PRODUCTION OF LIPASE BY IMMOBILIZED *BACILLUS THURINGIENSIS* AND THEIR BIODEGRADATION POTENTIAL ON PREMIUM MOTOR SPIRIT

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ABSTRACT

The synthesis of lipase by immobilized Bacillus thuringiensis was described in this work. Bacteria isolates were tested for oil degradation on Bushnell-Hass Mineral Salt medium with 1% v/v PMS. Bacillus thuringiensis was identified as the potent isolates utilizing 16S rRNA. The isolates were immobilized in gelatin matrix and grown in a submerged medium for lipase synthesis. The crude lipase extracted was used for degradation of PMS. Lipase from immobilized Bacillus thuringiensis achieved optimal PMS degradation of 50.9% in 20 days at pH 7 and 35. The chemicals decomposed within 20 days, according to GC-MS analyses. As a result, our study demonstrated that using immobilized bacterial lipase to degrade PMS is a simple and effective method.

KEYWORDS: *Bacillus thuringiensis*, Immobilization, Biodegradation, Lipase, PMS, GC-MS, Bushnell-Hass Mineral Salt medium

INTRODUCTION

Petroleum hydrocarbons are the primary source of energy and materials for a variety of industrial use (Varjani *et al*., 2016). Their uses in it is used in coastal oil refining, transportation, shipping activities, offshore oil production and accidental spills pose several dangers and results into environmental pollution (Arulazhagan et al., 2010). Various human activities, such as agriculture, the petroleum industry, nuclear technology, and the petrochemical sector, all contribute to major environmental issues. The release of wastes created as a result of those activities is of special environmental importance (Eskander and Saleh., 2017). Petroleum hydrocarbon contamination is caused by human activities such as municipal run-offs, liquid releases, and industrial processes, which have an influence on the environment and offer a direct or indirect health risk and threat to living things (Sajna et al., 2015). Carcinogens and neurotoxic organic pollutants have long been associated with hydrocarbon components. Because the volume of leakage is modest, on-site cleanup, treatment, or recovery of contaminants is facilitated in an unintentional leak, but contaminants in petrol stations and spills may persist. Petroleum leakage occurs frequently as a result of the unintentional and illegal discharge of oil waste at sea, endangering a variety of ecosystems. Petroleum hydrocarbons are very harmful substances that have been designated as priority pollutants (Costa et al., 2012). This is why various ways for degrading these chemicals into non-toxic or less hazardous compounds must be devised. A biological treatment is an alternate way of pollutant removal because it has no negative effects on the environment. It's also possible that it'll be less expensive than other methods. Chemical and mechanical treatments, which are commonly utilized to address hydrocarbon contamination on oil-polluted sites, are ineffective and expensive.

Biodegradation involves biological or enzymatic activity of a material which results in a significant change in the chemical structure of the material exposed, as well as the production of carbon dioxide, water, mineral salts, and new microbial cellular constituents (biomass) (Bandyopadhyay-Ghosh et al., 2015). Hazardous toxic pollutants are converted into less toxic or non-toxic compounds through this naturally normal process. Secondary metabolites, intermediate molecules, or any degradation

products from one creature can be used as a source of carbon and energy for other organisms. They can continue to decompose the leftover organic debris.

Biodegradation plays a critical role in the treatment of petroleum-based hazardous pollutants. Bioprocesses for treating hazardous wastes are a promising technology since they are cost-effective and can result in total hazard elimination and mineralization. Nature's approach of recycling wastes or breaking down organic matter into nutrients that can be utilised by other creatures or changed into less harmful or non-toxic compounds is known as biodegradation. Some forms of environmental toxins can be minimized or eliminated entirely by leveraging these natural processes of biodegradation (Eskander and Saleh, 2017). However, using microorganisms directly in the biodegradation of oil-polluted locations may be technically difficult, thus microbial enzymes are being examined as an alternative (Kareem et al., 2003). In addition, the use of immobilized cells has been reported to be used in repeated cycles in biodegradation process. Given the importance of the lipase enzyme, the goal of this study was to extract, identify, and immobilize lipase-producing bacteria from several sources of oil-contaminated soil, as well as to investigate their activity. The aim of this study was to produce bacterial lipase from immobilized bacteria species and then use it to degrade PMS.

