Quantitative Assessment of Hydrolytic Potentials of Fungal Isolates from Crude Oil Impacted Soil Ecosystem in Varied Media Formulations

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Authors’ contributions

This work was carried out in collaboration among all authors. Author JMM designed the study, performed the statistical analysis, managed the literature searches and wrote the first draft of the manuscript. Author AIO managed the analyses of the study. Author CICO wrote the protocol. All authors read and approved the final manuscript.

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ABSTRACT

Background of the Study: A quantitative assessment of different enzymes that produce the best of hydrolyses of starch, skim milk, carboxymathcellulose (CMC) and groundnut oil which were further assessed for the production of extracellular enzyme.

Aim: The study aimed at assessing some of the fungal isolates for their abilities to produce the following hydrolytic enzymes; amylase, protease, cellulase and lipase using different medium.

Material and Methods: About 19 fungal species associated with crude oil impacted soil ecosystem. Soil parameters taken were pH, organic matter, water holding capacity and moisture content. Analysis of variance were used to test the effects at statistical significance of $P \leq 0.05$ among the treatments and tukey post hoc tests were used to rank the means.

Results: A. fumigatus, A. niger, A.terreus and Basipetospora has the highest frequency of occurrence. A. fumigatus 2 has the highest amylase activity (80mm) while A. clavatus recorded the least (10mm) amylotic activity. A. fumigatus 2 recorded the highest hydrolytic zone of 66.67 mm, followed by A. fumigatus 3 (65 mm) and Curvularialunata which recorded 60mm, respectively. It was
revealed that pH of polluted soil sample from the three plots were more acidic than the control (non-polluted soil) 4.81 and 5.72 for plot 1, 5.58 and 6.08 for plot 2 and 5.15 and 6.57 for plot 3 respectively. The water holding capacity, organic matter and moisture content in the polluted soil ranged from 15.02-17.27%, 7.34-8.99 mgkg-1 and 1.23-4.60%, respectively.

**Conclusion:** It was concluded that aspergillus species exhibited maximum hydrolytic potentials of the fungal isolates using different media formulations and these results could provide basic data for further investigations on molecular characterization of fungal extracellular enzymes.

**Keywords:** Fungi; media; amylase; cellulase; protease; lipase.

1. INTRODUCTION

The industrial revolution has caused increase in immense usage of petroleum products and coal derivatives which had led to huge detrimental effect of climate change on the ecosystem. Crude oil is a composite of hydrocarbons of different molecular weights and structures. The exploration of crude oil has caused a huge pollution/contamination to the ecosystem which has resulted to loss and depletion of biotic systems. Crude oil pollution is a global phenomenon affecting all aspects of the environment. The major problems associated with oil and gas industry are accidental and deliberate release of oil spills/effluents. In Nigeria, most especially in the Niger-Delta region, oil contamination has been a major threat to the survival of living things which has made life unbearable to the community [1]. The use of microorganisms to remove, reduce, or ameliorate pollution from the environment is termed bioremediation. Bacteria and fungi constitute the most important part of the microflora present in soils that are polluted with oil hydrocarbons.

Fungi play an important role in decomposition of plant residues, releasing nutrients that sustain and stimulate plant growth [2]. Decomposition of complex mixtures can be performed by mixed microorganism cultures with varied activities and the ability to use hydrocarbons as a source of carbon and energy [3,4]. Several methods had been deployed as a tool for cleaning up crude oil polluted soils using physical, chemical, thermal and biological techniques of processing. The rationale is that microbial populations may be weak in degrading the wide range of substrates present in complex mixtures. Therefore, this study attempted to assess the hydrolytic potentials of fungal isolates from crude oil impacted soil ecosystem in varied media formulations.

2. MATERIALS AND METHODS

2.1 Collection and Processing of Soil Samples

The method of [5] were adopted in this study. The soil samples were collected from 3 locations in each site and a total of 10 soil sample were collected in each location totaling 30 soil samples from a depth of 1-10cm. The soils were collected with clean shovel. The different soil samples were stored in sterile cellophane bags on ice and then transported to Applied Microbiology Postgraduate Laboratory of Department of Plant Science and Biotechnology, University of Jos, Jos.

2.2 Physicochemical Parameters of the Soil Samples

The parameters of the soil samples that were assessed included pH, temperature, soil moisture, organic matter content (%), soil water holding capacity (g) and soil colour.

