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Abstract

Microbial biodegradation of oil pollutants and their derivations has become the most environmental-friendly method in the developing world. Lipolytic enzyme; *Aspergillus niger* extracted from oil polluted soil has been studied, examined and found to degrade Polyaromatic Hydrocarbons found in petroleum contaminated soil. A. *niger* can produce Bio-Surfactant at 25 °C and 35 °C that can biodegrade oil hydrocarbons. 24 hours at 35 °C it can reduce surface tension from 55 Nm/m to 25.3 Nm/m at different percentages of crude oil.

Keywords: *Aspergillus niger*, Biodegradation, Petroleum contaminated soil, Bio-surfactant, Polyaromatic Hydrocarbons.

INTRODUCTION

Our landmass, seas, oceans and coastal zones are under great stress; and pollution, particularly by crude oil, remains a major threat to the sustainability of planet Earth. Efforts to alleviate these pollutants remain focus to environment researchers. Of importance is biodegradation involves treating the petroleum pollutants with aliphatic-degrading microorganisms possessing many kinds of enzymes, notably lipases. It has been shown to be a viable, relatively low cost, low-technology technique, break down the pollutants in a shorter time and its use is widespread [5, 8]. Lipolytic enzymes are one of the most important groups of biocatalysts for biotechnological application since lipases can be naturally and readily discovered from the earth's flora and fauna. However, most commercial lipases are produced from microbes [6]. *Bacillus spp*. was the main sources of lipolytic enzymes, until the discovery of Aspergillus fungi as another potential source of lipase enzymes [10]. Lipase-producing fungi have been found in diverse habitats such as soil contaminated with oil, dairies, industrial wastes, oilseeds and decaying food, compost heaps, coal tips and hot springs [3, 12]. Studies have shown fungi metabolize crude oils, converting them to carbon dioxide or into secondary metabolites; hence, the enzymatic systems produced by fungi are keys to converting crude oil [15]. Current industrial fat splitting employs the energy expensive colgate-emery process, the biochemical properties of lipases have been reviewed to contribute to most of the industrial production [7].

Polysaccharide carbon sources such as starch or bran, triglycerides or fatty acids, nitrogen sources such as soyabean meal, groundnut, peptone, and yeast extract, casein hydrolysate, lipid substances etc. These substances have been reported to be effective stimulants of lipase enzyme, the lipase synthesis mostly occur through substrate induction and catabolic repression [9, 13]. The lipase production by *Penicillium restrictum* in a bench-scale fermenter revealed that specific air flow rate and agitation beneficial factors besides carbon and nitrogen and lipid substrates such as olive oil (1%) and the C: N ratio [2]. The optimum culture conditions for lipase production according to Chander are a cultivation period of 6 days, cultivation temperature of 30°C and at pH 7.0 in the presence of olive oil (0.15%). *Aspergillus oryzae* produces little lipase in solid cultures in contrast to large amount in a shake-flask culture with temperature exerting a tremendous influence on lipase synthesis [4].

Oil spills have a number of effects on the environment, economy, marine life and plants and animals on the land, human's health, when petroleum products, or the waste associated with use of these products, are released into the environment, they chemically and biologically interact with the soil, groundwater, and microorganisms. Petroleum contains benzene, xylene, toluine, napthalene, PAHs and metals [5]. Several studies following major oil spills like the *Amoco Cadiz* measured oil degradation in the

environment and confirmed previously published results from laboratory studies [12]. In this study we investigate and cross check biodegradation ability of *Aspergillus niger* on petroleum contaminated soil collected from a busy motor vehicle garage. Bioremediation was achieved by manipulation of the lipase media using techniques such as aeration or temperature control [7, 8].

MATERIALS AND METHOD

This study involved several phases, including soil sampling in the garage area, isolation of Fungi from the petroleum contaminated soil [14], selection of fungal pathogens from bacteria, culturing of fungi, isolation of pure culture of *Aspergillus niger*, measuring the activities level of fungal surface tension, and finally chemical analysis in order to be sure about biodegradation of *Aspergillus niger*. Petroleum contaminated soil samples were collected from Nairobi Ngara garage (latitude of 1° 18'S, longitude of 36°45' E, attitude/elevation of 1798m/5899 ft). Soil samples were taken to the laboratory in standard condition of 27°C. Different mediums were used in this study, culturing of fungi was done on Potato Dextrose Agar (PDA), Sabouraud dextrose agar mixed with 50mg of cycloheximide, 50mg of chloramphenicol and 20mg of gentamicin was used in selection of fungal from bacterial pathogens, Czapek – Dox Agar medium which contains 3gm of $NaNO_3$, 1gm of k_2HPO_4, 0.5gm of KCl, 0.5gm of $MgSO_4.7H_2O$, 0.01gm of $FeSO_4.7H_2O$, 30gm of sucrose, 15gm of Agar.was used to identify *Aspergillus* species, lastly modified lipase medium was prepared to make suspension and mass culturing of the *Aspergillus niger* fungi, it involved addition of 10.0 g peptone, 10.0 g of sterilized glucose and 1.0 g yeast extract to 1 L 0.1M phosphate buffer, pH 7.0 [2]. Lipase enzyme production will be tested on Rhodamine –B agar medium [9]. Fungal identification was done microscopically by use of Lactophenol cotton blue stain.

