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Optimal Physical and Nutrient Parameters for Growth of *Trichoderma virens* UKMP-1M for Heavy Crude Oil Degradation

(Pengoptimuman Parameter Fizikal dan Nutrien bagi Pertumbuhan *Trichoderma virens* UKMP-1M untuk Degradasi Minyak Mentah Berat)

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ABSTRACT

This study was carried out to determine the optimal parameters for the production of biomass of Trichoderma virens *UKMP-1M, a fungus isolated from oil-polluted wastewater. The isolate showed maximum growth at day six after incubation in Mineral Salt Medium (MSM) in the presence of 3% (v/v) heavy Khefji Sour crude oil. Although it grew at pH between 5.0 and 7.0, it grew best at pH 5.5.* T. virens *UKMP-1M grew at temperatures between 25°C and 35°C, with its highest growth at 30°C. Aeration by agitation at 200 rpm was shown to yield the greatest biomass. Peptone at concentration of 1.5% (w/v) was determined to be a better nitrogen source than urea, potassium nitrate (KNO_3), yeast extract, ammonium sulphate ($(NH_4)_2SO_4$) and ammonium chloride (NH_4Cl). Addition of 1% (v/v) crude oil to the MSM medium led to higher biomass production than the addition of 3%, 5%, 7% and 10% (v/v) crude oil. The result also revealed that 40% of total petroleum hydrocarbon (TPH), 100% of pristane and 74% of phytane compounds were degraded after 9 days of incubation at optimal physical and nutrient parameters.*

Keywords: Crude oil; optimisation; total petroleum hydrocarbon; Trichoderma virens

ABSTRAK

Kajian ini dijalankan untuk menentukan parameter optimum bagi menghasilkan biojisim Trichoderma virens *UKMP-1M iaitu kulat yang dipencilkan daripada air kumbahan tercemar minyak. Pencilan tersebut menunjukkan pertumbuhan yang maksimum pada hari keenam selepas pengeraman di dalam medium garam mineral (MSM) dengan kehadiran 3% (i/i) minyak mentah berat Khefji Sour. Walaupun ia tumbuh pada pH antara 5.0 dan 7.0, namun ia tumbuh dengan terbaik pada pH 5.5.* T. virens *tumbuh pada suhu antara 25°C dan 35°C, dengan pertumbuhan tertinggi pada 30°C. Pengudaraan dengan cara penggoncangan pada 200 psm menunjukkan penghasilan biomass yang terbaik. Kepekatan pepton pada 1.5% (b/i) telah ditentukan sebagai sumber nitrogen yang terbaik berbanding urea, kalium nitrat (KNO_3), ekstrak yis, ammonium sulfat ($(NH_4)_2SO_4$) dan ammonium klorida (NH_4Cl). Penambahan 1% (i/i) minyak mentah ke dalam medium MSM mengarah kepada penghasilan biojisim yang tertinggi berbanding penambahan 3%, 5%, 7% dan 10% minyak mentah. Hasil menunjukkan 40% komponen jumlah hidrokarbon petroleum (TPH), 100% sebatian pristin dan 74% sebatian fitin telah didegradasikan selepas 9 hari pengeraman pada parameter fizikal dan nutrien optimum.*

Kata kunci: Jumlah hidrokarbon petroleum; minyak mentah; pengoptimuman; Trichoderma virens

INTRODUCTION

Petroleum hydrocarbons are among the most common anthropogenic contaminants and have become a global environmental problem, with a wide variety of contributing sources. They include at least four types of hydrocarbon: alkane, aromatic hydrocarbon, resin and asphaltine. The effectiveness of bioremediation of petroleum hydrocarbons is significantly affected by the inherent capabilities of the microorganisms, by their ability to overcome the bioavailability limitations in multiphase environmental scenarios and by environmental factors such as pH, temperature, nutrients and electron acceptor availability (Mukherji et al. 2004; Trindade et al. 2005).

Various microbial genera have been detected in petroleum-contaminated soil or water, suggesting that each plays a role in hydrocarbon transformation (Mancera-López et al. 2008). Some microbes have been found to produce enzymes that can degrade or adapt to petroleum hydrocarbon. Some of the microbes degrade alkenes, aromatic or both paraffin and aromatic hydrocarbons (Atlas 1995). Other than bacteria, fungi such as *Rhizopus* sp. (Okerentugba & Ezeronye 2003), *Aspergillus* sp., *Penicillium* sp. and *Trichoderma* sp. have also been shown to degrade hydrocarbons (Chaîneau et al. 1999).

Sources of carbon and nitrogen and the balance of the two sources as well as physical parameters such as pH, temperature and agitation are also important factors in fungal growth. Although fungi need nitrogen to produce cells and their inner structure via the synthesis of nucleic acid, protein and chitin, they are not able to

assimilate the nitrogen directly from the atmosphere, so they rely on compounds such as nitrate, ammonia, amino acids, amine, polypeptide and other proteins as nitrogen sources (Shewfelt et al. 2005). Although it is generally agreed that ammonium-nitrogen is the preferred form for microbial metabolism as it requires less energy to be assimilated, there is some disagreement on the most effective form of nitrogen for hydrocarbon degradation and its enhancement.