2.3 Soil pH Determination

The method of [6] was used for the assessment. A weight 30 grams was weighed out from each soil sample. The soil samples were separately mixed with sterile water to form suspension.

2.4 Moisture Content Determination

The method of [6] was adopted for the assessment. A weight of 30 grams of soil from each soil sample was dried to constant weights in hot air oven set at 105°C. The percentage moisture contents of the soil samples were determined in triplicates using the expression below:

\[
\frac{x - y(g)}{x} \times \frac{1000}{1}
\]
Where, initial weight of soil = x (g)
Weight of soil after drying to constant weight = y (g)

2.5 Soil Water Holding Capacity

The method of [6] was also used for the assessment. The soil samples were saturated with water in glass funnel and allowed for free drainage for 48hrs. It was covered with perforated filter paper to prevent evaporation and to maintain atmospheric pressures on the soil.

2.6 Fungi Isolation

The method of [6] for fungi isolation from different soil samples. A total of 36 Petri dishes were used and weight of 0.03 g of the soil sample was put in each sterile Petri dish and then covered with 15 ml of molten Starch Agar (SA). Each soil sample was also plated out on Yeast Starch Agar (YSA), Eggnis and Pugh Cellulose Agar (E&P) and Czapek-Dox Agar (CZA). The plates were swirled in order to allow for even mixing of the particles with the agar medium. A volume of 1 ml of gentamycin (40 mg/ml) was added into each culture plate to suppress the growth of bacteria with a label on each plate. The resultant culture plates were divided into three batches of nine each for each of the sites and incubated at 25°C, 37°C and 45°C as reported by Ogbonna and Pugh [6]. The plates were examined after 4-7 days for fungal growths. The mixed cultures were sub-cultured severally in order to obtain pure culture.

The plate was re-examined after 14 days for the development of more fungal colonies. Such fungal colonies that developed were sub-cultured several times until pure cultures were obtained. The fungal isolates were then subjected to microscopic examinations for their morphological details and for their ultimate identifications with lactophenol cotton blue using both low and high-power objectives.

2.7 Characterization of the Fungal Isolates

Identification on each isolate was done based colonial and cultural properties and microscopic features of its sporulating structures. Other features were shape, colour, size, texture, elevation and outline of the colony. The nature of the hyphae such as the length, colour and texture as well as the fruiting bodies was useful in the identification of the isolates.

2.8 Screening of Fungal Isolates for Hydrolytic Enzyme Production

The fungi were screened for production of the following hydrolytic enzymes namely; amylase, protease, cellulase and lipase. The enzymatic activities determinations were done by initially growing the isolates on Potatoes Dextrose Agar (PDA) for 72hrs. After that, 5 mm plug of the actively growing pure fungus was inoculated on the specific culture media for each enzyme to be investigated. The production of enzyme was determined within the incubation period, according to González et al. [7].

2.9 Amylolytic Activity of the Test Fungi

Amylolytic activity of the test fungi were determined by method of [8]. Starch agar plates were inoculated with 5 mm mycelia disc from the edge of an actively growing 4-day old culture of the test fungus on Potato Dextrose Agar (PDA). The Starch agar plates were incubated at 25°C for four days and were flooded with Lugol's iodine solution for two minutes. Observation was done for clear zone of hydrolyzed starch against a blue background of unhydrolyzed starch in the plate. The diameters of the clear zone were measured and averages were recorded as the measure of amylase activity. The same experiment was repeated for Yeast Starch Agar (YSA) medium.

2.10 Proteolytic Activity of Test Fungi Using Skim Milk Agar

The methods of [8] were employed to determine the proteolytic activities of the test fungi. One percent (1%) skim milk agar plates were prepared and inoculated with 5 mm mycelia disc from the edge of actively growing 4-day old culture of the test fungi grown on Potatoes Dextrose Agar (PDA). After four days of incubation at 25°C, the plates were observed for growth. One percent Congo Red solution was used to flood the plates and then were observed for clear zones around the inoculated culture. Mean diameter were recorded for each fungal species.

