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Fungi from Coastal Sediments as Potential Agents in Biodegrading Used Engine Oil

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ABSTRACT

The use of microorganisms to decontaminate the environment loaded with oil pollutants is a beneficial option for bioremediation. This study aimed to isolate, characterize and identify oil-degrading fungi from the coastal sediments of the port area of Ormoc City, Philippines, and to evaluate and compare the oil-biodegrading capabilities of these fungi. Fungal isolates were subjected to an *in-vitro* biodegradation assay using used engine oil as substrate. Four species of filamentous fungi were isolated and identified down to genus level. The fungal isolates include three *Aspergillus* spp. and one *Penicillium* sp. *Aspergillus* spp. 1 & 2 were the most efficient in degrading and utilizing the used engine oil for growth. *Aspergillus* sp. 1 & 2 and *Penicillium* sp.had mean pH values of 6.286, 6.136 and 6.32, respectively. Highest mean percent fat loss on the media and highest mean dry weights were exhibited by *Aspergillus* sp. 2 (mean fat loss = 77.12% and mean dry weight = 0.24g) and *Aspergillus* sp. 1 (mean fat loss = 69.4% and mean dry weight = 0.21g). Filamentous fungi from the coastal sediments in the vicinity of Ormoc City port area could therefore be used as potential bioremediation agents in areas contaminated with petroleum oils.

Keywords: filamentous fungi, characterization, bioremediation, oil pollution

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INTRODUCTION

The growing demand for petroleum as a major energy source plays a very significant role in the contamination of marine environments around the world (Sepahi *et al.*, 2008). Petroleum, including all fossil fuels (i.e., motor or engine oils) primarily consists of a complex mixture of hydrocarbons and some organometallic constituents. In large concentrations, the hydrocarbon molecules that make up the petroleum products are highly toxic and mutagenic to many organisms, including humans (Lotfinasabasl *et al*., 2012; Pimda & Bunnag, 2012; Ojumu *et al.*, 2004).

These fuel oil contaminants should be degraded to keep the environment clean. The most rational way considered by scientists is through biodegradation, a method based mainly on the metabolic activity of microorganisms. It is favored because other methods including surfactant washing and incineration lead to production of more toxic compounds and are non-economical (Leahy & Colwell, 1990; Sepahi *et al.*, 2008).

Various microorganisms with biodegradative ability are widely distributed in marine, freshwater and soil habitats (Sepahi *et al.*, 2008). However, research focused more on the use of bacteria and less attention has been given to fungi (Husaini *et al*., 2008). It is only in recent years that advantages associated with fungal bioremediation were explored (Okafor *et al.*, 2009). Fungi have the ability to synthesize relatively unspecific enzymes involved in degradation of many molecules including aromatic structures and convert them into harmless, tolerable, or useful products (Al-Ghamdi, 2011). This study was conducted to describe the mycoflora of the sediments in the vicinity of a port area in Leyte which could be a potential source of microbes that can degrade petroleum oils. It focused on the isolation, identification and characterization of the oil-degrading fungal species and on the evaluation and comparison of their biodegrading capabilities.

MATERIALS AND METHODS

Collection of Sediments and Oil Preparation

Composite sampling method was done by establishing six sampling stations in the intertidal area with in the vicinity of the port area in Ormoc City (11° 0' 14.79" N and 124° 36' 27.10" E) where indications of oil contamination were observed. The six sediment samples (cored to about 10 cm deep) were mixed thoroughly to distribute the fungal cells. Approximately 500 g of composite sample was placed in a sterile 500-ml glass beaker and covered with aluminum foil. The beaker was placed in a bucket and transported immediately to the laboratory for microbiological analyses. Used engine oil for gasoline vehicles from the motor pool facility of the Visayas State University, Visca, Baybay City, Leyte was used as substrate for the study. The used engine oil was filtered and stored in a sterile beaker until used.

Fungi Isolation

Oil agar medium was used in the isolation and preliminary identification of oil-degrading fungi. It was prepared according to the modified mineral salts medium (MSM) composition of Obire *et al.* (2008). Ten (10) ml of the oil agar medium was added to each of 12 test tubes and autoclaved at 121°C, 15 psi for 20 min, mixed thoroughly, pour-plated into sterile Petri dishes and allowed to cool and solidify under aseptic condition. To the solid media, 0.1 ml of 50 mg/ml tetracycline was spread-plated on the surface to prevent bacterial growth and permit selective isolation of yeasts and molds (Obire *et al.*, 2008).

