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Bioremediation of Diesel Oil Spill by Filamentous Fungus *Trichoderma reesei* H002 in Aquatic Environment

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Abstract: Bioremediation of aquatic environment could be a response to the oil spills threats. In this paper, *Trichoderma reesei* H002, a filamentous ascomycete fungus isolated from a polluted site in an orchard garden, Johor, Malaysia, was experimented for its biodegradation ability to degrade diesel oil. Varying nitrogen and carbon sources, pH, agitation on diesel oil by *Trichoderma reesei* H002 in liquid media were examined to find their impacts on TPHs, alkane, aromatic and NSO fractions of diesel oil degradation. Glucose and yeast extract were the most suitable nutrients for the development of *T. reesei* H002 and increased the degradation of total petroleum hydrocarbons (TPHs up to 94.78% at the end of the study (40 days) at 25 ^{0}C. The degradation of TPHs were performed by gravimetric analysis and degradation of alkane and aromatic fractions were confirmed by GC-FID analysis. Based on the findings of *T. reesei* H002 for the biodegradation of diesel oil, it can be proposed that *T. reesei* H002 can be applied to bioremediate diesel oil spill in aquatic environment, therefore protect the ecosystem.

Keywords: Oil spill treatment, *Trichoderma reesei* H002, bioremediation, TPHs.

1. Introduction

Most of the worldwide oil production is transported by aquatic environment specially sea, therefore marine and coastal environments are prominently exposed to accidental oil spill incidents. The detrimental effects after an oil spill are incalculable, not only affecting human health but also threatening environmental ecosystem [9]. Once the oil has been released in the environment, oil prevents oxygen penetration and diffusion of light. In addition, its mutagenicity and carcinogenicity has been reported in many literature [8]. Therefore, it needs high priority to find an effective technology for eradicating contaminants to reduce oil pollution.

Nowadays, different techniques like using chemical dispersant or in situ burning are applied for oil removal but they possess ecological, economic or technic drawbacks: for example, using dispersant it only separate oil into another phase which is difficult to remove from the environment and sometimes the dispersants are seen more harmful than the spilled oil itself [14]. Such drawbacks can be overcome by microbial removal which avoid recontamination by secondary contaminants originated from chemical and physical remediation process. However, diesel like heavy fuel ranged from C8 to C25 are recalcitrant to remove naturally or in aerobic condition under limited nutrients [5]. Therefore, bioengineers and microbiologists working in related fields are facing a challenge to develop technologies which will ensure a good relationship among relevant microorganism and hydrocarbon and thereby adding the inadequate nutrients [9].

The term bioremediation means applying microorganism which are capable to degrade toxic pollutants for restoration of any polluted site [9]. Mostly microorganism like fungi and bacteria are used based on their ability to use hydrocarbon oil including its derivatives as carbon source. Their robustness and adaptation skills to withstand in extreme environments, lead them towards bioremediation process. Oil spill bioremediation is a common practice for treatment of the contaminated terrestrial environments [10]. However, the knowledge regarding marine oil spill treatment is still not smooth and its majority focused on the prokaryotic organisms. In addition, nutrients supply play an important role in biodegradation by microorganisms. Supply of nutrients like carbon or nitrogen source have been reported to enhance the biodegradation rate due to accelerated production of enzymatic action [6, 12]. Fungal species Trichoderma belong to filamentous group of fungi classified as a part of Ascomycetes. This species of fungi is distributed in grassland, agricultural system,

deserts, forests as well as aquatic ecosystems. Also, nutrient requirements are considered low as these fungi can survive in adverse conditions [2]. A study done by [4], showed that few *Trichoderma* species have the ability to degrade some of the fractions of petroleum hydrocarbons. Hence, it can be say that saturated hydrocarbons are easily degraded than that of PAHs. For example, *Trichoderma* S019 strain have the ability to degrade n-eicosane about 73% with the addition of glucose as subsidiary carbon source [7]. *Trichoderma* species like *T.inhamatumson*, *T. longibrachiatum* or *T. harizanium* have been reported to withstand 100 mg/L of pyrene or phenanthrene. Moreover, fungi degrading PAHs are reported recently, PAHs like benzo(a) fluoranthene, benzo(a)anthracene, benzo(a)pyrene, chrysene are reported to be degraded by the species *Trichoderma* [2]. Unfortunately, the ability of the filamentous fungal species associated to oil spill treatment in aquatic environment is limited, being the key topic of few researchers [1, 3].