In premilinary studies, a total of 48 isolates were isolated from oil samples taken from a refinery oxidation pond in Melaka, Malaysia. Of these, only four isolates were selected for investigation based on their ability to grow on mineral salt medium (MSM) agar with addition of four types of crude oil. These four isolates achieved a 5 cm colony diameter within 24 to 48 h of incubation. They were identified as *Trichoderma virens* UKMP-1M, *Trichoderma virens* UKMP-2M, *Trichoderma virens* UKMP-3M and *Trichoderma virens* UKMP-4M based on macroscopic and microscopic observations and polymerase chain reaction (PCR) using universal primers ITS1/2. Generally, the soilborne filamentous fungus *Trichoderma virens* is a biocontrol agent with a well-known ability to produce antibiotics, parasitize pathogenic fungi, and induce systemic resistance in plants (Djonović et al. 2006), but because these isolates can grow on MSM oil agar, this study was based on the assumption that *T. virens* UKMP-1M isolated from oil-contaminated wastewater may contribute to the biodegradation of crude oil and therefore merits study of its growth factors.

The aim of this study was to determine the parameters for optimal growth of isolate *T. virens* UKMP-1M and to determine its rate of TPH, pristane and phytane degradation of heavy crude oil.

Materials and Methods

Media and Culture Condition

Two types of medium were used: for cultivation, Potato Dextrose Agar (PDA) and for the optimisation study, mineral salt medium (MSM) (Zajic & Supplisson 1972) with addition of 3% (v/v) heavy Khefji Sour crude oil as a sole carbon source. The pH of the medium was adjusted to 5.5 with 1M HCl or 1M NaOH prior to sterilisation. The medium was sterilised at 121°C for 15 min before addition of the crude oil.

Preparation of Standardised Inoculums

Spore suspensions were prepared by adding 15 mL of sterile distilled water to mature (4-5 days) fungal colonies on PDA plates to dislodge the spores from the mycelium. The spores were counted using a haemacytometer (Neubauer, Germany) to obtain a spore concentration of about 10^5 spores/mL. These suspensions were then used to inoculate 100 mL MSM containing 3% (w/v) glucose in 500 mL Erlenmeyer flasks (Kendrick & Ratledge 1996). The cultures were incubated at 30°C in an incubator shaker operating at 180 rpm for 48 h. The resultant active growing cultures were aseptically washed three times with 300 mL of sterilised distilled water to remove remaining glucose. This resulting culture was then used as standard inoculum for further experiments.

Determination of Optimal Growth

The optimisation study covered physical (pH, temperature and speed of agitation) and nutrient (nitrogen source, nitrogen concentration and crude oil concentration) parameters. A total of 10% (v/v) of standard inoculum was inoculated in each experiment and performed in triplicate. Biomass production (g/L) was used as an indicator for growth after 6 days of incubation. MSM medium with crude oil (without inoculation with fungus) was used as a control.

Physical Parameters

1. pH
   The influence of initial medium pH on fungal growth was investigated at pH 5.0, 5.5, 6.0, 6.5 and 7.0. A 10% (v/v) standard inoculum was inoculated in a 500 mL Erlenmeyer flask containing 100 mL of MSM with addition of 3% (v/v) heavy Khefji Sour crude and incubated at 30ºC in an orbital shaker at 180 rpm for 6 days. The pH that promoted the highest biomass production was used for subsequent steps of the investigation.
2. Temperature
   The effects of temperature on fungal growth were studied at 25, 30 and 35ºC in MSM medium with 3% (v/v) heavy Khefji Sour crude at the determined optimum pH and incubated in an orbital shaker at 180 rpm for 6 days. The temperature that promoted the highest biomass production was used for the subsequent steps of the investigation.
3. Speed of agitation
   The effects of agitation during incubation on growth was carried out in MSM medium with 3% (v/v) heavy Khefji Sour crude at optimum pH using an orbital shaker at 150, 180, 200 and 250 rpm. Incubation was conducted at the determined optimum pH and temperature. The agitation speed that promoted the highest biomass production was used for the subsequent steps of the investigation.

Nutrient parameters

1. Nitrogen source
   The effects of different nitrogen sources on fungal growth were studied by replacing NH_4C_l in MSM broth with one of five alternative nitrogen sources: peptone, urea, yeast extract, KNO_3 and $((NH_4)_2SO_4)$. Yeast extract, urea and peptone represented organic nitrogen sources while $((NH_4)_2SO_4)$ and KNO_3 represented

inorganic nitrogen sources. The replacement was made so that the total amount of nitrogen used would be the same as the total amount of nitrogen in the NH_4C_1 which is typically used in MSM medium at 0.0075 mol/L MSM. The inoculate was grown in the oil-added MSM with one nitrogen source replacement and incubated for 6 days at the optimum pH, temperature and speed of agitation as determined in the physical parameter investigation. The nitrogen source found to induce the highest growth of the fungus was used in the subsequent steps of the investigation.

2. Nitrogen concentration
   The next step of the investigation was the determination of the optimum concentration of the determined optimum nitrogen source. The inoculum was grown in MSM with 3% (v/v) crude oil at concentrations of the selected nitrogen source from 0.01% to 2.0% (w/v) at 0.05% intervals and incubated for 6 days at optimum pH, temperature and agitation speed as determined in the physical parameter investigation.
3. Concentration of crude oil
   The isolate was grown in MSM prepared in accordance with the optimum nutrient parameters but supplemented with 1%, 3%, 5%, 7% and 10% (v/v) concentrations of heavy Khefji Sour crude and incubated at optimum pH, temperature and agitation speed for 6 days.