Fungi in the sediments were isolated by serial dilution method (Obire *et al.*, 2008). Four preparations of sterile physiological saline (0.85% w/v NaCl) were used as diluents. Ten grams (10 g) of composite sediment samples were aseptically transferred to a sterile bottle containing 90 ml of the diluents to give 10^{-1} dilution. The bottle with the sediments was shaken thoroughly for 5 min then the sediments were allowed to settle. Subsequently, 3 serial dilutions were prepared from the 10^{-1} dilution.

Aliquots of 0.1 ml were aseptically spread-plated on well-dried oil-agar plates in triplicates. The cultured plates were then incubated at 28 ± 2˚C for 30 days. After incubation, colonies that have developed zones of clearance of oil on the oil-agar plates were identified as oil-degrading fungi.

Preparation of Pure Cultures

Colonies were purified on plates and slants of SDA for identification. Purification was done twice a week to avoid contamination and to maintain pure cultures. The plate cultures were used as source of inocula for the biodegradation assay.

Identification and Characterization of Fungal Isolates

For preliminary identification, morphological examination of the colony shape, color, spore formation as well as the texture of fungal growth was done.

To observe fungal morphology, slide mounts were made using lactophenol cotton blue as the mounting medium (Tagaytay, 2007). The slides were viewed under an electric compound microscope (True Vision Microscope USA) at high power objective (400X) and oil immersion objective (1000X). All initial identifications were verified by a plant pathologist from the Department of Pest Management at the Visayas State University, up to the genus level only. A digital camera (Sony cyber-shot 4.1 megapixels) was used to photograph cell and colony morphology of the oil-degrading fungal isolates.

Biodegradation Assay

The biodegradation assay was carried out by combining the methods described by Miranda *et al.* (2007) and the enrichment procedure (minimal salt broth preparation) described by Adekunle and Adebambo (2007).

The prepared minimal salt broth was autoclaved at 121˚C under 15 psi for 20 min. Five (5) Erlenmeyer flasks (125 ml) for each fungal isolate were sterilized while another five were used for the control. Forty (40)milliliters of the minimal salt broth (MSB) and four (4) ml of the filter sterilized used engine oil were mixed inside each Erlenmeyer flask.

Fungal isolates on plates were removed using a cork borer (1 cm diameter). Two core samples were inoculated to each of the flasks leaving the control flasks uninoculated. The flasks were closed with sterile cotton plugs to ensure maximum aeration and prevent cross contamination. All the flasks were then incubated at room temperature (28˚C-31˚C) for 30 days. Shaking them constantly to facilitate oil-cell phase contact (Adekunle & Adebambo, 2007).

Data Gathering

The ability to degrade used engine oil was based on the growth of organisms in the MSB medium, change of pH in the media (Miranda *et al.*, 2007), and percent fat loss of the media.

Growth of the organisms in the medium was estimated through dry weight measurement. MSB medium with fungal inoculates was centrifuged twice at 500 rpm. The collected fungal mycelia were placed in previously weighed aluminum foil pans and placed for 1 h in a pre-heated oven at 80˚C. The foil pans, with the collected fungal mycelia, were then dried in an oven overnight (12 hr) at 80˚C and weighed again. It was done only at the end of the incubation period. pH readings were determined at the start of the experiment (day 0) and every week during the incubation period using Hanna Instruments 8520 pH meter. The percent fat loss on the media was determined at the end of the assay by taking a 0.1 ml of aliquot from each replicate (constantly shaken to evenly distribute oil on the media). These were placed in slides and stained with Sudan III. A glass cover slip was immediately placed over the stained aliquots avoid disintegration of the fat globules. These were then viewed under low power objective (100X) to count the fat globules present. These were done before and after the biodegradation assay. The fat loss on the media was then expressed in percentage.

Experimental Design and Statistical Analysis

Evaluation and comparison of the biodegrading capabilities of fungal isolates were done in completely randomized design (CRD) using fungal species as treatment for the data on dry weight and percent fat loss. The oil-degrading fungal species and the period of incubation (week 0, week 1, week 2, week 3, and week 4) were considered as the independent variables for pH measurements. Five replications per treatment were employed.

ANOVA for a completely randomized design (CRD) was done to test any significance effect of the different treatments. Comparison of means was done using Tukey's Honestly Significant Difference test (HSD). All statistical analyses were done using Statistical Analysis System (SAS, JMP v 7.0).

