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ORIGINAL PAPER

The efficiency of *Penicillium commune* for bioremoval of industrial oil

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Abstract Among all environmental contaminations, industrial oil is one of the major pollutants of soil, water, and air. There are different chemical, physical, and biological methods to remove all types of oil pollutions. One of the common biological methods is to utilize the microorganisms like yeast, fungi or bacteria. Previous studies concerning the biodegradation of an aromatic compound in industrial waste water by *Aspergillus niger* have been reported. In this study, we tried to identify an oil-derived microorganism and evaluate its efficacy on self-removal of industrial oil. Firstly, the strain of isolated fungus from various bulks of used oil was defined via colonial identification and DNA sequencing. Secondly, bioremoval activity of defined fungus (*Penicillium commune*) was evaluated using gas chromatography–mass spectrometry. The optimum conditions in biological elimination of oil including the incubation time, pH level of culture, and amount of reagents were determined. In the best condition, a removal rate of 95.4 % was obtained.

Keywords Bioremoval · Industrial oil · *Penicillium commune* · Fungus

Introduction

Oil pollutants such as lubricants, cutting fluids, and other types of heavy products such as tar, grease, crude oil, and diesel oil as well as light hydrocarbons are considered as a global disaster and a common problem in oil-bearing and industrial regions (Merkel et al. 2004).

In our research, the biodegradation of 1-naphthol in industrial waste water was examined. We separated *Aspergillus niger* in the soil of industrial waste water and its biodegradation by the same fungi was further investigated by us (Esmaeili and Fazeli 2012).

In a field study, fugal strains were isolated from oil-contaminated sites of Arak refinery (Iran) and their growth ability was checked in potato dextrose agar media containing 0–10 % v/v crude oil, the activity of three enzymes was evaluated in the fungal colonies and bioremediation ability of the fungi was checked in the experimental pots containing 3 kg sterilized soil and different concentrations of petroleum (0–10 % w/w) (Mohsenzadeh et al. 2012). In recent years, a number of natural biodegradable sorbents have been found as one of the most cost-effective and capable means for the oil spill cleanup, and a number of works have been studied for utilizing these materials in the removal of oil spill, e.g., barley straw (Husseien et al. 2009), raw sugarcane bagasse in different particle sizes was used for the sorption of layer of crude oil from surface of sea water (Behnood et al. 2013).

During recent years in our country, we have encountered with the increased destroying effects of oil pollution on ecosystem similar to the other oil-rich countries. Recently, the amazing effects of fungi on removal of pollutants or their transformation to harmless or useful products have been reported. In this process, many toxic components in oil industry wastewater can be changed to novel substances applicable in biotechnological procedures (Esmaeili and Fazeli 2012). Industrial oils as by-products of mineral-based oils are derived from crude oil in refineries. These oils include hydrogen and carbon which forms numerous compounds called hydrocarbons that are not heavy enough

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to change from a gas into a solid state. The most important application of industrial oil is to lubricate the metal parts of car engine or other types of machines (Dieter 1984). Hydrocarbons as oil industrial derivatives can be decomposed by soil fungi as a natural phenomenon. The living condition of soil-bacteria or fungi makes them suitable for bio-removal of hydrocarbon contaminations in soil. Noticeably, *Penicillium* is one of the most abundant fungal floras with the intention that there are 10^6–10^8 spores in one gram of normal soil and 10^4 spores in one milliliter of unpolluted groundwater (Gallegos Martinez et al. 2000). Recently, a considerable attention has been focused on using physical, chemical, and biological methods to remove or modify the environmental contamination created by petroleum products. (Chehregani and Malayeri 2007). According to previous studies, biological processes are the best options because of being cost-effective and less hazardous to both human and natural environment. They are also a proper replacement of expensive engineering techniques (Chehregani et al. 2009; Merkel et al. 2004; Smith et al. 1981). From a biological point of view, the aim of this study was to evaluate the potential of a fungus (*Penicillium commune*) isolated from wastewater oil on the elimination of oil industrial derivatives. More importantly, a probable metabolic pathway was defined using product analysis.