DRY WEIGHT MEASUREMENT

The biomass produced was recovered by filtration using Whatman filter paper (No. 4). The biomass was washed with 100 mL chloroform to remove residual oil, then dried in the oven at 60°C overnight, and cooled in a desiccator for 10-20 min prior to weighing.

DETERMINATION OF BIODEGRADATION ACTIVITY

The determination of the biodegradation activity of *T. virens* UKMP-1M was carried out in 100 mL MSM medium supplemented with the optimal concentration of crude oil in 500 mL Erlenmeyer flask and incubated at 30°C and agitated at 200 rpm for 9 days. The residual petroleum hydrocarbon was recovered by chloroform extraction at a ratio of 1:1 (MSM medium:chloroform) (Chaillan et al. 2004). MSM without fungal inoculation was used as control, to quantify abiotic loss due to evaporation.

Analysis of the fungal biodegradation activity was made using a computerised capillary gas chromatography with flame ionised detector (GC-FID) (Perkin Elmer-Auto System) equipped with HP 3390A Integrator, split injector (split ratio 20/1) and flame ionisation detector set at 300°C. The carrier gas was nitrogen at flow rate of 1.5 mL/min. The column was polydimethyl siloxane (length 30 m, internal diameter 0.32 mm, film thickness 0.25 μm). The temperature was programmed to increase from 60 to 320°C at 4°C min^{-1}. Individual compounds present in the fractions were determined by matching the retention time with authentic standards.

The total petroleum hydrocarbon (TPH) degradation by this isolate was calculated according to the following equation:

$$\%B = \frac{100\ (TPHC - TPHI)}{TPHC}, \quad \text{(i)}$$

where B is biodegradation, TPHC is the total petroleum hydrocarbon in the sterile control (without fungal inoculation) and TPHI is the total petroleum hydrocarbon with inoculation in this case with *T. virens* UKMP-1M.

STATISTICAL ANALYSIS

The results obtained were analyzed statistically using the MINITAB software for Windows package. The means were compared using oneway ANOVA to indicate any significant difference among parameters and the variables. The result was considered significant if $p<0.05$.

RESULTS

DETERMINATION OF OPTIMAL GROWTH

Physical parameters Physical parameters including initial medium pH, incubation temperature and aeration play important roles in enhancing biomass production (Figure 1). Therefore, they need to be optimised. For optimisation of initial medium pH for growth, the results showed that *T. virens* UKMP-1M was able to grow on a wide range of pH from 5.0 to 7.0 with significant difference value at p=0.001. However, the maximum biomass production (0.24 g/L) was achieved at pH 5.5. The production of biomass in MSM medium decreased as pH value increased from 6.0 to 7.0 (Figure 1(a)). Statistical analysis showed no significant difference between pH 6.0, 6.5 and 7.0 with p value at 0.394.

The production of biomass varied in *Trichoderma* cultured at different temperatures. Isolate *T. virens* UKMP-1M produced maximum biomass (0.23 g/L) when incubated at 30°C compared to incubation at 25°C and 35°C which resulted in the production of 0.17 g/L and 0.12 g/L biomass, respectively. There was no significant difference between 25°C and 35°C with p value at 0.120 (Figure 1(b)).

As for the effects of aeration, *T. virens* UKMP-1M showed an increase of biomass as the rate of agitation increased up to 200 rpm, then reduced when the speed of agitation increased up to 250 rpm (Figure 1(c)). Statistical analysis showed no significant difference between speed of agitation of 180 and 250 rpm with p value at 0.546, although isolate *T. virens* UKMP-1M produced higher biomass at 180 rpm than at 250 rpm.

Nutrient parameters Replacement of ammonium chloride (NH_4Cl) at 4.0 g/L in MSM with other nitrogen sources showed that fungal growth was more enhanced in the presence of peptone than with urea, yeast extract, KNO_3