RESULTS AND DISCUSSION

Identification and Characterization of Fungal Isolates

Four species of fungi were isolated from the coastal sediments in the vicinity of Ormoc City port area. Their identity was verified and confirmed by a mycologist from the Department of Pest Management to genus level only. These were three species of *Aspergillus* and one *Penicillium*.

Aspergillus sp. 1 (Fig. 1A) had colonies which were initially white, and became black with conidial production. The underside of the plate was pale yellow and colonies produced radial fissures on the agar plate. Its microscopic features include hyphae which were septate and hyaline, conidiophores which were smooth, and hyaline, darker at the apex and terminating in a globose vesicle (Fig. 1B). The metulae and phialides covered the entire vesicle.

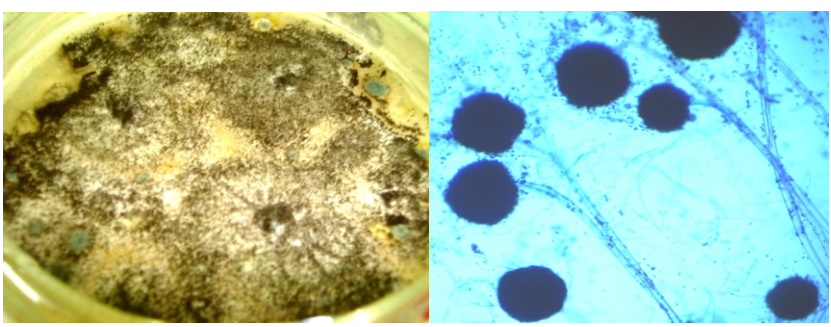

Figure 1. (A)Seven-day-old colonies of *Aspergillus* sp. 1 grown on Sabouraud dextrose agar (SDA) plate showing black conidial production (arrow), (B)Microscopic morphology of *Aspergillus* sp. 1(400X) showing its vesicle with conidiospores (arrow)

Aspergillus sp. 2 (Fig. 2A) colonies had powdery texture, reddish brown exudates and the underside was reddish. It has septated and hyaline hyphae,

loosely radial conidial heads, and variably shaped vesicles, with metulae and phialides. It had conidial structures resembling those seen in *Penicillium* sp. (Fig. 2B).

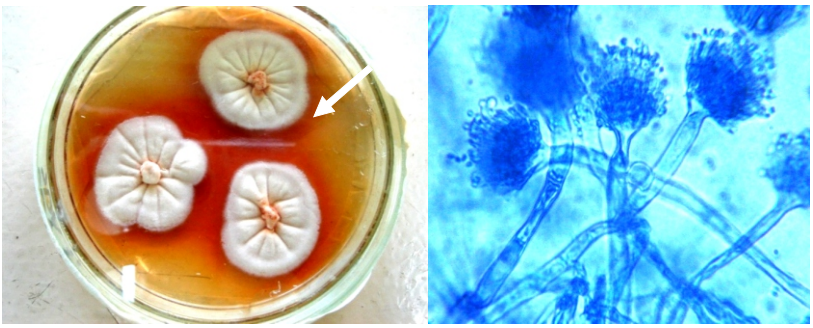

Figure 2. (A)Seven-day-old colonies of *Aspergillus* sp. 2 cultured on SDA plate showing reddish brown exudates (arrow), (B) photomicrograph of *Aspergillus* sp. 2 (1000X) showing conidiospores (arrow)

Aspergillus sp. 3 (Fig. 3A) had cottony colony texture. The color was initially gold but became pinkish after a week. The underside coloration of the plate was pale. Microscopic features include hyaline and septated hyphae. The metulae and two layers of phialides were visible. Conidiophores were branched and rather short which ended up in a vesicle. The vesicles were covered entirely of metulae and phialides (Fig. 3B).

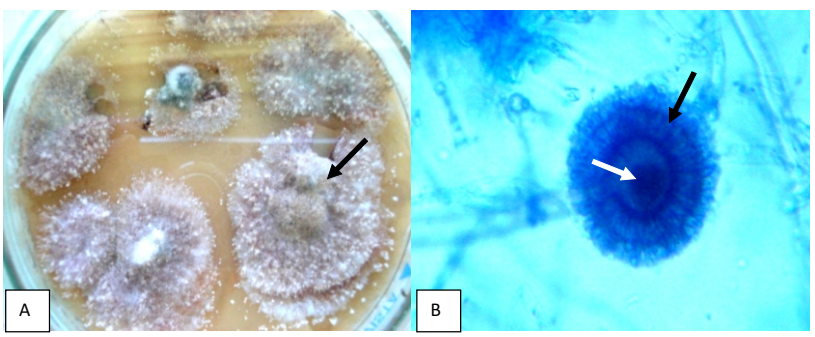

Figure 3. (A) Seven-day-old colonies of *Aspergillus* sp. 3 cultured on SDA plate showing the cottony texture (arrow), (B) photomicrograph of *Aspergillus* sp. 3 (400X) showing the vesicle (white arrow) and the metulae and phialides (black arrow)