3.0 MATERIALS AND METHODOLOGY:

3.1 SAMPLE COLLECTION: Samples were collected from oil contaminated Atlantic Seawater and Sediment sites. The collected samples were packed in sterile bottle to the laboratory. The entire sample was stored at refrigeration temperature before the experimental work.

3.2 ISOLATION OF BACTERIA:

Soil and water sample were serially diluted and plated on Nutrient Agar medium, pH 7.0 by spread plate method. Plates were incubated at 37°C for 48 hours. Pure cultures of the isolates were maintained on nutrient agar slants and were sub-cultured every 15 days.

3.3 SCREENING OF HYDROCARBON DEGRADING BACTERIA

The isolated bacteria were inoculated on an enrichment medium that contains the autoclaved mineral salt medium (MSM) supplemented with single hydrocarbon compound as sole carbon source (1% petrol). The MSM composition was made up of basal salt medium and trace element solution. The basal medium contain (g/L): K_2HPO_4, 1.8; KH_2PO_4, 1.2; NH_4Cl, 4.0; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; yeast extract, 0.1 and $FeCl_2.4H_2O$, 0.05 and trace elements solution contain: H_3BO_3, 0.1; $ZnSO_4.7H_20$, O.1; $CuSO_4.5H_2O$, 0.05 and $MnSO_4.H_2O$, 0.04 with the pH of 6.5 (Balogun and Fagade, 2010).

3.4 IDENTIFICATION OF BACTERIA

Identification of isolates was performed by Gram staining and biochemical tests.

3.4.1 MOLECULAR STUDIES

3.4.2 ISOLATION OF GENOMIC DNA FROM BACTERIA:

DNA was extracted from 1ml of bacterial culture, the culture was pelleted by centrifuging at 12,000rpm for 2min, the pellet was treated with lysis solution and proteinase k and incubated at 60°C for 30min. Nucleic acids was precipitated with isopropanol by centrifuging at 10,000 rpm for 10min, washed with 1ml of a 70% (v/v) ethanol solution and dissolved in 0.1ml of a TE buffer. The purity and quantity of DNA was examined by recording its UV absorption spectrum and running on 1% agarose gel electrophoresis.

3.4.3 SEQUENCE DETERMINATION OF 16S RDNA:

The DNA isolated was amplified using 16S rRNA universal primers and sequenced for the identification of bacterial strain at molecular level. Amplification of the PCR products of expected size was confirmed by electrophoresis. The sequence of the 16S rRNA was determined using a Dye terminator sequencing kit (Applied Biosystems), and the product was analysed with an ABI Prism

DNA sequencer (ABI). The gene sequences of each isolate obtained in this study will be compared with known 16S rRNA gene sequences in the GenBank database.

3.7.1 EXTRACTION OF LIPASE ENZYME

The extracellular Lipase enzyme production will be studied by standard method (Kumar *et al*., 2011). The extracellular Lipase enzyme is extracted from the production medium (isolates cultures will be inoculated on Nutrient medium) after desired incubation time (48h) by centrifugation at 10000 x g for 30min in a refrigerated centrifuge. The resulting supernatant contained extracellular lipase enzyme.

Lipolytic organisms (extracellular Lipase supernatant) were screened by qualitative plate assay according to (Singh *et al*., 2006). The medium was sterilised at 121°C for 15min and while warm, sterile Tween 80 (1%) was added and mixed properly. Sterile plates were poured and inoculated with pure isolates and incubated at 30°C for 72h. Lipase production was detected by a colour change around the colonies. Zone of hydrolysis were measured.

3.7.3 PRODUCTION OF LIPASE BY BACILLUS SPP:

The quantitative enzymatic assay of lipase activity was done using olive oil as the substrate. 1ml of TrisHCl buffer was taken along with 3ml of olive oil substrate, mixed by swirling and equilibrated at 37°C. The pH was adjusted to 7.7. To this 1ml of the extracellular Lipase supernatant enzyme solution was added. It was mixed and incubated at 37°C for nearly 30minutes. Then, 3ml of 95% ethanol was added to the mixture. 4 drops of 0.9 % Thymolphthalein indicator solution was added later. It is immediately titrated with standardized 50mM Sodium Hydroxide (NaOH) solution. Appearance of pale blue colour served as the end point. The procedure was repeated for concordant values and burette reading was noted. The blank was titrated in the same manner and the readings were tabulated.