2.11 Hydrolysis of Carboxymethyl Cellulolose (CMC) Agar

The method of [9] which involve measurement of cellulytic activity on a medium containing Carboxy Methy Cellulose (CMC) as
sole carbon source. Freshly prepared Carboxymethylcellulose (CMC) agar was dispensed into sterile Petri dishes and was allowed to solidify. The plates were then inoculated aseptically with 5 mm mycelia disc from the edge of actively growing 4-day old culture of the test fungi grown on Potatoes Dextrose Agar (PDA). The cellulose agar plates were incubated at room temperature for four days after which they were flooded with 1% Congo red stain for 15 minutes. This was followed by rinsing with 1 M NaCl solution. The plates were observed for clear zones against a pink background of un-hydrolysed cellulose. Diameter measurements of the clear zones were made and the means were recorded as the measure of cellulase activity. The experiment was replicated three times for each fungal species.

2.12 Lipolytic Activity of the Test Isolates Using Groundnut Oil Agar Medium

Lipase assay method of [10] which involves measurement of lipolytic activity on a medium containing 1% groundnut oil as sole carbon source was employed. Freshly compounded groundnut oil agar medium was dispensed into sterile Petri dishes. The plates were inoculated with 5 mm mycelia discs from the edge of actively growing 4-day old culture of the test fungi grown on Potatoes Dextrose Agar (PDA). After five days of incubation at 25°C, the plates were flooded with 1% Sudan III reagent on the colonized area of growth of the test isolates.

The plates were allowed to stand for 15 minutes and were observed for halo zones and the results were recorded. The experiment was replicated three times for each fungal species.

2.13 Data Analysis

Analysis of variance were used to test the effects at statistical significance of P ≤ 0.05 among the treatments. Tukey post hoc tests were used to rank the means using the statistical software package for Windows (Statistica version 13.3; Dell Inc., Tulsa, OK, USA).

3. RESULTS AND DISCUSSION

3.1 Physiochemical Parameters of the Soil

The soil parameters analysed were pH, organic matter, moisture content, water holding capacity and soil colour as shown in Table 1. Table 1 revealed that pH of polluted soil sample from the three plots were more acidic than the control (non-polluted soil) 4.81 and 5.72 for plot 1, 5.58 and 6.08 for plot 2 and 5.15 and 6.57 for plot 3 respectively. These might be as a result of the non-sorption of metals in the soil [11]. Acidic soil inhibits soil fertility and plant growth. The pH values obtained in this study are similar to the reports of several researchers [11,12].

The highest pH recorded in the surface polluted sites was 5.58. This is lower than the range of 6.20-6.88 reported by Ibeh and Omoruyi [13] in the physicochemical parameters of a contaminated effluent from a University Teaching Hospital based in Southern Nigeria. The pH range in this study were within the [14] permissible guideline value of 6.5-8.5. The pH range for the side-sediments and water could influence the mobility rate of the metal within the sites of sampling and also between the suspended particles and water. pH is among several properties which affect the availability, retention and mobility of nutrients and heavy metals in soils. Some organisms are unaffected by a rather broad range of pH values, while others exhibit considerable intolerance to even minor variations in pH [15,16]; pH can affect the accumulation factor of soil among others. It was observed that the accumulation factors for metals vary inversely with pH of soil, having great effects on solute concentration and absorption in soil. At low pH, metals are more bioavailable to plants, and hence could pose severe toxicity problems [17]. The highest organic matter content of the soil was recorded as plot 1 (8.99 mgkg-1), while the lowest values in was 7.17 mgkg-1 in the control soil. The water holding capacity, organic matter and moisture content in the polluted soil ranged from 15.02-17.27%, 7.34-8.99 mgkg-1 and 1.23-4.60% was higher than values reported by [16,17]. Higher organic matter contents were observed in the polluted plot as compared to the control; this could be due to the lower soil moisture contents in the polluted soil which retards the activities of the microorganisms involved in the organic matter decomposition.