Materials and methods

Chemicals and culture media

This study has been conducted in Garmsar, Semnan Province in Iran. Industrial oil with the flash point of 160–350 °C was provided from the second refinement oil industrial company. Samples were diluted (4 %) and their pH adjusted to normal (pH 7) using 1 M HCl/1 M NaOH.

The microbiological culture medium used in this study was SGA 4 % containing 5 g casein peptone, 5 g meat peptone, 40 g glucose and 15 g agar which were solved in 1 l distilled water and adjusted to pH 5.6 ± 0.2 at 25 °C (Malik 1996; Kachuei et al. 2009; Sherman et al. 1991).

Isolation and cultivation of fungus

In order to cultivate microorganism, a sample of used oil was collected from a barrel in an auto service using a sterile 10 ml pipette and was kept in a glass bottle at 4 °C. This sampling procedure was repeated three times in different auto service shops. Sample preparation was as follow: 1 ml of used oil was mixed with 10 ml of sterile distilled water and centrifuged 15 min at 1,000 rpm. After removing the supernatant, the pellet was resuspended in 1 ml distilled water and cultured on Sabouraud Glucose Agar (SGA) 4 %. Plates were kept at 27 °C incubator to colonize the microorganism. After a 7-day culture, fully colony growth plates were stored at a temperature of 4 °C (Cruickshank et al. 1975).

Identification of microorganism derived from used oil

The following procedures were done to identify the strain of oil-removal fungus: Colony formation on SGA was applied to find the type of fungus as previously reported by Kachuei et al. (2009), special staining of *Penicillium* on the slide was done using lacto phenol blue. Finally, we could detect *P. commune* in our samples.

DNA extraction

DNA extraction was performed according to the method published by Kachuei et al. (Glass and Donaldson 1995) with a few modifications. Briefly, the harvested mycelial mass was frozen in liquid nitrogen and ground to a fine powder. The mycelia powder was suspended in a DNA extraction buffer containing 50 mM Tris–HCl (pH 8), 50 mM EDTA, 3 % sodium dodecyl sulfate, and 50 µl of proteinase-K (20 mg/ml). The suspension was incubated at 65 °C for 1 h, and the cellular debris was removed by centrifugation at 3,000 rpm for 5 min. The suspension was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). DNA was precipitated by addition of an equal volume of ethanol and sodium acetate (3 M) followed by centrifugation at 12,000 rpm for 10 min, frozen at −20 °C for 2 h, and then centrifuged at 12,000 rpm for 30 min. Finally, the DNA pellet was rinsed with 70 % ethanol and suspended in TE buffer (Fig. 1) for further evaluations.

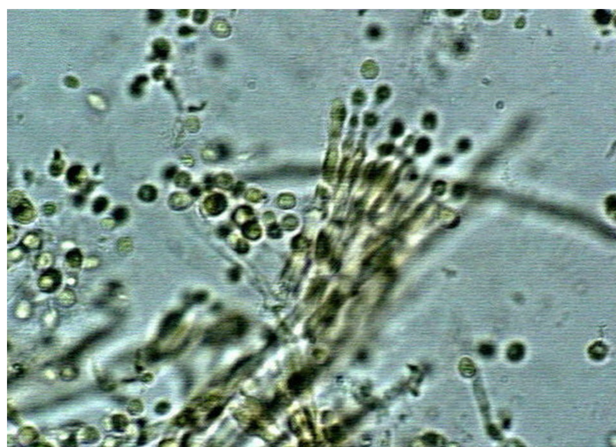

Fig. 1 Bright field microscopic examination of *P. commune* fungus

PCR and DNA sequencing

Isolated DNA was used in PCR. β-Tubulin gene and the internal transcribed spacer (ITS) regions were amplified using universal primers Bt2a, Bt2b, ITS5, and ITS4 (White et al. 1990).

