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ORIGINAL ARTICLE

Biodegradation of diesel fuel hydrocarbons by mangrove fungi from Red Sea Coast of Saudi Arabia

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Abstract Mangrove sediments were collected from major mangrove stands on the Red Sea Coast of Saudi Arabia. Forty five isolates belonging to 12 genera were purified and five isolates as well as their consortium were found to be able to grow in association with petroleum oil as sole carbon source under *in vitro* conditions. The isolated strains were identified based on internal transcribed spacer (ITS) rDNA sequence analysis. The fungal strains with the greatest potentiality to degrade diesel oil, without developing antagonistic activity, were identified as *Alternaria alternata*, *Aspergillus terreus*, *Cladosporium sphaerospermum*, *Eupenicillium hirayamae* and *Paecilomyces variotii*. As compared to the controls, these fungi accumulated significantly higher biomass, produced extracellular enzymes and liberated larger volumes of CO_2. These observations with GC–MS data confirm that these isolates displayed rapid diesel oil bioremoval and when used together as a consortium, there was no antagonistic activity.

1. Introduction

Diesel fuel obtained by the distillation of crude oil has a carbon range between C_8 and C_{26} (Adam and Duncan, 1999) with high content of polyaromatic hydrocarbons (Wang et al., 1990). The toxicity of diesel to ecosystems is more as compared to crude oil, because of diesel's higher content of light-hydrocarbons. Although diesel is a commonly used fuel for vehicles and machines, it is recognized as a serious threat to ecosystems (Jagtap et al., 2014).

Diesel hydrocarbons can accumulate in food chains at various levels where they disrupt biochemical or physiological processes of many organisms; thus causing carcinogenesis of some organs, mutagenesis in the genetic material, and impairment in reproductive capacity, and hemorrhages in exposed population (Janani Prathiba et al., 2014).

Bioremediation has proved to be the most promising, practical and economical method for the complete mineralization of hydrocarbons to carbon dioxide and water (Wang et al., 2015).

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The need for remediating polluted areas has induced development of new technologies to detoxify contaminants not only through chemical or physical methods, but through biological techniques as well. Bioremediation comprises a set of technologies that make the removal of contaminants possible or render them less harmful by means of biological activity (Silva et al., 2015).

Many isolated bacterial and fungal species have been reported to be capable of biodegrading petroleum hydrocarbons and even polynuclear aromatic hydrocarbons effectively (Márquez-Rocha et al., 2005). Laboratory studies provide greater control and manipulation in providing a basis to distinguish between biotic and abiotic processes, and to determine the optimal conditions for biodegradation (Li et al., 2008).

It is known that petroleum hydrocarbons can be removed by microorganisms such as fungi belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium,* *Amorphotheca*, *Neosartorya*, *Paecilomyces*, *Talaromyces*, *Graphium*, yeasts which includes *Candida*, *Yarrowia* and *Pichia* and microalgae (Chaillan et al., 2004). This study attempted to assess the ability of fungi isolated from mangrove stands on the Red Sea Coast of Saudi Arabia to degrade diesel oil under *in vitro* conditions.

2. Materials and methods

2.1. Reagents

Clean diesel oil was procured from Saudi Aramco. Diethyl either (BDH) used as solvent for preparation of samples for GC–Mass analysis was obtained from the local market.

2.2. Collection of samples

Samples for isolation of fungi were collected form mangrove stands located near Jeddah and Jazan cities on the Red Sea Coast of Saudi Arabia and Farasan Island 40 km off the shore. Floating debris, tidal water, and sediment under the mangrove plants were collected as samples for isolation of fungi. The debris consisted of dead fallen leaves, pieces of pneumatophores, bark and wood of mangrove plants. The samples were collected in sterilized specimen tubes and were brought to lab in ice.