The aim of this research is to investigate diesel oil spill treatment by fungi *Trichoderma reesei* H002 in marine environment. To find out the optimum treatment for oil spill, a pH ranges from 4 to 8 and agitation ranges from 80 rpm to 120 rpm has been studied. In addition, three carbon sources (galactose, fructose, glucose), four nitrogen sources (yeast extract, ammonium nitrate, ammonium chloride, ammonium sulphate) and different concentration of oil were applied for better understanding the role of fungal species in marine oil spill incidents.

2. Materials and Methods

Fungi

A filamentous fungus designated as H002 was isolated from a contaminated site in UTM orchard area, Johor, Malaysia and stored in a refrigerator to maintain their growth. Prior to use, tissue of the collected fungi was cut into small piece and cultured on malt extract agar (MEA). Based on 18 s rRNA identification and macroscopic morphological characteristics, phylogenetic tree was constructed, and H002 was classified as belonging to the *Trichoderma reesei* (Fig 1).

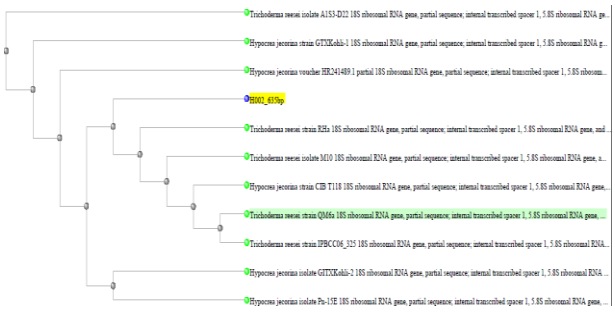


Fig 1. Phylogenetic tree of *Trichoderma reesei* H002 species.

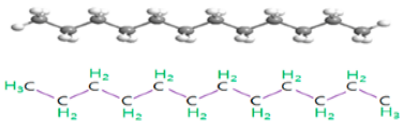
Chemicals and Reagents

MEA was procured from Difco (Detroit, USA). Glucose, yeast extract as well as other nutrients and chemicals were highly analytical grade and were supplied by Sigma Aldrich supplier company from Milwaukee, USA. From BHPetrol station in skudai, Johor, diesel oil was bought. Physical and chemical characteristics of diesel oil are listed in Table 1.

Fungal Inoculum and Culture Conditions

The strain of *T. reesei* H002 was maintained on MEA containing 20 g/L glucose, 20 g/L malt extract agar, 300 mg/L chloramphenicol to avoid bacterial growth and 4 g/L diesel oil in a glass petri dish at a dark place at room temperature and observed on daily basis for seven days. To study biodegradation of oil, three agar plugs of fungal inoculum were cut out from MEA plate fungal mycelium and transferred to liquid medium containing 20 g/L of both glucose, yeast extract and 300 mg/L of chloramphenicol. All the reagents were mixed with distilled water until it made a final volume of 30 ml in a 100-ml flask. After 4-5 days of incubation to get a homogenous distribution of fungal growth, finally a predetermined concentration of diesel oil containing 0.2% Tween 80 (1000 ppm-4000 ppm) has been added and kept in incubation or at 100 rpm for 20 and 40 days at room temperature. Each sample flask was prepared in triplicate to get an accurate result. All glass wares including liquid medium was autoclaved for 20 min at 121 ^{0}C. In addition, control flask containing diesel oil without inoculum was also maintained parallelly.