The quantitative activity of the enzyme can be calculated by the following formula:

$$Lipase\ Activity = \frac{Volume\ of\ Alkaline\ consumed\ X\ Normality\ of\ NaOH}{Time\ of\ Incubation\ X\ Volume\ of\ Enzyme\ Solution}$$

3.8.0 CELL IMMOBILIZATION

3.8.1 IMMOBILIZATION OF *BACILLUS THURIGIENSIS* WITHIN A GELATIN MATRIX

Immobilization of bacteria isolates was done within gelatin matrix using a modified method of Osho *et al*. (2001). Aqueous solution of gelatin (7-10.0% w/v) was crossed linked by adding ethanolic formaldehyde to give a final formaldehyde concentration of 2% (v/v) and incubated for 20min at 37ºC. Bacillus sp was added to the crossed-linked gelatin under vigorous stirring at 35ºC. The mixture was poured into a burette and added dropwise into a beaker containing cold liquid paraffin to obtain droplets of gelatin (beads). The beads were hardened by suspending in the cross linking agent for 24h. They were washed with sterile distilled water and stored in the refrigerator at 4ºC.

For optimal bead size determination, an aqueous solution of gelatin (8%w/v) with 1ml of the cell culture was entrapped at 35ºC under vigorous stirring. Each preparation gel was poured through an improvised laboratory dropper (2.5 mm diameter) and a syringe (4.0 mm diameters) into a cold paraffin liquid respectively at a constant flow rate (2ml/min). The gel bead were hardened by suspending in the cross linking agent for 24h. They were washed with sterile distilled water and stored in the refrigerator at 4ºC prepared.

3.9.0 DETERMINATION OF LIPASE BIODEGRADATION OF PMS:

The degrading activities of each enzyme was obtained by using Mineral salt broth (MSB) in which 40 mL of each hydrocarbon (PMS) was added and incubated at room temperature for 20 days. The enzyme activity was measured by taking the optical density (O.D) readings at 600nm after 20days against mineral salt medium as blank.

3.9.1 OPTIMIZATION STUDIES ON DEGRADATION

Optimization studies of the enzymes on degradation of hydrocarbons was done, effect of temperature (20 ºC, 25 ºC, 30 ºC, 35 ºC, 40 ºC, 45 ºC, 50 ºC), effect of pH (5, 5.5, 6, 6.5, 7, 7.5, 8) and degradation time (5 days, 10 days, 15 days, 20 days)

3.9.2 GRAVIMETRIC ANALYSIS

The amount of oil in culture was estimated using the Gravimetric method. Diethyl ether and acetone were taken in 1:1 ratio and was mixed with culture. The mixture was allowed to vaporize at room temperature. The oil residue obtained was weighed and taken as the gravimetric value for further calculation. (Marquez-Rocha *et al*., 2001).

$$Percentage\ of\ PMS\ degraded = \frac{Weight\ of\ PMS\ degraded}{Weight\ of\ PMS\ present\ original} \quad X\ 100$$

Where, the weight of PMS degraded = (original weight of PMS –weight of residual PMS obtained after evaporating the extract).

3.9.3 EXTRACTION AND ANALYSIS OF RESIDUAL OIL

Biodegradation of petroleum hydrocarbon in liquid culture was collected for analysis by gas chromatography (GC HP 680 series GC system, US90704303) (Marquez-Rocha *et al*., 2001).

3.9.3 DATA ANALYSIS

All data obtained were analyzed using Statistical package for social sciences Version 17.0. Means were separated using Duncan multiple range test.

RESULT AND DISCUSSIONS

Table 1 Biochemical tests of the isolates

Code	Grain staining	Catalase	Oxidase	Indole	Motility	Methyl Red	Voges P	Citrate	Urease	Starch hydrolysis	Probable organisms
1	GPB	+	-	-	+	-	+	-	-	+	*Bacillus thuringiensis*
2	GPC	+	+	-	-	-	-	+	+	NA	*Staphylococcus aureus*
3	GNB	+	+	+	-	-	+	+	+	-	*Klebsiella oxytoca*
4	GPB	+	-	-	+	-	-	-	+	NA	*Corynebacterium striatum*
5	GPB	+	-	-	+	-	+	-	-	+	*Lysinibaccillus sphaericus*