3.2 Isolation of Fungi

A total of nineteen species of both mesophilic, thermophilic and thermotolerant fungi were isolated from the soil samples. Aspergillus species were predominant which include among others; A. fumigatus, A. niger, A. parasiticus, A. oryzae and A. terreus. Other fungi isolates included species of Penicillium, Cladosporium, Cunninghamella, Thermomyces ibadanensis,
obtained three fungal isolates from oil-contaminated soil. The details of the fungal isolates are presented in Table 2. A. fumigatus, A. niger, A. terreus and Basipetospora has the highest frequency of occurrence with the most active ability. The presence of fungal species is an indication that petroleum-contaminated soil was rich in microbial flora. This is similar to the reports of [6]. Aspergillus spp (especially A. niger) and Penicillium spp were predominant and of public health importance. Their common occurrence could be linked to high sporulating nature and ability to grow well on organic substrates, which were in abundance in the oil-contaminated soil. Simister et al. [18] revealed two isolates, identified as Aspergillus sclerotiorum and Mucor racemosus, with an ability to degrade pyrene and benz[a]pyrene in north coast of Sao Paulo in Brazil. Interestingly, most of these species in this study are ubiquitous, indicating that soil-derived fungi may represent an untapped reservoir of hydrocarbonoclasts. This study supports the findings by April et al. [20] who reported 22 species of Penicillium and 5 species of Aspergillus isolated from the flare pit soils in Northern and Southern Canada. These isolates showed the ability to degrade hydrocarbons on solid medium amended with crude oil.

Table 1. Effect of plots on physiochemical parameters of polluted soil ecosystem

| Plots       | pH        | Water Holding Capacity (%) | Organic Matter (mgkg-1) | Moisture Content (%) | Soil Colour |
|-------------|-----------|----------------------------|-------------------------|---------------------|-------------|
| Plot 1      |           |                            |                         |                     |             |
| Polluted Soil | 4.81 ± 0.48 | 15.08±6.48                  | 8.99±4.85              | 4.60 ± 0.40         | Dark Brown  |
| Control Soil | 5.72 ± 0.23 | 17.22±5.56                  | 7.17±4.95              | 6.55±0.092          |             |
| Plot 2      |           |                            |                         |                     |             |
| Polluted Soil | 5.58 ± 1.11 | 15.02±2.00                  | 7.34±0.05              | 4.21 ± 0.17         | Dark Brown  |
| Control Soil | 6.08 ± 0.32 | 17.22±5.56                  | 7.17±4.95              | 6.55±0.02           |             |
| Plot 3      |           |                            |                         |                     |             |
| Polluted Soil | 5.15 ± 0.78 | 17.27±10.80                 | 8.93±0.71              | 1.23±0.58           | Dark Brown  |
| Control Soil | 6.57 ± 0.39 | 17.22±5.56                  | 7.17±4.95              | 6.55±0.02           |             |

*One-way ANOVA conducted the mean with same superscript letters are similar meaning there is no significant difference*

Table 2. Species of fungi isolates from crude oil polluted sites

| S/N | Fungal Isolates       | Plot 1 | Plot 2 | Plot 3 | Total | % Fo |
|-----|-----------------------|--------|--------|--------|-------|------|
| 1   | Aspergillus candidus  | _      | _      | +      | 1     | 33.33|
| 2   | A. Clavatus           | +      | _      | +      | 2     | 66.67|
| 3   | A. Flavuslinis        | _      | _      | +      | 1     | 33.33|
| 4   | A. Fumigatus          | +      | +      | _      | 3     | 100  |
| 5   | A. Niger              | +      | +      | +      | 3     | 100  |
| 6   | A. Oryzae             | _      | _      | +      | 1     | 33.33|
| 7   | A. Parasiticus        | +      | _      | _      | 1     | 33.33|
| 8   | A. Terreus            | +      | +      | _      | 3     | 100  |
| 9   | Basipetospora sp      | +      | +      | +      | 3     | 100  |
| 10  | Cladospora sp         | +      | _      | +      | 2     | 66.67|
| 11  | Cunnibghmellalenasa   | +      | +      | +      | 3     | 100  |
| 12  | Curvelialunata        | +      | _      | +      | 2     | 66.67|
| 13  | Mucorisp             | +      | _      | +      | 2     | 66.67|
| 14  | Pencilliumsp          | +      | _      | +      | 2     | 66.67|
| 15  | Scopulariosissp       | +      | _      | +      | 2     | 66.67|
| 16  | Sporotrichumthermoplia| _      | _      | +      | 1     | 33.33|
| 17  | Thermomceslanginosus  | _      | _      | +      | 1     | 33.33|
| 18  | T. ibanoesis          | _      | _      | +      | 1     | 33.33|
| 19  | Trichoderma wide      | +      | +      | _      | 2     | 66.67|
| Total |                      | 13     | 6      | 17     | 37    |      |