Forward (ITS1): 50TCCGTAGGTGAACCTGCGG30

Reverse (ITS4): 50TCCTCCGCTTATTGATATGC30

Amplification was performed in a final volume of 25 µl containing 25 µl of 10× PCR buffer, 0.5 µl deoxynucleoside triphosphate (dNTP) at 0.2 mM, each forward and reverse primer at 20 pmol, 1.25 µl of template DNA, and 2.5 U of qDNA polymerase (0.5 µl). The PCR condition consisted of the initial denaturation at 94 °C (20 min), 35 cycles each of denaturation at 94 °C (45 s), 58 °C for annealing (1 min) and finally at 72 °C for 7 min. A temperature of 58 °C was found suitable for the PCR process. Approximately, 500–600-bp of the PCR product was obtained and sequenced by Pishgam Biotechnology Co. The result of the sequence analysis revealed that the isolated fungus was *P. commune*.

Evaluation of industrial oil

To evaluate the industrial oil, 7 ml of oil were reached to the volume of 25 ml using dichloromethane solvent in a volumetric flask and assessed by gas chromatography–mass spectrometry (GC–MS) device. The levels of individual components were determined using the measurement of specific spectral peaks with units of parts per million (ppm).

Determination of oil-removal capacity of fungus

After completing the fungal growth, industrial oil was added to the plates with an area of 1 cm using a pipette. After 10-day culture at 27 °C, the samples were collected and the oil-removal ability of contained fungus was studied using GC–MS device. Mass of harvested sample from each plate was about 0.2 g. Thereafter, the samples were solved in an appropriate solvent (ethyl acetate) and filtered. The filtered samples were diluted to a volume of 25 ml in the flask balloon. The formulae for the assessment of oil-removal activity and the percentage of removing were as follow:

$$Y = (X_t/X_o) \times 100$$

$$\text{Percentage of removing} = (100 - Y)$$

X_o and X_t were the ppm values of initial and final volumes of oil, respectively. Bioremoval process was assessed by measuring different parameters including injection volume of oil to each plate, pH level of plates' contents and the removal time. The tests were conducted in various time intervals between 0 and 10 days. In addition, the effects of a range of pH values (4, 5, 5.6, 6, and 7), concentration of oil (1, 3, 5, 7, 10, 15, 25, 50, and 75 µl), and biomass content were studied in culture medium. All the assessments were repeated five times and the mean ± SD values were reported.

Statistical analysis

One-way ANOVA and LSD were used to evaluate differences between groups. All statistical analyses were carried out using SPSS 17 (SPSS Inc.). The significance level of 0.05 was considered in this study.

Results and discussion

The present study aimed to investigate the potential ability of wastewater-derived fungus on bioremoval of industrial oil. A fungus from Penicillin family named *P. commune* was isolated from oil in an effluent disposal area near the second refinement oil industrial company of Garmsar. The strain of fungus was also confirmed using gene sequence analysis of 18s Ribosomal RNA. In addition, bioremoval activity of *P. commune* to degrade the hydrocarbon compounds was about 95.4 %. A morphological study of *P. commune* was done after smearing on slides and staining them with lactophenol cotton blue wet mount preparation. Slides were assessed by optical microscopy and the conidiophores specifically basipetal conidia were observed (Fig. 1).

Effect of initial dye pH on the process

The optimum conditions for some important parameters in this biological process such as final injection volume of oil, the pH level of culture, and removal time were also determined (Fig. 2). According to the recent studies, the changes in pH influence the oil-removal efficiency of fungi. As shown in Fig. 2, the most bioremoval activity of *P. commune* was found in pH 5.6 (83.59 %) which was considerably lower than normal condition (pH 7). The statistical analysis of these changes has been shown in Table 1. In contrast to acid-resistant microorganisms (e.g., *P. commune*), the suitable pH for living and higher activity of some other kinds of microorganisms is near neutral pH. However, considerable decrease in soil pH level has a great impact on the ability of microbial population to reduce hydrocarbons (Bossert and Bartha 1984). In addition, Verstraete et al. indicated that the biological elimination of gasoline will be doubled if some modifications perform to maintain the acidity of soil when it increases from 4.5 to