2.3. Isolation and purification of fungi

Fungi were isolated from wood debris by splitting the specimen into smaller pieces and directly laying on a synthetic mineral salt medium (MSM) formulated to simulate seawater conditions based on the compositions of Ameen et al. (2014) with some modification. Composition of the medium per liter was: $MgSO_4$ 246.5 mg/l, $FeSO_4{\cdot}7H_2O$ 5.56 mg/l, $ZnSO_4{\cdot}7H_2O$ 0.29 mg/l, $MnSO_4{\cdot}H_2O$ 0.34 mg/l, $CuSO_4{\cdot}5H_2O$ 0.025 mg/l, NH_4Cl 5.35 mg/l, KCl 7.46 mg/l, $CaCl_2{\cdot}2H_2O$ 1.47 mg/l, NaCl 5.84 mg/l, $COCl_2{\cdot}6H_2O$ 0.027 mg/l, KH_2PO_4 136 mg/l, Na_2MoO_4 24 mg/l, and dextrose 20 g/l. The medium was gelled with 15 g/l agar and the pH adjusted to 5.5 before autoclaving. Tidal water and sediment suspension were layered on the same medium by the standard dilution method. The plates were incubated at 30 ± 2 °C for 5–7 days. Pure cultures were obtained by sub culturing inoculum from young colonies developing on the initial medium.

2.4. Identification of fungal isolates

The selected pure fungal isolates were subcultured from the MSM agar plates and inoculated into the potato dextrose agar (PDA) to harvest more mycelia. Total genomic DNA of the isolates was extracted according to the method of Wiese et al. (2011). The isolated strains were identified on the basis of internal transcribed spacer (ITS) rDNA sequence analysis. For sequence analysis, the ITS1-5.8S-ITS4 rDNA gene of the fungus was amplified using PCR with primer set pITS1 (5′ TCCGTAGGTGAACCTGCCG-3′) and pITS4 (5′ TCCTCCGCTTATTGATATGC-3′) (Al-Nasrawi, 2012). The 550-bp amplicon obtained was cloned and sequenced. DNA sequence of the positive clones with 18S rDNA gene fragment was compared with those available on the database using the BLAST program at the National Center for Biotechnology Information (NCBI) and analyzed.

2.5. Biomass determination on diesel fuel hydrocarbons

Fungal biomass was determined by filtering the culture broth through Whatman No. 1 filter paper. Recovered biomass samples were weighed and dried in pre-weighed aluminum foil at 60° C to a constant weight and the l dry mass was obtained by subtraction. Three replicate flasks were maintained for each fungal isolate. For each treatment, three flasks with corresponding inoculum but without diesel fuel substrate were retained as controls. Gain in biomass under each treatment and the corresponding control was recorded; and difference between gain in treatment and control was considered to be due to biodegradation activity of the fungus.

2.6. Enzyme assays

Five fungal isolates, which showed an increase in total dry weight under treatment were co-cultivated with diesel fuel in replicates along with the controls as mentioned above. As a separate treatment, consortium of the five above isolates was also tested for enzyme activity. After 4 weeks of incubation, enzyme level in the medium was assayed for controls and treatments. Cultures were centrifuged at 10,000 rpm, 4 °C; the pellet consisting of fungal cells was discarded and enzyme level was determined in the extracellular fluids. Catalase activity was determined according to Aebi (1983) by measuring decomposition of H_2O_2 and decline in absorbance at 240 nm in a 3 min period. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 15 mM H_2O_2, and 0.1 ml of enzyme extract in final volume of 3 ml. Laccase was estimated by oxidation of 2,2-Azino-bis-3-ethyl-benzthiazoline-6-sulfonic acid (ABTS) according to Novotny et al. (1999) using 0.1 mM ABTS in the reaction buffer of 100 mM sodium tartrate (pH 4.5) with 50 µl culture filtrate. One unit (U) of laccase activity was defined as the production of 1 µmol product per min at 30 °C and pH 4.5. Manganese-dependent peroxidase (MnP) was estimated by using 0.01% phenol in the presence of 0.1 mM H_2O_2 and 1 mM $MnSO_4$ in 100 mM sodium tartrate (pH 4.5); while lignin peroxidase (LiP) was determined by