

Isolation of fungi from water and sediment samples by plating technique

After sampling, within 24 hrs the water samples from each station were subjected to appropriate dilutions (10^{-2} to10^{-5}) and 0.1 ml of sample was aseptically transferred into the plates containing Potato dextrose agar/ Czapek dox agar/Corn meal agar/Rose Bengal agar with addition of mixture antibiotics, Tetracycline and Penicillin (Spread plate method) The plates were

incubated at room temperature (28°C) for 4-5 days. Control plates were also maintained. Sterilization of glasswares and preparations of media were carried out as per the method described by Booth (1971).

Identification of fungi

The identification of fungal taxa was based on illustrated Genera of imperfect fungi (Barnett, 1965), Hyphomycetes (Subramanian, 1971), Dematiaceous Hyphomycetes and More Dematiaceous Hyphomycetes (Ellis, 1971, 1976), Marine Mycology (Kohlmeyer and Kohlmeyer, 1979), Micro fungi on land plants (Ellis and Ellis, 1985) Micro fungi on Miscellaneous substrate (Ellis and Ellis, 1988), Illustrated key to the filamentous higher marine fungi (Kohlmeyer and Volkman - Kohlmeyer, 1991) and Manual of soil fungi (Gilman, 1957, 1998).

Biodegradation of hydrocarbon study

Totally, 19 species of fungi were selected based on frequency of occurrence for hydrocarbon degradation.

Confirmatory Test for Hydrocarbon Utilization Potentials of the Isolated Fungi

The enrichment procedure as described by Nwachukwu (2000) was used in the estimation of hydrocarbon utilizers. A minimal salt broth containing 2.0g of NA_2HPO_4, 0.17g of K_2SO_4, 4.0g of NH_4NO_3, 0.53g of KH_2PO_4, 0.10g of $MgSO_4$, $7H_2O$ and 5.0g of agar – agar dissolved in 1000ml of distilled water was prepared.

The solution was sterilized by autoclaving. Twenty-eight test tubes were sterilized and placed in test tube racks, where there were four test tube racks containing seven test tubes each. 10ml of the minimal salt broth (MSB) was measured into each of the test tube. 2ml of either crude-oil or diesel or engine oil or spent engine oil or the seed oil extract was measured and added to the 10ml of minimal salt broth in the first 6 test – tubes in each rack respectively with the exception of the last test – tubes, that is, the seventh test tube in each rack, making 12ml in twenty – four test tubes and 10ml of only minimal salt broth in four test tubes which served as controls.

Three fungi which were isolated from marine samples were added to the test tubes in three racks with the test tubes in the last rack serving as control without fungi. Each of the test tubes was plugged with sterile cotton wool wrapped with Aluminium foil so as to ensure maximum aeration and prevent cross – contamination. All the test tubes were then incubated at room temperature (28° C - 31° C). The test tubes were shaken constantly throughout the duration of the experiment to facilitate oil (cell phase contract). The ability to degrade the petroleum products (based on the growth rate of the organisms in the MSB medium) was measured upto 5 days using the visual method which is based on the turbidity of the MSB. The turbidity was measured using the photoelectric colorimeter.

Results and Discussion

A total of 135 fungal species were isolated and enumerated by plating, baiting and direct observations techniques. Among these, 67 species were represented in Sanguthurai (S1), 61 in Chothavilai (S2), 53 in Vivekananda Rock (S3), 63 in Vattakottai Fort (S4) and 56 in Chinnamuttom (S5). In this study, 98 species of fungi were recovered from sediment samples whereas water samples yielded 87 species and natural substrates with 66 species. From the sea foams, a total of 40 fungal species were recorded. Among the Hyphomycetes, *Aspergillus* was the common genus represented by 31 species followed by *Alternaria* (10 sp.), *Penicillium* (12 sp.) and *Curvularia* (12 sp.).