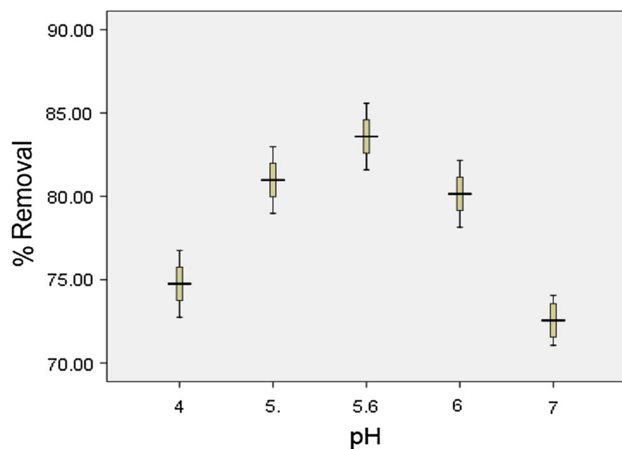

Fig. 2 Removal of injection volume (7 µl), 0.2 g of biological weight, 10 days, at different pH

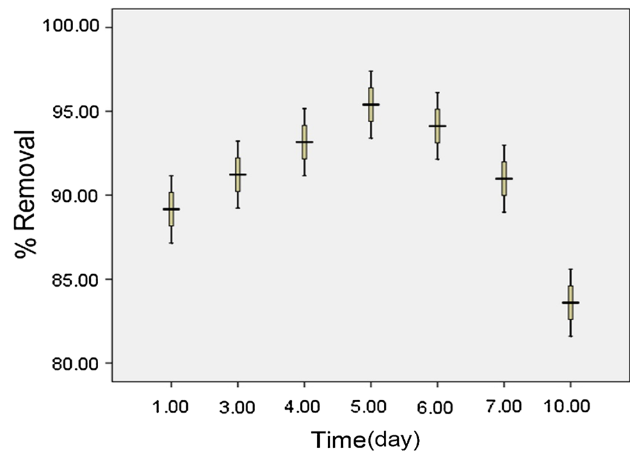

Fig. 3 Removal of injection volume (7 µl), 0.2 g of biological weight, 10 days, at different time

Table 1 Injection volume, pH, time: ANOVA test statistical study, 0.2 g of biological weight

	Between group	Within groups	Total
Sum of squares			
Volume	392.185	50.000	442.185
pH	459.601	69.920	529.521
Time	41,258.556	90.000	41,348.556
df			
Volume	4	20	24
pH	6	28	34
Time	8	36	44
Mean square			
Volume	98.046	2.500	–
pH	76.600	2.497	–
Time	5,157.319	2.500	–
F			
Volume	39.218	–	–
pH	30.675	–	–
Time	2,063	–	–
Sig.			
Volume	0.000	–	–
pH	0.000	–	–
Time	–		–

7.4 (Walker and Colwell 1974; Verstraete et al. 1976; Spain et al. 1980).

Effect of different time on the process

In order to find the optimum incubation time, this process was done in different time intervals (1–10 days). As shown in Fig. 3, the ideal length of time for oil degradation is 5 days. A gradually increasing in the efficacy of fungus to remove the oil was seen from 89.16 % in the first day to 95.4 % in the fifth day. The study continued to day 10 and the results have been shown in Fig. 3.

The statistical analysis of parameters related to the 10-day evaluation of oil-fungus co-culture has been also shown in Table 1. The biomass of fungus in this study was about 0.2 g per 1 cm^2. According to a research conducted in Ardabil, East Azerbaijan Province in Iran, the maximum bio-removal function of *A. niger* derived from the soil of industrial waste water was 75 % after 5-day co-culture with the fungal concentration of 75 mg/l (Esmaeili and Kalantari 2012).