The colonies of *Penicillium* sp. (Fig. 4A) were filamentous and velvety in texture. These were initially white then became bluish-green during conidial production and eventually blue-green at maturity. The plate reverse was pale to yellowish in color. Septated hyaline hyphae, branched conidiophores, metulae, phialides, and conidia were observed. Metulae carried flask-shaped phialides which formed brush-like clusters which were also referred to as penicilli. The conidia were round, unicellular, and unbranching chains at the tips of the phialides (Fig. 4B).

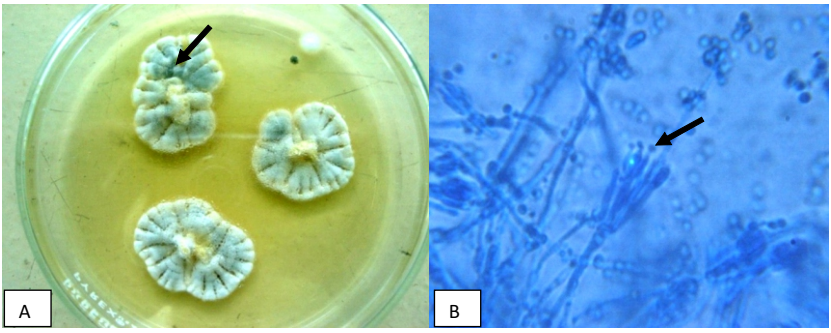

Figure 4. (A) Sevenday old colonies of *Penicillium* sp. cultured on SDA plate (a), slant (b) showing bluish green conidial production (arrow), (B) photomicrograph of *Penicillium* sp. (1000X) showing its reproductive structure (arrow)

Aspergillus and *Penicillium* spp. have frequently been reported in recent biodegradation researches. They possess some attributes that enable them to serve as potential agents of degradation like easy ramification on the substratum and digestion through the secretion of extracellular enzymes (Al-Ghamdi, 2011; Husaini *et al*., 2008; Lotfinasabasl *et al*., 2012; Okafor *et al*., 2009).

Atlas (1981) reviewed the taxonomic value of the property of fungi to assimilate hydrocarbons, that is, whether the ability of fungi to utilize hydrocarbons was a useful diagnostic test for defining different fungal genera or species. It was found out that the ability of fungi to utilize hydrocarbons was shown mainly in two orders, the Mucorales and the Monilales with *Aspergillus* and *Penicillium* as the genera found to be rich in hydrocarbon assimilating strains.

Biodegradation Assay

The pH of the media was significantly affected by the specific fungal species and the period of incubation. There were no interaction effect of the fungal species and the period of incubation (weeks). Figure 5 shows that the media inoculated with fungi had significantly lower pH compared to the control (uninoculated) except for the media inoculated with *Aspergillus* sp. 3. On the other hand, Figure 8 shows that all fungal species had near-neutral pH values in the range of 5.7 and 6.7. Leahy and Colwell (1990) observed that pH values between 6.0 and 8.0 were favorable for the action of microorganisms to degrade petroleum, although fungi were more tolerant of acidic conditions.

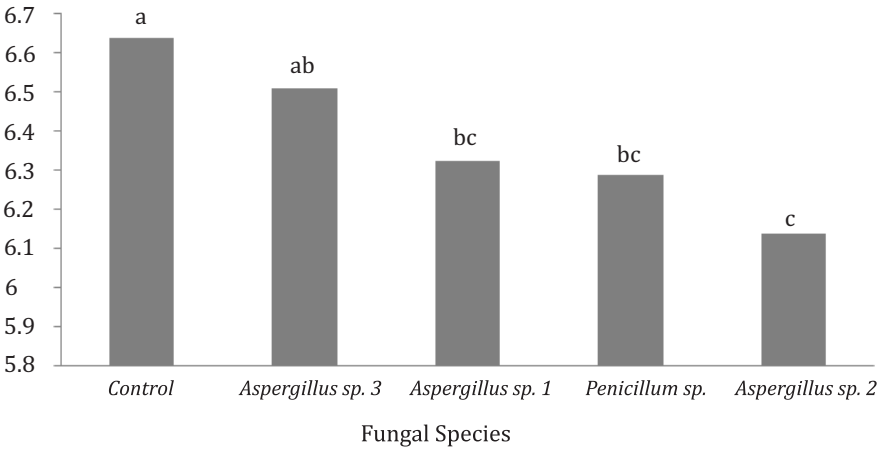

Figure 5. Mean pH measurements of the culture media with fungal species within four weeks of biodegradation assay. Levels with same letter are not significantly different from each other, HSD