Key: GNB-Gram negative Bacilli, GPB-Gram positive Bacilli, NA-Not applicable

Table 2 Primary Screening and enzyme activity of the isolates

Isolates	Zone of hydrolysis	Enzyme activities(u/ml)
Bacillus thuringiensis	5.67 ± 1.45^{cd}	2.63 ± 0.04^{e}
Staphylococcus aureus	1.77 ± 0.13^{a}	1.25 ± 0.03^{ab}
Klebsiella oxytoca	4.68 ± 0.20^{cd}	1.53 ± 0.04^{d}
Corynebacterium striatum	5.36 ± 0.52^{cd}	1.73 ± 0.04^{d}
Lysinibaccillus sphaericus	6.40 ± 0.46^{d}	1.96 ± 0.07^{d}

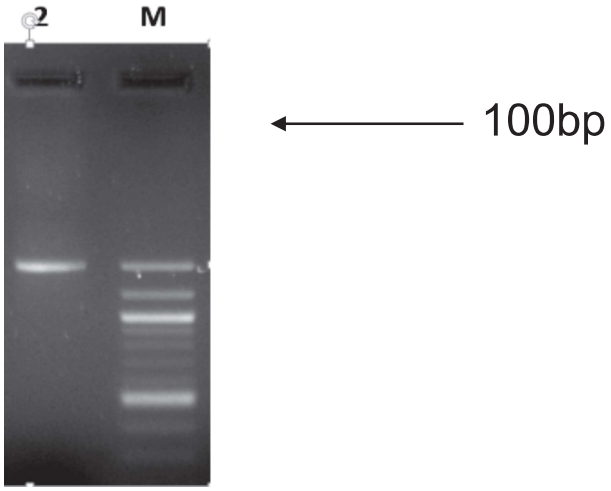

Plate 1: Gel electrophoresis of DNA extraction of bacteria isolates

1- Sample 1- Sequencing result showed it to *Bacillus thuringiensis*

2-

Table 3 Biodegradation of PMS by lipase from Immobilized *Bacillus thuringiensis*

	Bacillus thuiringiensis	
Test enzyme + Hydrocarbons	OD	Oil consumption (%)
Free lipase + Petrol	0.44	44.5
Lipase From Immobilized *Bacillus thuiringiensis* + Petrol	0.58	50.9
Petrol with no enzyme(Control)	1.98	0.0