Effect of inoculated oil volume on the process

Although there is a report of higher removal efficacy of *P. commune* than *A. niger* following 10 days cultivation, but another study revealed that the extended duration time could improve the removal efficacy no more than 75 %. On the other hand, the effect of different volumes of oil from 1 to 75 µl was evaluated in a period of 10 days. As shown in Fig. 4, our results pointed to a gradually decrease in the removal activity due to increased amount of oil from 7 µl (83.6 %) to 75 µl (3.33 %). Outcomes of related statistical analysis have been also shown in Table 1. The peaks of industrial oil analysis in an optimized condition before and after bioremoval process were obtained by GC (Fig. 5). As a result of this study, *P. commune* is a suitable, highly cost-effective and eco-friendly bioremoval for elimination of industrial-oil pollution (Atlas et al. 1980; Esmaeili and Kalantari 2012).

There are some evidences on the abundant amounts of bacteria and fungi in soil and their potential role in the elimination of hydrocarbons (White et al. 1990). Effluent of oil water waste into the soil increases the bacteria population and makes it easy to collect a large amount of

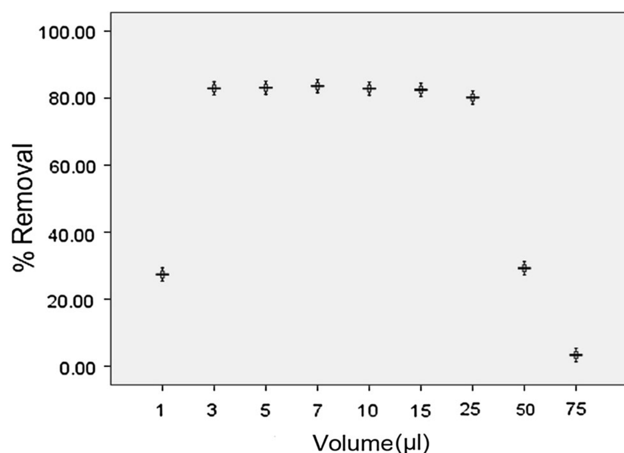

Fig. 4 Effect of injection volume on industrial oil by *P. commune*. 0.2 g of biological weight, 10 days

bacteria from the soil (Bossert and Bartha 1984; Jensen 1975; Atlas et al. 1980). In contrast to our study, Song et al. showed that bacteria (82 %) had a more potent activity to eliminate *n*-hexadecane in sandy environments than fungi (13 %) (Mohsenzadeh et al. 2012). In a research study, the possible role of different fungi to eradicate the contamination of soil has been compared. Among them, *Aspergillus terreus*, *Penicillium* sp., *Alternaria* sp., *Acromonium* sp. could efficiently remove 10, 8, 8, and 2 % of crude oil, respectively (Kirk and Gordon 1988). Although, the environmental oil contaminations can be removed by bacteria and fungi (Atlas et al. 1980), but the biological degradation of hydrocarbon compounds is mostly done by both *Penicillium* sp. and *Aspergillus* (Fusey and Oudot 1984). The highest rate of bioremoval is associated with saturated hydrocarbons and the lowest rate to light aromatic, with high molecular and polar compounds (Kirk and Gordon 1988; Fusey and Oudot 1984).

In this study, decreasing in peak area of GC–MS after passing the time of incubation indicates the successful ability of *P. commune* to remove or modify the industrial oil. In optimum condition (5 days, pH 5.6, 7 μl of oil), the remove of 95.4 % industrial oil is possible to wrap up, it is possible to efficiently use of natural metabolic function of microorganisms to eliminate the environmental pollutants such as industrial oil and, in fact, it will be as a part of biological self-removing.