Biodegradation of hydrocarbon studies

Totally, 19 species of fungi were isolated from marine samples and they are as follows: *Aspergillus, Penicillium, Rhizopus, Cladosporium, Neurospora, Mucor and Fusarium*. The growth pattern of fungi in diesel and minimal salt broth is represented on Table.1. *P.janthinellum* had a maximum growth peak on the 5th day at an optical density of 2.05 while *R.nigricans* had the least growth peak on the 5th day at an optical density of 0.88. On the 7th day, *P.janthinellumand A.candidus* attained a growth peak of 0.51 while *A.luchensis* fell to a growth peak of 0.06.

The growth pattern of fungi in petrol and minimal salt broth is shown in table 2. *P.janthinellum* had maximum growth peaks on the 5th day at an optical density of 0.34 with *A. carbonarius* and *A. flavus* earlier having this minimum growth peak on the 5th day (0.08). On the 7th day, *P.janthinellum* had the highest optical density at 0.19 followed by *R.nigricans* (0.15) and *F.semitectum* (0.12). *P.janthinellum* had the highest ability to degrade petrol while *Aspergillus* had the least ability.

Table – 1 Biodegradation of hydrocarbon – Diesel

Name of the fungi	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
Aspergillus candidus	0.01	0.02	0.07	0.08	1.02	0.05	0.51
A. carbonarius	0.01	1.06	0.08	0.08	1.09	0.11	0.08
A.erythrocephalus	0.01	0.03	1.07	1.02	1.02	0.09	0.13
A. flavus	0.01	0.05	0.08	0.08	2.00	0.10	0.11
A. fumigatus	0.02	1.01	1.06	1.04	1.05	0.07	0.18
A. luchuensis	0.02	0.02	0.09	0.06	1.03	0.05	0.06
A. nidulans	0.01	1.01	0.08	1.00	1.04	0.06	0.13
A. niger	0.01	1.01	0.23	1.04	1.07	0.06	0.09
A. ochraceus	0.03	1.02	1.07	1.03	1.02	0.12	0.22
A. sulphureus	0.02	1.34	1.48	0.80	1.04	0.01	0.15
Pencillium citrinum	0.01	0.06	1.04	0.09	2.00	0.06	0.15
P. janthinellum	0.01	0.06	0.20	0.07	2.05	0.04	0.51
Rhizopus nigricans	1.2	0.54	0.83	0.73	0.88	0.08	0.18
R.oryzae	0.01	0.02	0.09	1.04	2.03	0.14	0.27
R.stolonifer	0.02	1.0	1.09	1.01	1.02	0.09	0.20
Cladosporium britannicum	0.01	1.03	1.02	1.06	1.06	0.26	0.14
Neurospora crassa	0.01	0.05	1.00	1.03	1.02	0.06	0.08
Mucor sp	0.01	1.0	1.08	0.05	1.08	0.09	0.11
F. semitectum	0.02	0.03	0.07	1.01	1.09	0.14	0.15

The growth pattern of fungi in Crude oil and minimal salt broth is represented in table 3.*R.stolonifer* had a maximum growth peak at optical density 0.48 on the 5th day. *A.fumigatus and A.nidulans*also had this growth peak at 1.25 on the 4th day. *P.janthinellum*had the lowest growth peak at optical density 0.08 on the 7th day.

The growth pattern of fungi in kerosene and minimal salt broth is represented on Table.4. *P.janthinellum* had a maximum growth peak at optical density 0.56 on the 4th day while *R.oryzae* had the lowest optical density at 0.03 on the 2th day. On the 7th day of incubation, *A.erythrocephalis, A.sulphureus* and *F. semitectum* had the highest optical density at 0.20 and lowest with *A.luchensis* (0.06).