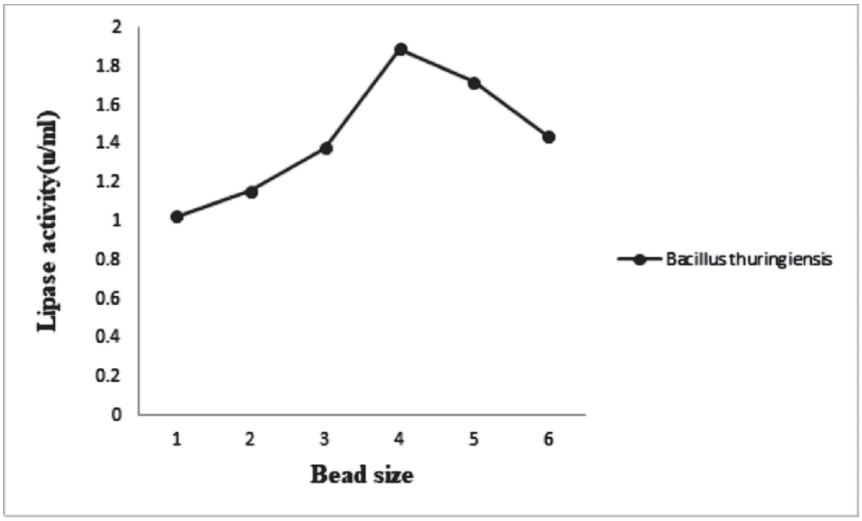

Figure 1: Effect of bead size on lipase activity by *Bacillus thuringiensis*

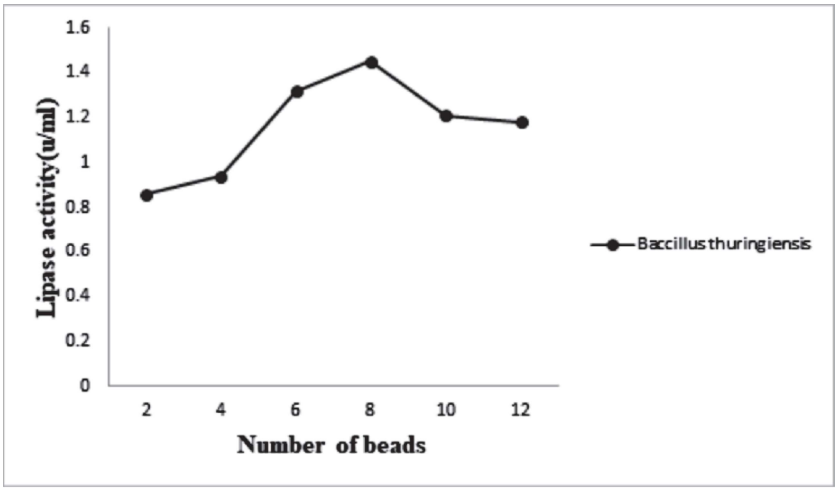

Figure 2: Effect of number of beads on lipase activity *Bacillus thurigiensis*

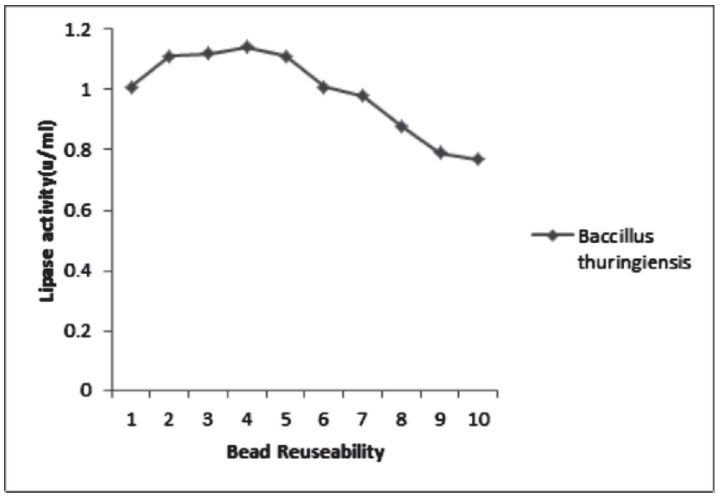

Figure 3: Bead reusability on lipase activity *Bacillus thuringiensis*

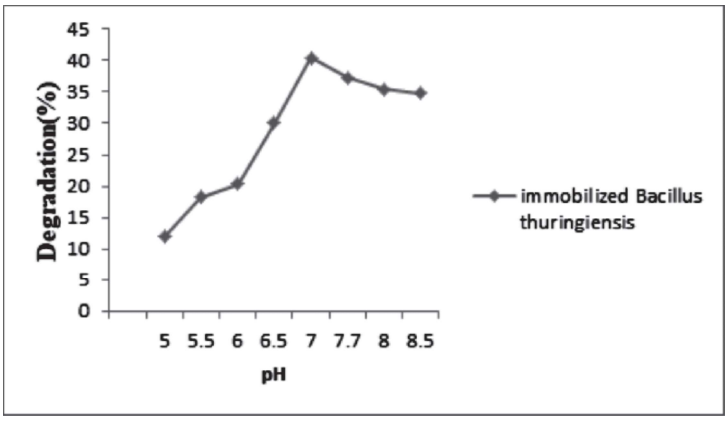

Figure 4: Effect of pH on degradation of Petrol using lipase from immobilized *Bacillus thunrigiensis*

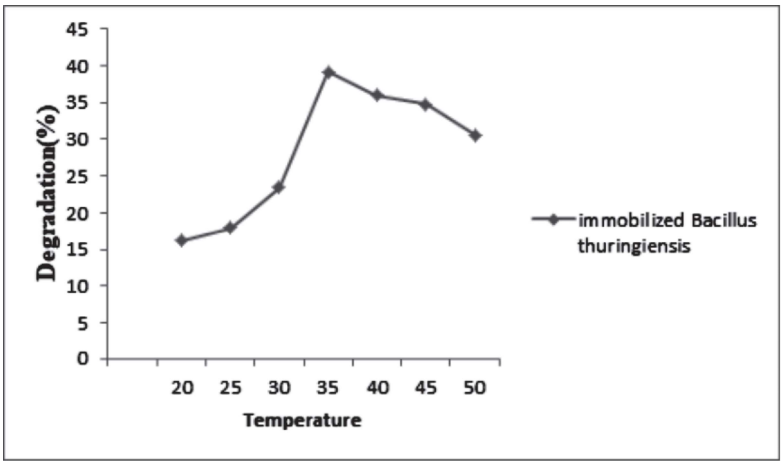

Figure 5: Effect of temperature on degradation of PMS using lipase from immobilized *Bacillus thunrigiensis*