Table 1. Physical and chemical characterization of diesel oil.

Structure	
Molecular formula	$C_{10}H_{20}$ to $C_{15}H_{28}$
Appearance	Slightly brown viscous liquid
Molecular weight	178.6
Elemental analysis	carbon 85.9% (ww-1), hydrogen 13.1% (ww-1), nitrogen 0.3 % (ww-1)
Boiling point	180-360 °C
Aqueous solubility (15 °C)	0.005 g/L
Density	0.87 to 0.96 g/cm^3

TPH quantification

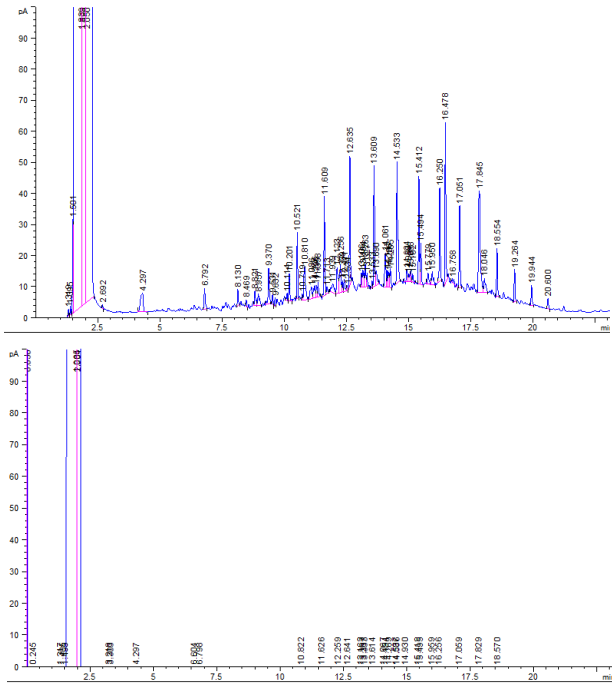
Samples were withdrawn at zero day (just before the bioremediation started), 10-day, 20 days and 40 days. Extraction procedure was followed from the method

provided by [9]. Briefly, TPH content in each cultured flask was extracted by equal volume of hexane followed by dichloromethane and finally chloroform. These three extracts volume were dried using a rotary evaporator and nitrogen stream until it gets complete dry. Then amount of remaining TPH was calculated gravimetrically. After gravimetric calculation, remaining TPH was divided into alkane fraction, aromatic as well as NSO fractions using silica gel loaded column. Alkane and aromatic fractions were eluted 100 ml hexane and 100 ml toluene. Lastly, the NSO fraction part was eluted by 200 ml of 1:1 methanol and chloroform. The aromatic and alkane fraction were analyzed by GC-FID (Agilent), injecting 1 µl of each solvent. The temperature program was set to 35 0C for 2 min then rise to 290 0C at a rate of 12 0C/min covering a total run of 24 min. Gravimetric calculation of degradation of TPH was done by following equation,

% Degradation of TPHs = $(W_0-W_t)/W0$ X 100%.....(1)
W_0 and W_t are the weight of TPHs in dry round bottom flask at 0 day and after treatments.

3. Result and Discussion

The lowest rate (51%) of degradation of TPHs (mg/30 mL liquid media) was observed at highest concentration of diesel oil (4000 ppm) while 67%, 80%, 85% and 95% degradation of TPHs were observed at 3000 ppm, 2000 ppm, 1500 ppm and 1000 ppm oil (data not shown). Hence, a natural trend is seen showing the decrease of degradation with the increase of initial concentration of diesel oil. Alkane and aromatic fractions collected from 1000 ppm diesel oil by GC analysis after silica fractionation loaded on column is shown in Fig 2 (A, B, C, D). Table 2 summarizes all parameters of batch culture.