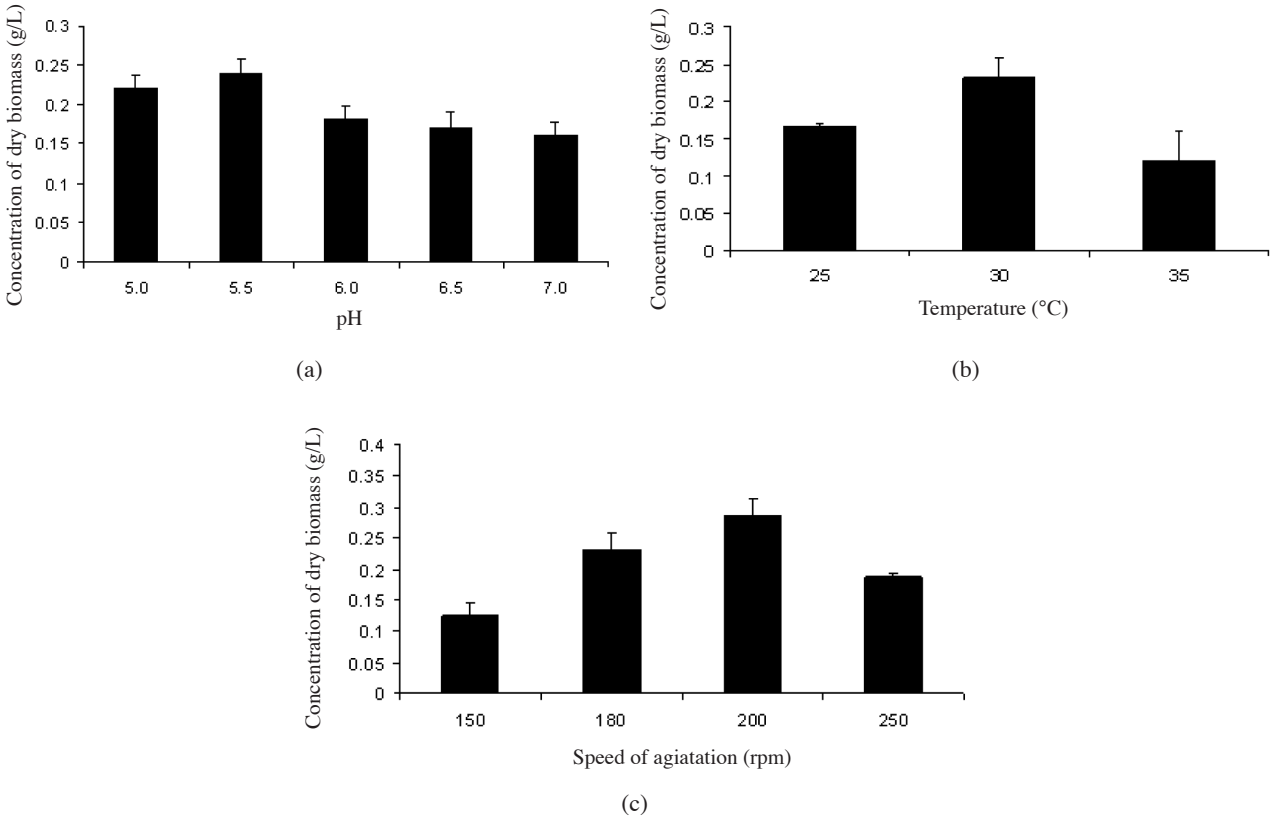

FIGURE 1. Biomass production of *T. virens* UKMP-1M after 6 days incubation (a) Isolate growth in MSM at different initial medium pH, incubated at 30°C and agitated at 180 rpm, (b) Isolate growth in MSM at pH 5.5, incubated at different temperatures and agitated at180 rpm and (c) Isolate growth in MSM at pH 5.5, incubated at 30°C with different agitation speeds

and $(NH_4)_2SO_4$ (Figure 2). The presence of peptone in MSM produced better biomass growth (0.49 g/L) than did NH_4Cl, whose biomass production was 0.29 g/L. On the other hand, the lowest biomass production for this isolate was produced when inoculated in MSM with urea as nitrogen source. *T. virens* UKMP-1M also showed an increment of biomass with increasing concentration of peptone. The optimum concentration of peptone was found to be 1.5% (w/v) with maximum biomass production at 1.99 g/L. The initial concentration of peptone (0.17% (w/v)) produced only 0.49 g/L biomass (Figure 3). The biomass was shown to decrease with peptone at 2.0% (w/v).

T. virens UKMP-1M was found to grow in a wide range of crude oil concentrations. The production of biomass increased to 2.25 g/L in the presence of 1% (v/v) heavy Khefji Sour crude oil. The production of

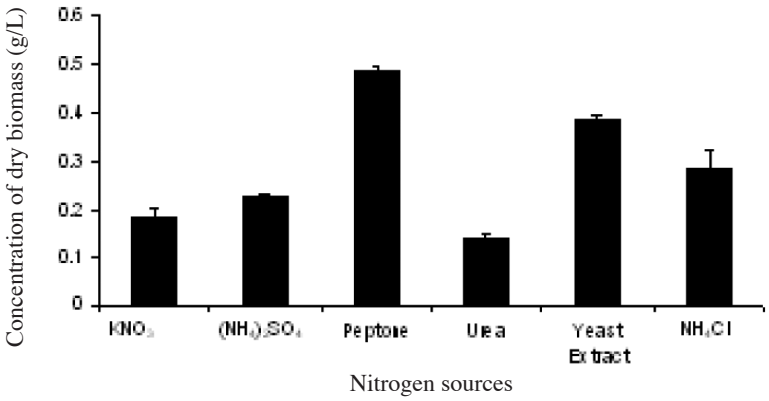

FIGURE 2. Biomass production of *T. virens* UKMP-1M in MSM medium at pH 5.5 with addition of different nitrogen sources incubated at 30°C and agitation at 200 rpm for 6 days. The concentration of total nitrogen use of each source was 0.0075 mol

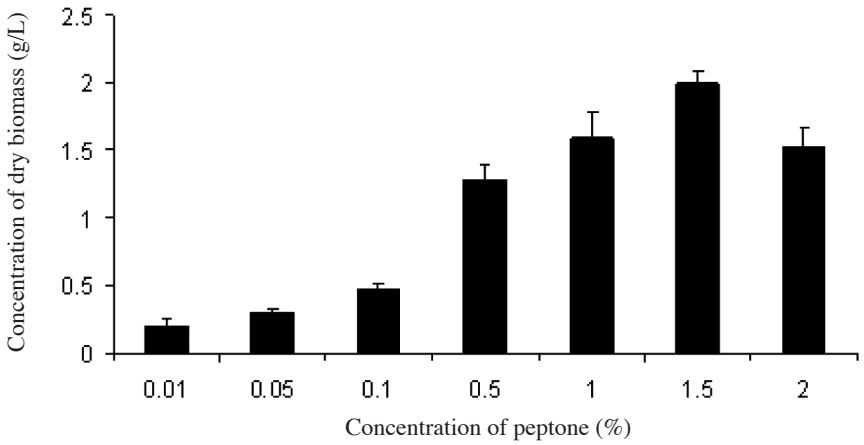

FIGURE 3. Biomass production of *T. virens* UKMP-1M in MSM at pH 5.5 with peptone at different concentrations, incubated at 30°C and agitated at 200 rpm for 6 days