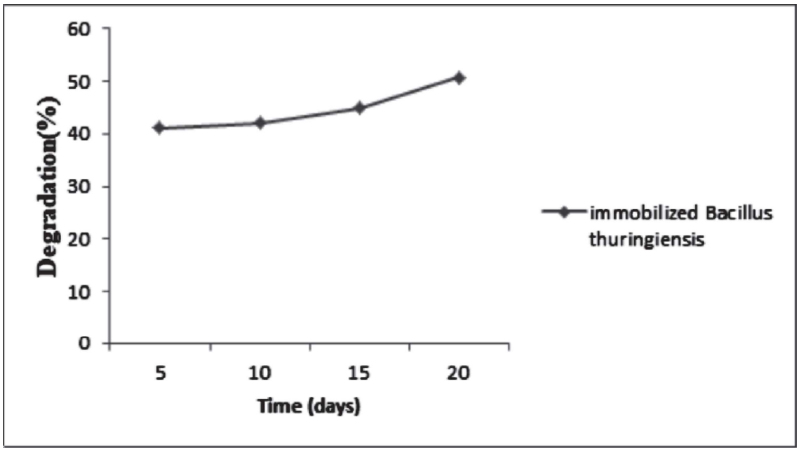

Figure 6: Effect of degradation time on degradation of Petrol using lipase from immobilized *Bacillus thunrigiensis*

Table 4: GC-MS results on degradation of PMS

	Compound	R.T(min)	Area	PPM	Area (%)
Control	Hexadecane	14.823	140545	6.71	100
	Eicosane	19.166	1672	0.08	100
	Tetracosane	22.902	663	0.03	100
	Hexacosane	24.504	435	0.02	100
	Octacosane	26.021	997	0.04	100
Bacillus thuringiensis	Hexadecane	14.800	639	0.03	0.4
	Eicosane	19.194	286	0.01	17
	Tetracosane	22.862	677	0.03	100
	Hexacosane	24.510	625	0.03	100
	Octacosane	26.015	92	0.00	9