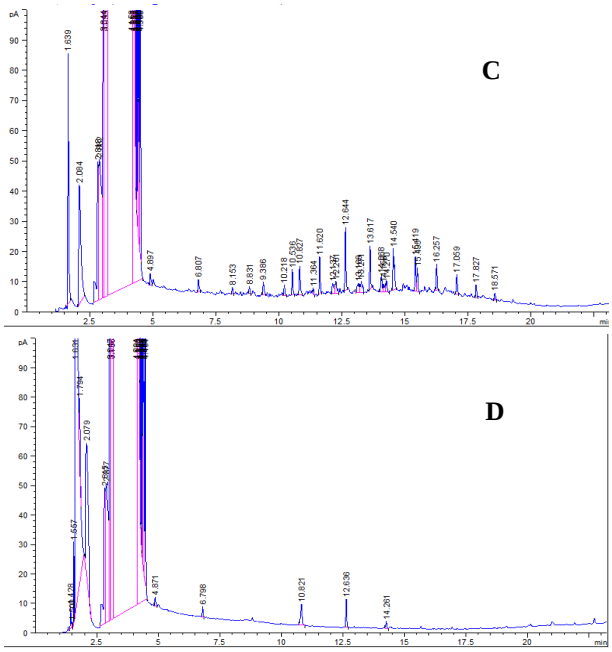


Fig 2. Degradation of alkane at 0 day (A), after 40 days (B), aromatic at 0 day (C) and after 40 days (D).

3.1 Effect of pH

Regulation of pH in liquid medium is practically crucial since it affects the enzyme activity including biodegradation procedure by *T. reesei* H002. In our experiment, pH 4, 5, 6 and 8 were studied (Fig. 3A). In room temperature, highest degradation of TPH was found at a range of pH 5 to 6 at the end of 40 day. However, at pH 8, the degradation was seen to reduce (52% only). Similar optimum value of pH (pH 5.5) was maintained constant to degrade crude oil and some aromatic compounds by *Trichoderma* strain [2].

3.2 Effect of Agitation

To figure out the optimum agitation limit, three distinct conditions with agitation (80, 100 and 120 rpm) were maintained placing the inoculated flask on mechanical shaker. From the graph In Fig. 3B, it is seen that at higher shaking rate (120 rpm) the degradation of TPH is lowest (only 56%) and projecting 84.4% degradation at 100 rpm. Sufficient agitation enhances the distribution of carbon and nitrogen source in liquid media which helps for the proper growth of inoculum, however, excessive agitation causes mycelium shearing leading to decrease in biomass yield [18].

3.3 Effect of Carbon Source

Carbon source is one of the fundamental nutrients for fungal growth. In oil spill bioremediation process, hydrocarbon oil can be used as sole carbon source [6] or with the presence of other carbon sources like glucose [9]. In this study, three different carbon sources like glucose, galactose and fructose were applied with yeast

extract as nitrogen source. Fig. 3D suggests the effect of carbon sources in an order like glucose (94.78%) > galactose (71.5%) > fructose (60.5%) at the end of 40 days culture.

3.4 Effect of Nitrogen Source

The highest degradation of oil was observed by using yeast extract as nitrogen source comprising more than 94% removal of TPH of diesel. Degradation rate after 40 days can be arranged in an order like yeast extract (94.8%) > ammonium chloride (84.4%) > ammonium sulphate (70%) > ammonium nitrate (51%) (Fig 3C). [13] reported the degradation of olive oil mill effluent by fungi *P. citrinopileatus* was best observed in the presence of yeast extract due to increase laccase enzyme production. A comparison showing diesel oil degradation by other species is listed in Table 3.