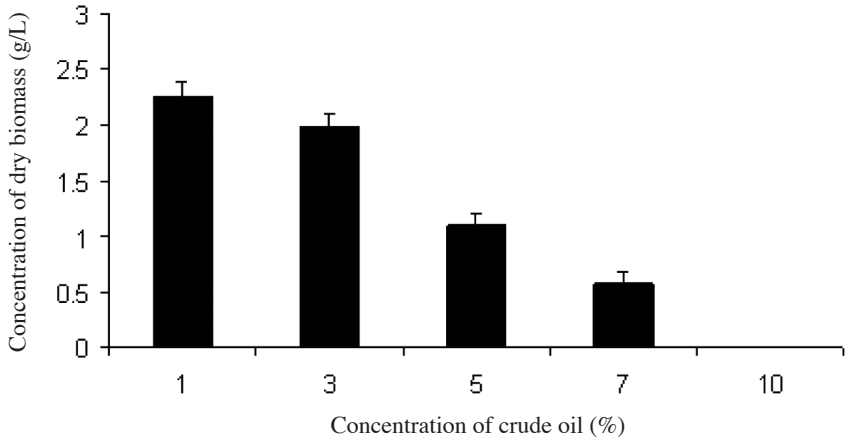

FIGURE 4. Biomass production of *T. virens* UKMP-1M with different concentrations of crude oil incubated at 30°C and agitated at 200 rpm for 6 days

biomass decreased when the concentration of crude oil increased beyond 1% (v/v) (Figure 4). Notably, no growth was found when the medium contained 10% (v/v) crude oil. Statistical analysis showed no significant difference between the presence of 1% and 3% (v/v) crude oil with p value at 0.053, although crude oil at 1% (v/v) produced the highest biomass.

DETERMINATION OF BIODEGRADATION OF CRUDE OIL

The degradation of crude oil was determined by cultivating the *T. virens* UKMP-1M culture in the optimum physical and nutrient parameters (pH 5.5, temperature of 30°C, speed of agitation at 200 rpm, addition of 1.5% (w/v) of peptone and 1% (v/v) of crude oil). The GC profile showed the percentage of TPH degradation by isolate *T. virens* UKMP-1M was 39.52±0.31% (Figure 5). The isolate also tended to degrade C_{21}-C_{38} hydrocarbons (66.70±4.16% degradation) more than C_{12}-C_{20} hydrocarbons (13.03±0.66% degradation) (Figure 5).

T. virens UKMP-1M also seemed to be selective to pristane and phytane because of its almost total degradation of these compounds after 9 days incubation at 30°C (100% and 73.85±2.04%, respectively).

DISCUSSION

PHYSICAL PARAMETER STUDY

This study represents the first steps toward the generation of an efficient bioremediation process. First, a suitable microorganism was chosen for its degradation potential, and then culture conditions were identified that optimise the growth of the microorganism (Ryan et al. 2007). As expected, this study confirmed that *T. virens* UKMP-1M grew better in acidic conditions. Previous studies also reported that several fungal isolates such as *Fusarium solani*, *F. oxysporum*, *Trichoderma viride* (Verdin et al. 2004) and *Aspergillus niger* (Srivastava & Thakur 2006) cultured in MSM medium at pH 5.5 also gave a good growth. However, although its growth was highest under acidic conditions, isolate *T. virens* UKMP-1M was able to grow in a relatively wide range of pH from 5.0 to 7.0, suggesting