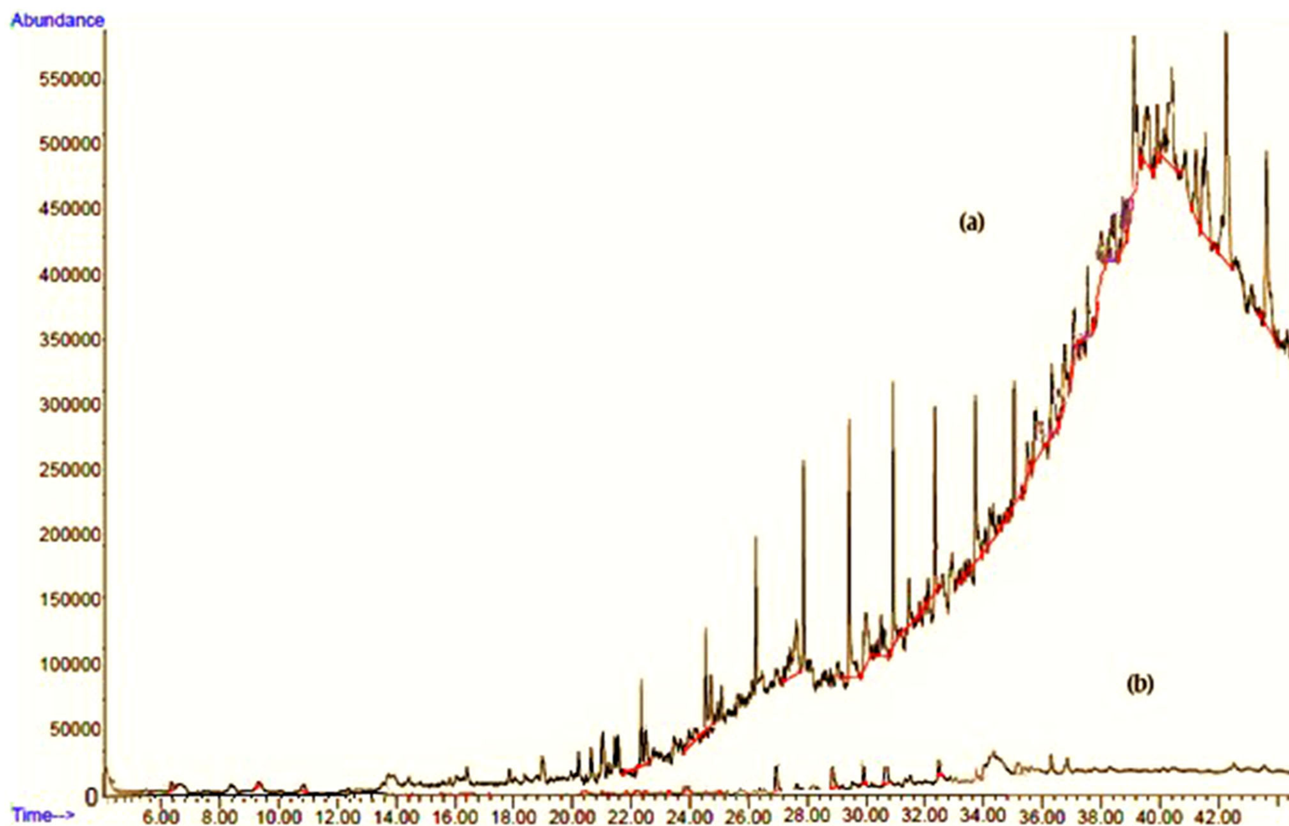

Fig. 5 GC spectrum relating to industrial oil before removing process (*a*) and after biological removing (*b*) by *P. commune* in optimized conditions

Conclusion

In conclusion, bio-removal of the oil waste water was carried out by a fungal strain named *P. commune* which was derived from oil and identified using biological tests. Effective identification of the isolated fungus was performed by 18s rRNA gene sequence analysis. GC–MS spectrums analyses confirmed the remove of oil industrial by *P. commune*. The bio-removal of the oil was dependent on the volume of oil, pH level of culture, and co-culture time point. The most efficiency of bioremoval process was up to 95.4 % under optimal condition.

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