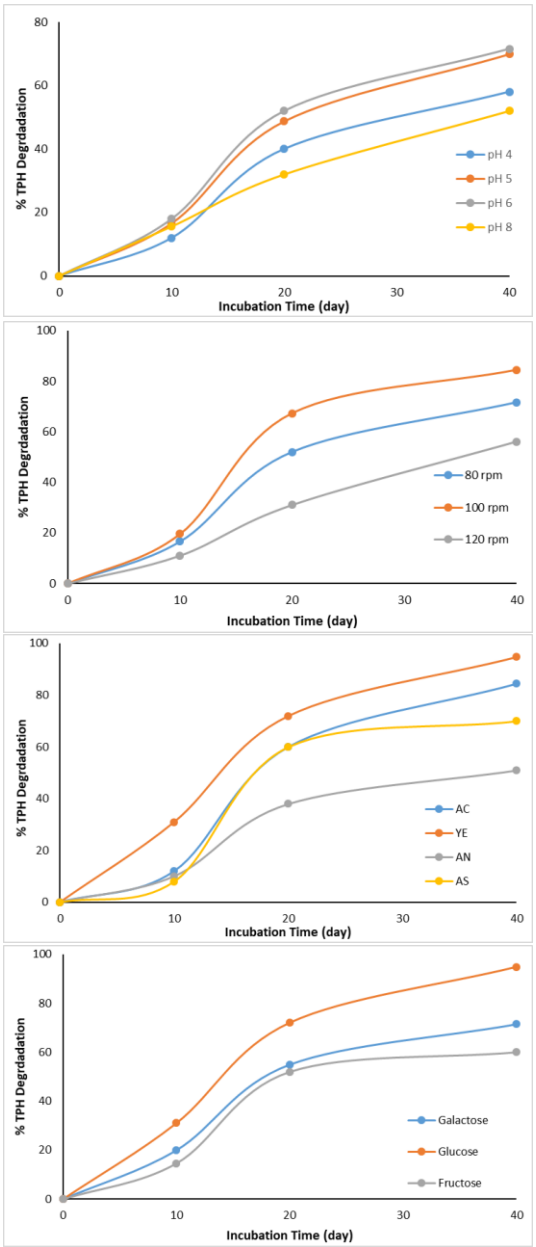


Fig. 3 Effect of pH (A), agitation (B), nitrogen source (C), carbon source (D) on 1000 ppm diesel oil. AC: ammonium chloride, YE: yeast extract, AN: ammonium nitrate, AS: ammonium sulphate.

Table 2. Summarizes for all experiments of batch culture.

Parameters	Initial pH	Agitation	Nitrogen Source	Carbon Source
Effect of initial pH	4 to 8	80	AC	Glucose
Effect of agitation	6	80 to 120	AC	Glucose
Nitrogen source	6	100	AC, YE, AN, AS	Glucose
Carbon source	6	100	YE	Glucose, Galactose, Fructose

* AC: ammonium chloride, YE: yeast extract, AN: ammonium nitrate, AS: ammonium sulphate.

Table 3. Some species used for degradation of diesel oil.

Species	% Degradation	Source
Cladosporium	34%	[10]
Trichosporon asahii	95%	[11]
T. longibrachiatum Evx1	54%	[12]
Trichoderma reesei H002	94.70%	This study

4. Conclusion

Oil spills are posing immense threat for wildlife biodiversity and aquatic ecosystem and the appropriate use of microorganisms capable to biodegrade this hydrocarbon oil constituents have demanded a real challenge for future researches. Until recently, Trichoderma reesei species fungi had been overlooked despite their potential hydrocarbon degradative abilities. The biodegradation ability of the *T. reesei* H002 has considerably boosted by optimizing physio-chemical parameters. Filamentous fungus *T. reesei* H002 degraded more than 90% TPHs of diesel oil of 1000 ppm with agitation 100 rpm, pH 6 and in the presence of glucose and yeast extract. This encouraging result established that *T. reesei* H002 is an effective candidate for the bioremediation of diesel oil spill in ocean and therefore, help to protect ecosystem. Further study to identify the excreted fungal enzymes that are responsible for degradation of oil is needed.

Acknowledgement

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