The results of this work indicate that many of the fungal species isolated from the marine system were capable of degrading petroleum hydrocarbons. Bartha and Atlas (1973) listed 22 genera of bacteria, 1 algal genus and 14 genera of fungi which had been demonstrated to contain members which utilize petroleum hydrocarbons; all of these micro organisms had been isolated from an aquatic environment. Also, Okerentugba and Ezeronye (2003) demonstrated that *Penicillium* spp., *Aspergillus* spp. and *Rhizopus* spp. were capable of degrading hydrocarbons especially when single cultures were used. These fungi had been isolated also from aquatic environments in the Niger Delta area of Nigeria. Batelle (2000) showed that fungi were better degraders than traditional bioremediation techniques including bacteria. The fungi used were wood-degrading fungi. Also, the ability of the white-rot fungus – *Pleurotus tuberregium* to ameliorate crude oil polluted soil has been reported. The isolation of fungal petroleum hydrocarbon utilizers from oil seeds was first documented by Adekunle and Oluyode (2002).

An interesting observation generated in this study was that the fungi isolated had increased growth rates in the media containing petroleum and petroleum products compared to only when minimal salt broth was used. This might be due to the fact that the fungi isolated were able to use the hydrocarbons as substrates for growth by probably releasing extra cellular enzymes and acids which are capable of breaking down the recalcitrant hydrocarbon molecules, by dismantling the long chains of hydrogen and carbon, thereby, converting petroleum into simpler forms or products that can be absorbed for the growth and nutrition of the fungi.

Shaw (1995) found that organisms break down hydrocarbons and use the energy to synthesize cellular components. After being completely broken down, the reaction releases Carbon (IV) oxide, water and energy used to create cellular biomass (Keeler, 1996).

Table – 2 Biodegradation of hydrocarbon - Petrol.

Name of the fungi	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
Aspergillus candidus	0.03	0.21	0.09	0.10	0.11	0.09	0.06
A. carbonarius	0.01	0.03	0.11	0.07	0.08	0.05	0.06
A.erythrocephalus	0.01	0.06	0.06	0.03	0.11	0.15	0.09
A. flavus	0.03	0.04	0.08	0.07	0.08	0.11	0.10
A. fumigatus	0.01	0.02	0.09	0.05	0.09	0.06	0.09
A. luchuensis	0.02	0.02	0.10	0.05	0.10	0.08	0.05
A. nidulans	0.01	0.06	0.09	0.11	0.09	0.12	0.11
A. niger	0.03	0.02	0.12	0.04	0.10	0.08	0.06
A. ochraceus	0.01	0.02	0.14	0.05	0.13	0.10	0.08
A. sulphureus	0.02	0.03	0.02	0.03	0.13	0.11	0.09
Pencillium citrinum	0.04	0.04	0.05	0.09	0.09	0.09	0.07
P. janthinellum	0.16	0.03	0.32	0.22	0.34	0.24	0.19
Rhizopus nigricans	0.01	0.06	0.16	0.07	0.12	0.13	0.15
R.oryzae	0.01	0.10	0.10	0.14	0.17	0.10	0.11
R.stolonifer	0.01	0.10	0.09	0.08	0.26	0.12	0.08
Cladosporum britannicum	0.03	0.02	0.13	0.13	0.14	0.14	0.08
Neurospora crassa	0.05	0.20	0.11	0.13	0.20	0.13	0.10
Mucor sp	0.06	0.11	0.12	0.10	0.11	0.12	0.08
Fusarium semitectum	0.01	0.02	0.07	0.11	0.16	0.15	0.12

Table – 3 Biodegradation of hydrocarbon – Crude oil.