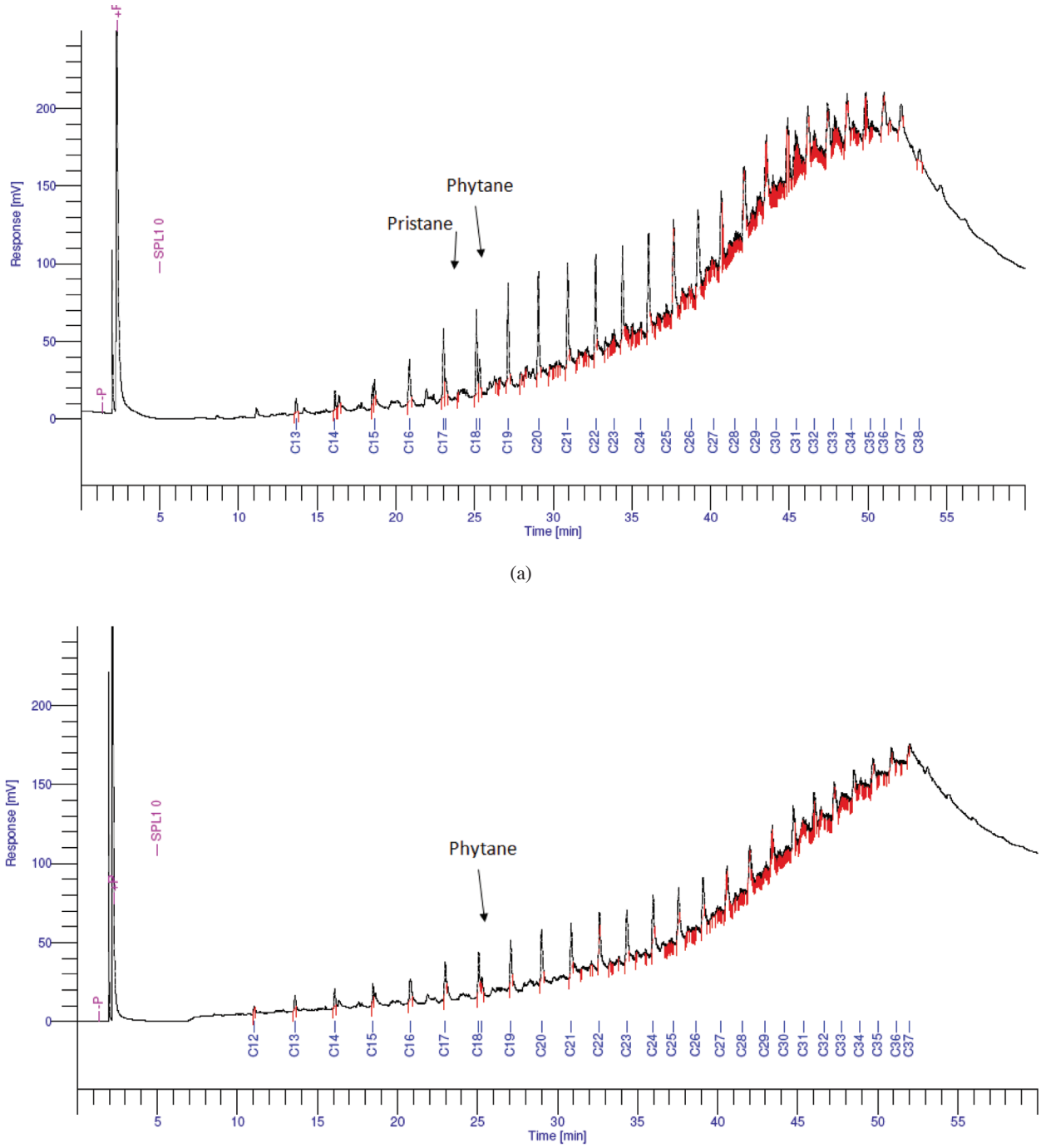

FIGURE 5. Gas chromatogram profile of residual oil after 9 days incubation at 30°C. (a) Control without inoculation and (b) Inoculated with *T. virens* UKMP-1M. TPH degradation was 39.52% with pristane and phytane degradation at 100% and 73.85%, respectively

that this isolate could degrade oil under not only acidic but also neutral conditions.

Among the parameters that could affect biomass production is temperature, generally considered the most important factor (Delille et al. 2004). The common incubation temperature for the growth of fungi such as *A. niger* (Delille et al. 2004), *Fusarium* sp., *Penicillium* sp. and *Graphium* sp. (Santos & Linardi 2004)) is taken to be 30°C. In this study, *T. virens* UKMP-1M was able to grow at up to 35ºC though with a slight reduction in biomass. This range of temperatures that encourage growth makes this isolate suitable for use in bioremediation in tropical climates, unlike *Trichoderma* sp., which has been reported to grow faster at the lower temperatures of 25 to 30°C (Samuel et al. 2007).

Agitation influenced the microbe to absorb more nutrients, increasing both the microorganism's surface area for degradation of the oil (Lee et al. 1996) and the amount

of dissolved oxygen in the cultivation medium (Purwanto et al. 2009). Agitation speed has also been proven to be a critical factor influencing mycelial biomass. In an earlier study by Kim et al. (2003), isolate *Paecilomyces sinclairii* showed an increment of biomass with speed of agitation at a range 50 to 250 rpm. Under optimum conditions up to 30.5 g/L biomass was produced when the isolate was shaken at 250 rpm (Kim et al. 2003). Similarly, this study found that production of biomass increased with the speed of agitation. Aeration could be beneficial to the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate, product or by-product and oxygen.

NUTRIENT PARAMETER STUDY

Nutrient addition is important to achieve the C:N balance and successful biodegradation of petroleum contaminants (Jin & Fallgren 2007). Among the tested nitrogen sources, the best biomass yield was recorded in the medium that contained peptone compared to those containing yeast extract, urea, ammonium chloride, potassium nitrate and ammonium sulphate. Urea has been considered as preferred nitrogen source for enhancing biodegradation because of its high nitrogen content (Jin & Fallgren 2007) but our results indicate that urea inhibited *T. virens* UKMP-1M growth. It may be that urea is toxic to the cells of microorganisms involved in petroleum degradation. All other inorganic nitrogen sources tested in this study showed poor growth; similar to the results of a previous study (Adejoye et al. 2006) where ammonium chloride, potassium nitrate and ammonium nitrate did not increase the isolate *Pleurotus florida* biomass production. Nitrate ions have been implicated in the inhibitory effect of some fungi where sulphate ion (SO_4^{2-}) is a large radical which may be difficult to transport across the fungal membrane where it can promote growth. Previous study also showed that for white rot fungi, the presence of 2.0% (w/v) of peptone was needed for good growth (Ruiz-Aguilar et al. 2002). It showed that the requirement of peptone varied for different isolates studied.