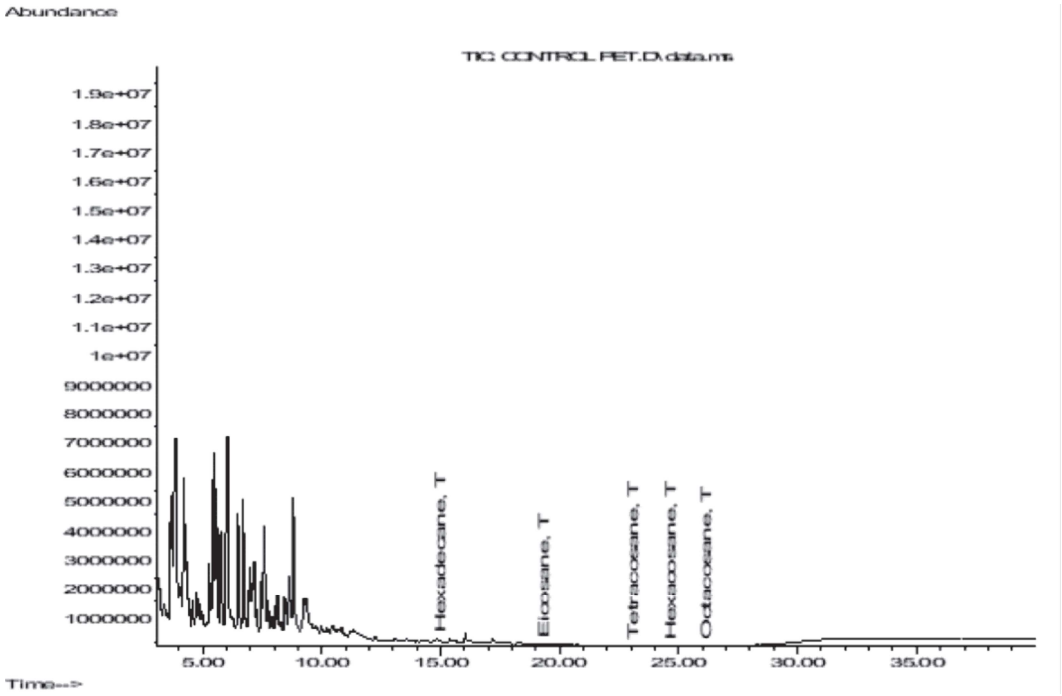

Figure 7: GC-MS of PMS without enzyme as the control

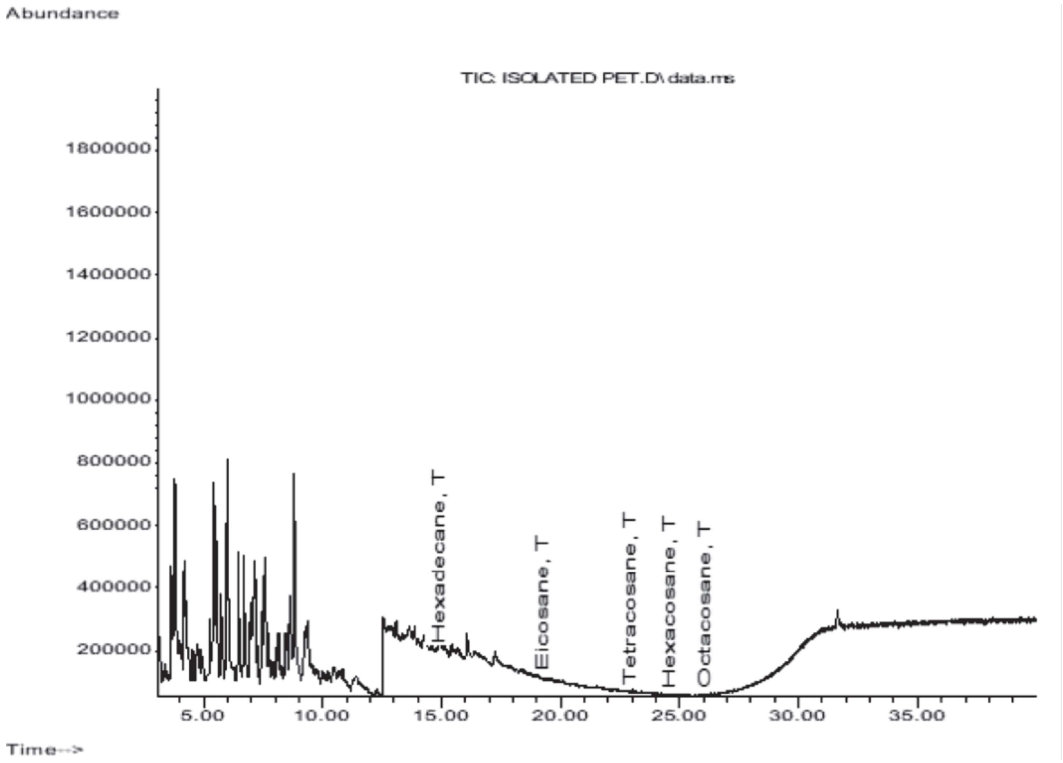

Figure 8: GC-MS result of PMS degraded by lipase produced from immobilized *Bacillus thuringiensis*