Name of the fungi	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
A.candidus	0.01	0.04	0.02	0.31	0.19	0.09	0.10
A. carbonarius	0.06	1.05	1.07	1.09	0.39	0.23	0.15
A.erythrocephalus	0.01	0.05	1.04	0.21	0.24	0.19	0.13
A. flavus	0.01	0.04	1.02	1.01	0.34	0.09	0.10
A. fumigatus	0.03	0.04	1.01	1.25	0.40	0.18	0.19
A. luchuensis	0.01	0.02	1.04	1.02	0.20	0.20	0.12
A. nidulans	0.01	1.03	1.09	1.25	0.24	0.15	0.17
A. niger	0.05	0.08	0.08	0.24	0.20	0.14	0.14
A. ochraceus	0.01	0.07	0.09	0.22	0.20.	0.16	0.19
A. sulphureus	0.04	1.00	1.04	0.20	0.19	0.06	0.17
Pencillium citrinum	0.01	1.02	0.08	1.06	0.24	0.14	0.18
P. janthinellum	0.01	1.03	0.06	0.19	0.19	0.11	0.08
Rhizopus nigricans	0.02	1.01	0.05	0.28	0.23	0.13	0.12
R.oryzae	0.04	1.06	0.07	0.20	0.25	0.17	0.17
R.stolonifer	0.02	0.07	2.00	0.31	0.48	0.21	0.21
Cladosporium britannicum	0.03	1.07	1.01	0.36	0.29	0.20	0.17
Neurospora crassa	0.02	1.04	1.05	0.21	0.29	0.15	0.12
Mucor sp	0.01	1.05	1.01	0.28	0.30	0.11	0.13
Fusarium semitectum	0.02	0.08	0.09	0.20	0.18	0.15	0.14

Table – 4 Biodegradation of hydrocarbon - kerosine.

Name of the fungi	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
Aspergillus candidus	0.04	0.06	0.10	0.12	0.17	0.09	0.13
A. carbonarius	0.06	0.10	0.10	0.18	0.16	0.10	0.12
A.erythrocephalus	0.05	0.07	0.08	0.08	0.16	0.09	0.20
A. flavus	0.02	0.08	0.10	0.09	0.11	0.04	0.13
A. fumigatus	0.04	0.04	0.06	0.29	0.21	0.09	0.07
A. luchuensis	0.02	0.04	0.08	0.14	0.16	0.06	0.06
A. nidulans	0.05	0.09	0.13	0.12	0.21	0.17	0.16
A. niger	0.04	0.11	0.08	0.16	0.20	0.08	0.11
A. ochraceus	0.04	0.10	0.14	0.11	0.14	0.05	0.09
A. sulphureus	0.08	0.13	0.15	0.17	0.15	0.11	0.20
Pencillium citrinum	0.10	0.07	0.07	0.24	0.19	0.07	0.15
P. janthinellum	0.29	0.34	0.50	0.56	0.02	0.05	0.07
Rhizopus nigricans	0.04	0.06	0.08	0.12	0.17	0.04	0.10
R.oryzae	0.02	0.03	0.10	0.03	0.23	0.18	0.18
R.stolonifer	0.08	0.06	0.11	0.13	0.18	0.13	0.14
Cladosporium britannicum	0.05	0.05	0.09	0.18	0.08	0.11	0.16
Neurospora crassa	0.03	0.05	0.08	0.19	0.23	0.10	0.13
Mucor sp	0.01	0.04	0.07	0.12	0.20	0.12	0.15
Fusarium semitectum	0.08	0.11	0.17	0.18	0.19	0.10	0.20

However, it must be noted that there were also nutrients present in the minimal salt broth though more of it could have been present in the oil which stimulated the growth of each fungus. In view of this then, the additional nutrients present in the minimal salt broth helped in overcoming nutrient limitation to microbial growth to a certain extent and also helped in creating a favourable environment for the rapid development of the fungi especially at the times when the fungi had not started breaking down the hydrocarbons into simpler molecules.

Microbial degradation of oil has been shown to occur by attack on the aliphatic or light aromatic fractions of the oil. Although some studies have reported their removal at high rates under optimal conditions, high molecular weight aromatics, resins and asphaltenes are generally considered to be recalcitrant or exhibit only low rates of biodegradation. Amund and Akangbou (1993) showed that crude oil fractions with lower amount of saturated hydrocarbons were more resistant to microbial degradation than the fraction(s) containing higher amount(s) of saturated hydrocarbons. The Escravos crude oil blend used in this experiment has been shown to contain 69.74% saturated hydrocarbons, 22.05% aromatics, 2.56% asphaltenes and 5.65% residue (Amund and Akangbou, 1993). This could possibly have accounted for the slow rate of degradation of the oil.In conclusion, the result here shows that fungi isolated from marine system can be exploited in the biodegradation of crude petroleum oil spill and bioremediation of the environment.

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