%FO = percentage frequency occurrence, + = present; _ = absent.
3.3 Enzymatic Studies on Some of Fungal Isolates

The determinations of amylolytic activity of the fungal isolates on Yeast starch agar medium revealed that *A. fumigatus* 2 has the highest amylase activity (80 mm) following by *A. fumigatus* 3 (75 mm). *Trichoderma sp* (73.3 mm) and *Cuninghamella sp* recorded (66.67 mm). All these fungal isolates showed strong amylolytic activities on Yeast starch agar medium (Table 3). The medium supported the growth of all the selected fungi.

3.4 Amylolytic Activities of the Fungal Isolates Using Soluble Starch Agar Medium

Amylolytic activities of the fungi isolates on the soluble starch agar were depicted by the presence of halo zones of clearing of the soluble starch agar after flooding with Lugol's iodine (Table 4). All the isolates hydrolyzed the soluble starch with the highest zone of clearing of 80 mm and were grouped as strongly amylolytic (+++).

Other fungi that were grouped as moderately amylolytic due to their high zones of clearing includes *A. fumigatus 3, A. niger, Curvlarialunata* and *Trichoderma viride* with zone of 81 mm, 78.33 mm, 60 mm and 66.67 mm respectively. *A. candidus, A. parasiticus* and *Penicillium* with zone of clearing 53.33 mm, 43.33 mm and 43.55 respectively and were grouped as moderately amylolytic.

3.5 Lipolytic Activities of the Fungal Isolates

The determination of lipolytic activity of some of the isolates on groundnut oil agar medium showed that the agar medium supported the growth of all the fungal isolates employed in the study (Table 5). All the test fungi were found to hydrolyze the medium. *A. fumigatus* 2 recorded the highest hydrolytic zone of 66.67 mm, followed by *A. fumigatus* 3 (65 mm) *Curvularialunata* recorded 60 mm. These fungi were strongly lipolytic while (+++) *A. fumigatus* 1, *Basipetospora sp* (46.67 mm), *A. terreus* (43.33 mm) recorded moderate lipolytic activity (+).

Table 3. Amylolytic activities of fungal isolates on yeast starch agar medium

| Test Organism  | Mean Diam. Clear Zone | Activity Level |
|---------------|-----------------------|---------------|
| *A. Candidus* | 35.0 ±13.23           | +             |
| *A. Clavatus* | 10.0 ±1.00            | +             |
| *A. Parasticus* | 46.07 ± 0.77       | ++            |
| *A. fumigatus 1* | 73.33 ±15.27         | +++           |
| *A. fumigatus 2* | 80±5.00              | +++           |
| *A. fumigatus 3* | 75±5.00              | +++           |
| *Cunninghamella sp* 1 | 70±10.00        | +++           |
| *Cunninghamella sp* 2 | 66.67±15.77      | +++           |
| *Penicillium* | 35±5.00              | ++            |
| *Trichoderma sp* | 73.33±5.77         | +++           |

Table 4. Amylolytic activities of the fungal isolates using starch agar medium

| Test organism  | Mean diameter clear zone | Activity level |
|---------------|--------------------------|---------------|
| *Aspergillus parasiticus* | 53.33± 5.77        | ++            |
| *A. fumigatus 1* | 80±10.00                 | +++           |
| *A. fumigatus 2* | 65±5.00                 | ++            |
| *A. fumigatus 3* | 81.67±2.88               | +++           |
| *A. candidus* | 43.38±5.77              | +             |
| *A. niger* | 78.33±7.63               | +++           |
| *Penicillium sp* | 40±10.00                | +             |
| *Curvularialunata* | 60±10.00             | +++           |
| *Curviasp* | 60 ± 5.00                | +++           |
| *Trichoderma viride* | 66.67±15.27       | +++           |
Table 5. Lipolytic potentials of the fungal isolates

| Test Organisms | Mean Diameter Clear Zone | Activity Level |
|----------------|--------------------------|----------------|
| A. fumigatus 1 | 46.67±5.77               | ++             |
| A. fumigatus 2 | 66.67±5.77               | +++            |
| A. fumigatus 3 | 65±5.00                  | +++            |
| A. niger       | 50±10.00                 | +++            |
| A. parasiticus | 51.67±2.88               | +++            |
| A. terreus     | 43.33 ±11.54             | ++             |
| Basipetospora sp | 46.67±5.77             | ++             |
| Curvularia lunata | 60±10.00              | +++            |
| Curvularia sp  | 46.67 ±5.77              | ++             |
| Trichoderma sp | 46.67±5.77               | ++             |