In the first week, there was a significant decrease in the pH of the media in all fungal species (Figure 6) indicating a potentially greater production of organic acids as intermediate products from the biodegradation of the hydrocarbons (Miranda *et al.*, 2007)

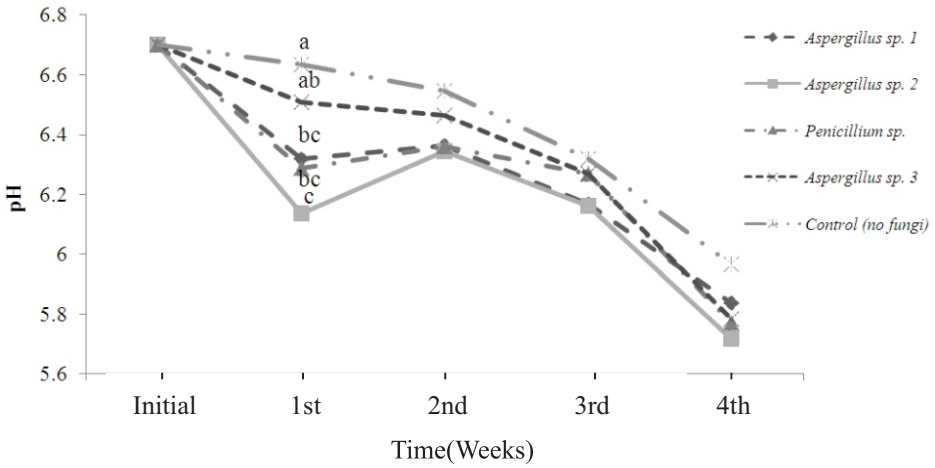

Figure 6. Mean pH measurements of the culture media during the biodegradation assay. Points with the same letter are not significantly different from each other, HSD

There was a highly significant difference in percent fat loss among fungal species. *Aspergillus* sp. 2 and *Aspergillus* sp. 1 had the highest mean percentage of fat loss at 77.12% and 69.4%, respectively (Figure 7). *Aspergillus* sp. 2 and *Aspergillus* sp. 1 also had the highest mean dry weights of 0.24g and 0.21g, respectively, whereas, *Aspergillus* sp. 3 was the least with 0.13g (Figure 8).

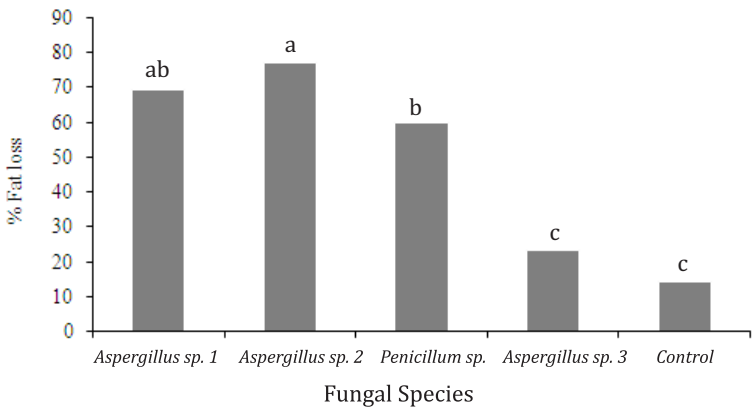

Figure 7. Mean percent fat loss of the culture media with fungal species after four weeks of biodegradation assay. Levels with the same letter are not significantly different from each other, HSD