Higher concentration of crude oil could have toxic effects on the cells and lead to decreased biomass production with the increment of concentrations of crude oil (Head & Swannell 1999). The toxicity of crude oil or petroleum products varies widely, depending on their composition, concentration, environmental factors and on the biological state of the organisms at the time of the contamination (Obire & Anyanwu 2009). Different species and different life stages of organisms have been demonstrated to have different susceptibilities to pollution. The decrease in biomass production with increasing concentration of crude oil is often attributed to oil toxicity. Some microorganisms are killed or inhibited by toxic components in the oil, while other heterotrophic organisms degrading the oil are increasing in number.

Of the various petroleum fractions, *n*-alkanes of the intermediate length (C_{10}-C_{20}) are the preferred substrates and tend to be the most readily degradable whereas shorter chain compounds are rather more toxic. Longer chain alkanes (C_{20}-C_{40}) are hydrophobic solids and difficult to degrade due to their poor water solubility and bioavailability and branched chain alkanes are also degraded more slowly than the corresponding normal alkanes (Balba et al. 1998). In the present study, isolate *T. virens* UKMP-1M showed favourably in degrading longer chain compared to intermediate length hydrocarbons. This isolate can potentially be one of the fungi that are useful in degrading difficult compounds present in the crude oil.

Isolate *Penicillium funiculosum* has demonstrated the greatest removal of TPH, polycyclic aromatic hydrocarbons (PAHs) and aromatic hydrocarbons (AH) which were at 86±6%, 75±0.5% and 92±5%, respectively, after 15 days treatment in mineral medium (Mancera-López et al. 2007). Meanwhile, isolate *Polyporus* sp. has shown maximal degradation (93%) of 0.1% crude oil after 60 days of incubation (Hadibarata & Tachibana 2009). These results showed *P. funiculosum* and *Polyporus* sp. had higher TPH degradation compared to isolate *T. virens* UKMP-1M but needed longer incubation time to achieve their highest percentages of TPH degradation of 15 days and 60 days, respectively compared to 9 days for isolate *T. virens* UKMP-1M.

Phytane is a diterpenoid alkane while pristane (2,6,10,14-tetramethylpentadecane) is a naturally occurring isoprenoid alkane that is probably derived from the phytyl moiety of chlorophyll, from thermal degradation of tocopherols, and/or from the catagenic decomposition of methyltridecylchromans (Bregnard et al. 1997). During bioremediation of mineral oil-contaminated sites, it is commonly observed that *n*-alkanes are biodegraded more rapidly than isoprenoid alkanes (Hess et al. 1996). The biodegradation of pristane and phytane is of particular interest, since it has often been used as a relatively inert biomarker in studies of oil degradation (Bregnard et al. 1997; Haven et al. 1988).

CONCLUSION

Trichoderma virens UKMP-1M which was isolated from crude oil wastewater showed maximum growth at day six of incubation with optimum growth condition of pH 5.5, temperature 30ºC, speed of agitation 200 rpm, 1.5% (w/v) of peptone and 1% (v/v) of heavy Khefji Sour crude oil. Total petroleum hydrocarbon degraded was almost at 40% with 100% of pristine and 74% of phytane compounds. The fungus preferably degrades longer chain hydrocarbons (C_{20}-C_{40}) after 9 days of incubation with optimal physical and nutrient parameters.

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REFERENCES

Adejoye, O.D., Adebayo-Tayo, B.C., Ogunjobi, A.A., Olaoye, O.A. & Fadahunsi, F.I. 2006. Effect of carbon, nitrogen and mineral sources on growth of *Pleurotus florida,* a Nigeria edible mushroom. *Afr. J. Biotechnol*. 5(14): 1355-1359.

Atlas, R.M. 1995. Bioremediation of petroleum pollutants. *Int. Biodeter Biodegr.* 35: 317-327.

Balba, M.T., Al-Awadhi, N. & Al-Daher, R. 1998. Bioremediation of oil-contaminated soil: microbiological methods for feasibility assessment and field evaluation. *J. Microbiol. Meth*. 32: 155-164.

Bregnard, T.P.A., Haner, A., Hohener P. & Zeyer, J. 1997. Anaerobic degradation of pristane in nitrate-reducing microcosms and enrichment cultures. *Appl. Environ. Microbiol*. 63(5): 2077-2081.

Chaillan, F., Fleche, A.L., Bury, E., Phantavong, Y.H., Grimont, P., Saliot A. & Oudot, J. 2004. Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms. *Res. Microbiol*. 155: 587-595.

Chaîneau, C.H., Morel, J., Dupont, J., Bury, E. & Oudot, J. 1999. Comparison of the fuel oil biodegradation potential of hydrocarbon-assimilating microorganisms isolated from a temperate agricultural soil. *Sci. Total. Environ*. 227: 237-247.

Delille, D., Coulan F. & Pelletier, E. 2004. Effects of temperature warming during a bioremediation study of natural and nutrient-amended hydrocarbon-contaminated sub-Antarctic soils. *Cold Reg. Sci. Technol*. 40: 61-70.

Djonović, S., Pozo, M.J., Dangott, L.J., Howell, C.R. & Kenerley, C.M. 2006. Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. *Am. Phytopathol. Soc*. 19(8): 838-853.

Hadibarata, T. & Tachibana, S. 2009. Microbial degradation of crude oil by fungi pre-grown on wood meal. *Interdiscipl. Studies Environ. Chem*. 317-322.