Du Nouy ring method and Tensiometer were used to measure surface tension [1, 14]. 25ml of 24 hours samples of culture were used. The experiment was repeated triple times for each sample. For each measurement, surface tension of distilled water and fungal-free culture environment were taken as control samples. Three 250ml Erlenmeyers which included 100ml sterilized Rhodamine –B agar medium and %1Modified lipase medium and %1sterilized crude oil. Also, a sample was taken as control and the amount of oil compound reduction (Biodegradation) by fungi was measured within 12 hours, 24 hours, and 48 hours and 72 hours by using High performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) [6].

RESULTS

From the fungal isolates *Aspergillus niger* was separated, screened and pure culturing was grown using modified lipase medium pH 7.0. Microbial growth was checked at Optical Density of 600nm (OD_{600}), whereby high multiplication was after 24 hours at 25°C and 35°C as shown in fig. 1 and fig. 2. Maximum amount of microbial growth occurred at 25°C with an optimum pH of 7 as shown in fig. 1and fig. 2 shows maximum microbial growth to occur at 35°C with an optimum pH of 8 for *A. niger*.

Lowest surface tension on oil wasat OD_{600} was 2.30 mN/m at pH of 4 displayed at 35°C. Another comparative low surface tension of 3.59 mN/m was seen at pH 8 at a temperature of 25°C as shown in table 1.

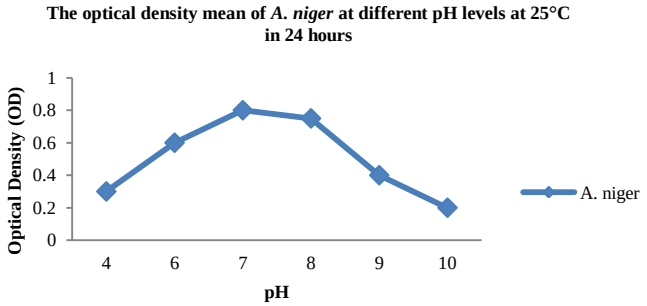

Figure 1: The optical density mean of *A. niger* at different pH levels at 25°C in 24 hours

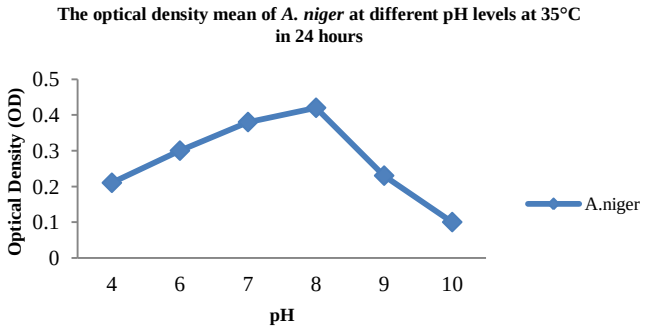

Figure 2: The optical density mean of *A. niger* at different pH levels at 35°C in 24 hours

Table 1: The amount of *Aspergilus niger* surface tension (mN/m) at 25 and 35°C in 24 hours

	Temp (°C)	**pH**	**4**	**6**	**7**	**8**	**9**	**10**
Aspergilus niger	25		7.01	8.42	8.94	3.59	7.33	10.03
Aspergilus niger	35		2.30	6.76	5.37	2.45	3.22	2.36
Blank			55	53	54	55	55	55

Table 2: The average surface tension (mN/m) of *Aspergillus niger* at various shaker rotations in %1 Concentration.

Temp (°C)	**Rotation of shaker (RPM)**	**50**	**100**	**150**	**200**
25	***A. niger***	39.4	32.2	29.7	26.8
35	***A. niger***	32.1	28.4	27.9	25.3
	Blank	55	55	55	55

Table 3: The degree of average surface tension (mN/m) and the Optical Density (OD_{600}) of *Aspergillus niger* strain at different temperature in 1% concentration

Temp (°C)		**20**	**25**	**30**	**35**	**40**	**45**	**50**	**55**	**60**
Surface Tension	**Blank**	55	55	55	55	55	55	55	55	55
	A. niger	32.1	29.7	29.9	25.4	30.3	31.6	32.4	33.8	35.8
***A. niger* OD_{600}**		0.357	0.372	0.381	0.395	0.402	0.411	0.415	0.418	0.419

Table 2 displays results of 100ml of sterilized Rhodamine –B agar medium and %1Modified lipase medium and %1sterilized crude oil when placed on a rotating shaker displayed lowest average surface tension of 25.3 mN/m at 35°C, 200 rpm. At 25°C *A.niger* showed a lower surface tension of 26.8 mN/m at 200 rpm of the rotating shaker. Table 3 shows that *A. niger* achieved the lowest surface tension at 35°C at an optical density of 0.395 mN/m which was followed by 25°C at an optimum density of 0.372 mN/m.