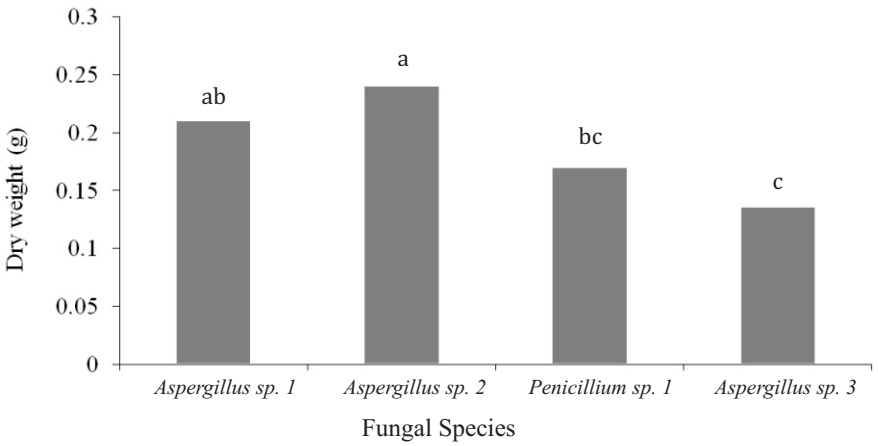

Figure 8. Mean dry weights (g) of fungal inoculates after four weeks of biodegradation assay. Levels with the same letter are not significantly different from each other, HSD

Hydrocarbons within the saturated fraction like used engine oil include n-alkanes, branched alkanes, and cycloalkanes (naphthenes). The n-alkanes are generally considered as the most readily degraded components in a petroleum mixture. Biodegradation of n-alkanes from mid- to higher molecular weights by filamentous fungi have been reported by Husaini *et al.* (2008). The biodegradation of n-alkanes normally proceeds by a monoterminal attack; usually a primary alcohol is formed followed by an aldehyde and a monocarboxylic acid. Further degradation of the carboxylic acid proceeds by fl-oxidation with the subsequent formation of two-carbon-unit shorter fatty acids and acetyl coenzyme A, with eventual liberation of $C0_2$. Fatty acids have been found to accumulate during hydrocarbon biodegradation (Atlas, 1981).

The findings by Okafor *et al.* (2009) support these results. Their study found that high biodegradation efficiency was exhibited by *A. versicolor* and *A. niger* at >98% against crude oil. The high efficiency could be attributed to the fungi's massive growth and enzyme production responses during their growth phases. These findings could explain similar mechanism of action of the two *Aspergillus* species on used engine oil as seen in this study.

Adekunle and Adebambo (2007) found that microorganisms break down hydrocarbons and use the energy to synthesize cellular components by releasing carbon (IV), water and energy.

Results of this study support earlier findings that fungal species were capable of degrading used engine oil using the hydrocarbons as substrates for growth. In the experiment by Adekunle and Adebambo (2007) using fungi isolated from *Detarium senegalense* seeds, it was shown that fungi release

extracellular enzymes and acids capable of breaking down the recalcitrant hydrocarbon molecules by dismantling the long chains of hydrogen and carbon, converting petroleum into simpler forms or products that can be absorbed for the growth and nutrition of the fungi. However, they also reported that there are also nutrients present in the minimal salt broth, (the same media used in this study) though some of it could have been present in the oil, which stimulated the growth of each fungus. So, the additional nutrients present in the minimal salt broth helped microbial growth to a certain extent and also in creating a favorable environment for rapid fungal development especially at times when the fungi have not started breaking down the hydrocarbons into simpler molecules.

Findings of this study are consistent with those reported by Gopinath *et al.* (2005) on fungal extracellular enzymes. From the 34 wild fungal species they isolated, *A. versicolor* exhibited high amylolytic and gelatinolytic activity while maximum *A. niger* cellulolytic activity against edible oil mill wastes.

In this study, the same action may have been exhibited by the two *Aspergillus* species (*Aspergillus* sp 1 & 2) against used engine oil but not for *Aspergillus* sp. 3 which had low efficiency in degrading and utilizing the used engine oil for growth. This could be due to the shorter length of time used for the assay. It could also be that biodegradation efficiency of the *Aspergillus* is a characteristic of the species, and not the whole genus.

CONCLUSIONS

Four species of oil-degrading fungi which include three species of *Aspergillus* and one species of *Penicillum* were isolated from the coastal sediments of the Ormoc Port Area. The main fungal species that exhibited bioremediation potential against used engine oil were *Aspergillus* sp. 2, *Aspergillus* sp. 1 and *Penicillium* sp. However, only *Aspergillus* sp. 1 and 2 were the most efficient in degrading and utilizing the used engine oil for growth. These findings support earlier reports on the potential of fungal species in detoxifying oil spills that threaten the health of the oceans and the various organisms in it. The development of possible application strategies for utilizing oil-degrading activities of fungi in the removal of petroleum oils from contaminated ecosystems as well as studies on the action of these microorganisms in consortium with other microorganisms however, need to be done.

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