Haven, H.L.T., Rullkotter, J., de Leeuw, J.W. & Damsté, J.S.S. 1988. Pristane/phytane ratio as environmental indicator. *Nature*. 333: 604.

Head, I.M. & Swannell, R.P.J. 1999. Bioremediation of petroleum hydrocarbon contaminants in marine habitats. *Curr. Opin. Biotech*. 10: 234-239.

Hess, A., Hohener, P., Hunkeler, D. & Zeyer, J. 1996. Bioremediation of a diesel fuel contaminated aquifer: simulation studies in laboratory aquifer columns. *J. Contam. Hydrol*. 23: 329-345.

Jin, S. & Fallgren, P.H. 2007. Site-specific limitations of using urea as nitrogen source in biodegradation of petroleum wastes. *Soil Sediment Contam*. 16(5): 497-505.

Kendrick, A. & Ratledge, C. 1996. Cessation of polyunsaturated fatty acid formation in four selected filamentous fungi when grown on plant oil. *J. Am. Oil. Chem. Soc*. 73(4): 431-435.

Kim, S.W., Hwang, H.J., Xu, C.P, Choi, J.W. & Yun, J.W. 2003. Effect of aeration and agitation on the production of mycelial biomass and exopolysaccharides in an enthomopathogenic fungus *Paecilomyces sinclairii*. *Appl. Microbiol*. 36: 321-326.

Lee, K., Weise, A.M. & St-Pierre, S. 1996. Enhanced oil biodegradation with mineral fine interaction. *Spill. Sci. Technol. B*. 3: 263-267.

Mancera-López, M.E., Casasola, M.T.R., Leal, E.R., Garcia, F.E., Gómez, B.C., Vázquez, R.R. & Cortés, J.B. 2007. Fungi and bacterial isolated from two highly polluted soils for hydrocarbon degradation. *Acta Chim Slov*. 54: 201-209.

Mancera-López, M.E., Esparza-García, F., Chávez-Gómez, B., Rodríguez-Vázquez, R., Saucedo-Castañeda, G. & Barrera-Cortés, J. 2008. Bioremediation of an aged hydrocarbon-contaminated soil by a combined system of biostimulation-bioaugmentation with filamentous fungi. *Int. Biodeter. Biodegr***.** 61: 151-160.

Mukherji, S., Jagadevan, S., Mohapatra, G. & Vijay, A. 2004. Biodegradation of diesel oil by an Arabian Sea sediment culture isolated from the vicinity of an oil field. *Bioresource Technol*. 95: 281-286.

Obire, O. & Anyanwu, E.C. 2009. Impact of various concentrations of crude oil on fungal populations of soil. *Int. J. Environ. Sci. Tech*. 6(2): 211-218.

Okerentugba, P.D. & Ezeronye, O.U. 2003. Petroleum degrading potentials of single and mixed microbial cultures isolated from rivers and refinery effluent in Nigeria. *Afr. J. Biotechnol.* 2: 288-292.

Purwanto, L.A., Ibrahim, D. & Sudrajat, H. 2009. Effect of agitation speed on morphological changes in *Aspergillus niger* hyphae during production of tannase. *World J. Chem*. 4(1): 34-38.

Ruiz-Aguilar, M.L.G., Fernández-Sánchez, J.M., Rodríguez-Vázquez, R. & Poggi-Varaldo, H. 2002. Degradation by white-rot fungi of high concentrations of PCB extracted from a contaminated soil. *Adv. Environ. Res*. 6: 559-568.

Ryan, D., Leukes, W. & Burton, S. 2007. Improving the bioremediation of phenolic wastewaters by *Trametes versicolor*. *Bioresource Technol*. 98: 579-587.

Samuels, G.J., Chaverri, P., Farr, D.F. & McCray, E.B. 2007. *Trichoderma* Online, Systematic Mycology and Microbiology Laboratory, ARS, USDA. Available from: http://nt.ars-grin.gov/taxadescriptions/keys /TrichodermaIndex.cfm. [16 November 2008].

Santos, V.L. & Linardi, V.R. 2004. Biodegradation of phenol by a filamentous fungi isolated from industrial effluents-identification and degradation potential. *Process. Biochem*. 39: 1001-1006.

Shewfelt, K., Lee, H. & Zytner, R.G. 2005. Optimization of nitrogen for bioventing of gasoline contaminated soil. *Environ. Eng. Sci*. 4: 29-42.

Srivastava, S. & Thakur, I.S. 2006. Evaluation of bioremediation and detoxification potentiality of *Aspergillus niger* for removal of hexavalent chromium in soil microcosm. *Soil. Biol. Biochem*. 38: 1904-1911.

Trindade, P.V.O., Sobral, L.G., Rizzo, A.C.L., Leite, S.G.F. & Soriano, A.U. 2005. Bioremediation of a weathered and a recently oil-contaminated soils from Brazil: a comparison study. *Chemosphere* 58: 515-522.

Verdin, A., Sahraoui, A.L. & Durand, R. 2004. Degradation of benzo[a]pyrene by mitosporic fungi and extracellular oxidative enzymes. *Int. Biodeter. Biodegr.* 53: 65-70.

Zajic, E. & Supplisson, B. 1972. Emulsification and degradation of "Bunker C" fuel oil by microorganisms. *Biotechnol. Bioeng*. 14: 31-43.

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