Table 4: The results of chemical analysis of samples by GC and HPLC in %1 concentration

Type of Analysis	**Sample**	**Blank (mg/L)**	**12^{th} hour**	**24^{th} hour**	**48^{th} hour**	**72^{nd} hour**
GC Analaysis	C13	8.7	8.6	8.1	8.0	8.2
	C16	6.6	6.4	6.1	6.1	5.8
	n-C 18	4.6	4.4	4.3	4.2	4.1
	n-C 10	2.0	1.9	1.7	1.7	1.5
	n-C 11	5.6	5.2	5.1	4.8	4.6
	C7	3.4	3.0	2.4	2.0	1.8
HPLC Analaysis	Naphthalene	220	185	139	110	73
	Acenaphthene	2.8	2.2	2.1	1.8	1.4
	Fluorene	13	9.8	8.4	7.1	6.3
	Phenanthrene	245	210	180	156	132
	Pyrene	14	10	7.2	5.1	3.4
	Chrysene	3.9	2.8	1.6	1.2	0.9
	Dibenz(a,h)	1.6	1.12	0.84	0.51	0.28
	Anthracene	7.2	7.4	6.5	4.2	3.9
	Benzo (ghi)	6.8	6.3	5.8	4.9	3.7
	Perylen	7.0	6.4	6.1	5.2	4.0

DISCUSSION

Aspergillus species are the main fungal groups which degrade hydrocarbon compounds as studied by Shoeb et al. in their studies on the biodegrading ability of aromatic hydrocarbons (PAH) by fungal microbes [14]. According to studies done by Margesin et al. *Aspergillus* species of fungi have the capability of surviving at alkaline pH and perform their metabolic activity at moderate basic pH of 7 to 9 and relative temperature of 24 ^{O}C to 38 ^{O}C [10]. In this study as displayed in fig.1 and 2, pH of 7 and 8 are suitable for multiplication of *A.niger* species of fungi and relative temperatures of 25 ^{O}C and 35 ^{O}C, high growth is experienced at an optimum temperature of 35 ^{O}C and at a pH of 8.

Biodegradation of crude oil can be measured by determining the surface tension; high surface tension indicates high bondage in the sample [1]. From this study the minimal surface tension was at an optical density of 2.30 mN/m at pH of 8 indicated at 35 ^{O}C, similar studies done on *B. Subtilis* bacteria showed a surface tension of 30.5 mN/m at OD_{600} performed at 37 ^{O}C with a pH of 7 [11]. In comparison it's relatively higher as to this study finding.

Temperature is one of the important environmental factors in biodegradation. Studies on *A. terrus* on crude oil %1 concentration, where by the strain was cultured in %1 Nutrient agar medium, %1sterilized crude oil which were placed on a rotary shaker at different temperatures and pH, later measured against their relative optimum density at 600nm, the degree of average tension attained was 0.57 mN/m, pH of 7, 37^{O}C at 200 rpm [3]. This was higher as compared to this study finding where the OD_{600} was 0.395 mN/m, 35^{O}C, pH 8 at 200 rpm and 0.372 mN/m, 25^{O}C, pH 7 at 200 rpm.

Degree of oil degradation has always been estimated by HPLC and GC. The obtained results confirm either biodegradation process is active or not. It is confirmed there is reduction in the components of oil as seein in table 4. From the table it can be analysed that there is sequential reduction of components at every 12 hours of time difference. At 24^{th} hour there was high breakdown of carbon bonds (C13, C16, n-C18, n-C10) at an average of 0.2 mg/L, Chrysene and Benzo (ghi) at 0.75 mg/L, Dibenz (a,h) at 0.33 mg/L, Anthracene at 1.17 mg/L, Perylen at 0.65 mg/L this is relatively higher when compared with studies done by Meintanis et al. on *B. Subtilis* and *B. cereus*, whereby at 24^{th} hour the breakdown of C13, C16, n-C18, n-C10 was at an average of 0.05 mg/L Chrysene and Benzo (ghi) at 0.65 mg/L, Dibenz (a,h) at 0.22 mg/L, Anthracene at 1.04 mg/L, Perylen at 0.31 mg/L [11]. This demonstrates

that fungi have higher biodegradability activity of both aromatic and aliphatic compounds as compared to bacteria.

CONCLUSION

Soil contamination with oil spills is the major global concern today. Soil contaminated with Petroleum has a serious hazard to human health, causes organic pollution of ground water which limits its use, causes economic loss, environmental problems, and decreases the agricultural productivity of the soil. A great part of the oil pollution problem results from the fact that the major oil-producing countries are not the major oil consumers. It has been known for 80 years that fungi microorganisms are able to degrade petroleum hydrocarbons and use them as a sole source of carbon and energy for growth. *Aspergillus niger* microorganisms are able to degrade petroleum hydrocarbons and use them as source of carbon and energy. Lipolytic enzymes have attracted an enormous attention due to their rise in demand and various industrial application like medical, pharmaceuticals, fine chemical synthesis, food Industry, domestic, environmental and in production of biofuels. Extracellular lipases are today the enzymes of choice for organic chemists, pharmacists, biophysicists, biochemical and process engineers, biotechnologists, microbiologists and biochemists.

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