++ = Moderately lipolytic, +++ = Strong lipolytic

3.6 Cellulolytic Activities of the Some of the Fungi Isolates

The determination of cellulolytic activity of the isolates on Carboxymethylcellulose (CMC) agar revealed that the agar medium supported the growth of most of the organisms (Table 6). The highest halo zone (76.67 mm) was recorded by *Penicillium* sp, followed by *A. fumigatus* 1 and *A. fumigatus* 3 (73.33 mm) each. *A. terreus* (73.33 mm) and *A. candidus* (70 mm). *Trichoderma viride* recorded 65 mm. *Cunninghamella* sp recorded 9.67 mm, the least clearing zone.

3.7 Proteolytic Activity of the Fungi Isolates on Skim Milk Agar Medium

For the proteolytic enzyme activity using skim milk, it was observed that *Aspergillus niger* had the highest clearing zone of 78.33 mm, followed by *A. parasiticus* with a clearing zone of 76.67 mm, *A. fumigatus* 1 (50 mm). These fungal isolates proteolytic (+++) on skim milk agar (Table 7). *A. fumigatus* 2 (48.3 mm), *Trichoderma* sp (36.67 mm) and *A. fumigatus* 1 (26.67 mm) were said to be moderately proteolytic (++) on skim milk agar.

*Aspergillus* sp was most effective in amylolytic, lipolytic, cellulolytic and proteolytic activities of the fungal isolates. This is connected to the fact that *Aspergillus* sp possess all component of the cellulase complex and also produce an extensive range of plant cell wall degrading enzymes [21,22]. *Trichoderma* sp has been listed as a common and effective cellulase producer [23,24] as seen in this study. Xylan and cellulose degrading enzymes have been used in food processing, detergent formulation, textile production, feed preparation, production of wine, beer, and fruit juice, and bioconversion of lignocelluloses to fuel ethanol [25,26].

Table 6. Cellulolytic activities of the test fungi carboxymethyl cellulose agar

| Test Organisms          | Mean Diameter clear Zone | Activity Level |
|-------------------------|--------------------------|----------------|
| Aspergillus fumigatus 1 | 73.33 ±7.64              | +++            |
| Aspergillus fumigatus 2 | 63.33±15.28              | +++            |
| Aspergillus fumigatus 3 | 73.33±11.55              | +++            |
| Parasiticus             | 61.67±10.44              | +++            |
| terreus                 | 70±10.00                 | +++            |
| Candidus                | 70±5.00                  | +++            |
| Basipetospora           | 53.33±5.78               | +++            |
| Cunninghamella sp       | 9.67±0.58                | +              |
| Trichoderma viride      | 65.00±5.00               | +++            |
| Penicillium sp          | 76.67±5.78               | +++            |

++=low lipolytic, ++ = Moderately lipolytic, +++ = Strong lipolytic
Table 7. Proteolytic activity of the fungi isolates on skim milk agar medium

| Test Organisms       | Mean Diameter clear Zone | Activity Level |
|----------------------|--------------------------|----------------|
| A. fumigatus 1       | 26.67±11.54              | ++             |
| A. fumigatus 2       | 48.33±7.63               | +++            |
| A. fumigatus 3       | 50±10.00                 | +++            |
| A. terreus           | 46.67±5.77               | +++            |
| A. parasiticus       | 76.67±7.63               | +++            |
| A. niger             | 78.33±2.88               | +++            |
| Trichoderma sp.      | 36.67±5.77               | ++             |

++ = Moderately lipolytic, +++ = Strong lipolytic

4. CONCLUSION

The findings of this research work revealed that aspergillus species exhibited maximum hydrolytic potentials of the fungal isolates using different media formulations. The results further revealed that some of such fungal isolates produce lipolytic enzymes (Lipases) needed for the degradation of crude oil. Aspergillus species appear has the most promising for bioremediation of crude oil spills.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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