DISCUSSION

Many bacterial species are naturally present in soil, adjusting to the conditions, and nearly all bacteria that decompose oil are present in soil contaminated with oil, assisting in the cleaning of the soil from oil products (Cesarini *et al*., 2014). *Bacillus sp*. is the most common bacteria discovered in oil-contaminated soil, which is consistent with previous results by Kumar et al. (2012), who found *Bacillus sp., Pseudomonas sp., Micrococcus, and Aeromonas spar* among microorganisms found in oil-contaminated locations. Table 1 shows the biochemical profiles of the various bacterial isolates. To test their biodegradative capabilities, dominant bacterial cultures were inoculated in mineral salts broth (MSB) medium with 1% PMS as a carbon source: The crude oil served as a carbon source for all of the isolates. This is consistent with the findings of Latha and Kalaivani (2012), who discovered that the isolates could use hydrocarbons as a carbon source. Five bacterial colonies were chosen and isolated in total. Only two of the five isolates in the olive agar medium with phenol red as an indicator showed a nice clean zone. Plate 1 shows 100bp marker gel electrophoresis of DNA extraction of bacterium isolates. *Bacillus thuringiensis* strain VITSJ-01 has a nucleotide sequence that was 99 % similar. *Bacillus thuringiensis* produces the most lipase in the screening medium, 2.6 U/mL, and was chosen for further study, whereas others produced less than 1.96 U/mL. (Table 2). *Bacillus coagulans* produced the most lipase at 72 hours, according to research by (PrasanthKumar *et al*., 2007). Gelatin matrix was used to immobilize Bacillus thuringiensis on solid surfaces. Figure 1 shows the effect of different bead sizes (1, 2, 3, 4, 5, and 6) on lipase synthesis by immobilized bacteria cells. Lipase activity increases progressively, but when the bead size went above 4, lipase activity decreased (Figure 4). Immobilized *B. thuringiensis* (1.89U/ml) had the highest lipase activity. Figure 1 shows the effect of changing the number of beads (5, 10, 15, 20, and 30) on lipase synthesis by an immobilized - bacteria cell. Lipase activity increases progressively, but as the number of beads climbed beyond 8, lipase activity decreased (Figure 5). Immobilized B. thuringiensis (1.45 U/ml) had the highest lipase

activity. Figure 2 depicts the effect of altering bead reuseability on lipase synthesis by immobilized bacteria cells. Lipase activity gradually increased, and greater bead reuseability beyond 4 resulted in a drop in lipase activity (Figure 6). Immobilized B. thuringiensis (1.14 U/ml) had the highest lipase activity. Contrary to popular belief, (Hung *et al*., 2003; Won *et al*., 2005) discovered that immobilized *C. rugosa* lipase entrapped in Ca-alginate gel beads retained 72 percent after three uses, and that repeated use of immobilized *C. rugosa* lipase on chitosan retained 74 percent after ten reuses. Similarly, Yi et al, (2009) found that after 10 times of reuse, the activity of immobilized lipase from *C. rugosa* on alanine chitosan beads retained 77 percent of its initial activity. The bacterial strain obtained in this investigation was found among crude oil hydrocarbon digesting microorganisms. The results clearly demonstrated that the lipase produced by this microbe was biodegradable, and the values of degraded PMS fluctuated after 20 days of incubation. Table 3 shows the breakdown of PMS using immobilized *Bacillus thuringiensis* lipase. PMS was digested 50.9 % by lipase from immobilized *Bacillus thuringiensis*. The effect of pH on PMS degradation revealed that degradation increased with increasing pH from 5-8.5 to a maximum of 7 for PMS degradation from immobilized *Bacillus thuringiensis* lipase (figure 1). According to Gupta *et al*. (2004), lipases' maximum activity has been seen in many cases at pH values greater than 7. The effect of temperature on lipase activity of the crude enzyme on PMS degradation revealed that degradation of PMS increased gradually as temperature increased from 20°C to 35°C for lipase of Bacillus thuringiensis (figure 2). Stringfellow and Aitken, (1994) found that 40°C was the best temperature for phenantheren breakdown. Figure 3 shows the effect of degradation period on crude enzyme lipase activity on PMS degradation; an increase in degradation was found when the duration was increased from 5 to 20 days. Individual n-alkane percentage degradation increases with time, according to Siddiqui *et al*. (2001), and was in the range of 43.5-53.9 % on the seventh day. The GC-MS analysis of PMS, as shown in Table 3, revealed that lipases from immobilized *Bacillus thuringiensis* reduced Hexadecane by 99.96%. PMS degradation from immobilized *Bacillus thuringiensis* lipase peaked after 20 days. Verma *et al*. (2006), on the other hand, evaluated the ability of *Bacillus sp*. SV9 to degrade the n-alkanes portion of oily sludge and found that *Bacillus sp*. SV9 could breakdown 88.91.24 percent of C12-C30n-alkanes in 5 days.

CONCLUSION

In this study, lipase from Immobilized *Bacillus thurigiensis* degraded PMS after 20 days. It can be concluded that enzymatic degradation of PMS using bacterial lipase is an effective and eco-friendly biotechnological approach

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