

## PRODUCTION OF LIPASE BY IMMOBILIZED *BACILLUS THURINGIENSIS* AND *LYSINIBACILLUS SPHAERICUS* AND THEIR BIODEGRADATION POTENTIAL ON DIESEL

Olaposi Adegoke<sup>\*1</sup>, Sarafadeen Kareem<sup>1</sup>, Saka Balogun<sup>1</sup> and Adeniyi Afolabi<sup>2</sup>

Address (es): Olaposi Adegoke,

<sup>1</sup>Department of Microbiology, Federal University of Agriculture, Abeokuta, P.M.B. 2240, Ogun State, Nigeria.

<sup>2</sup>Department of Chemistry, Federal University of Agriculture, Abeokuta, P.M.B. 2240, Ogun State, Nigeria.

\*Corresponding author: [adegokeolaposi@gmail.com](mailto:adegokeolaposi@gmail.com)

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### ABSTRACT

This study reported production of lipase by immobilized *Bacillus thuringiensis*. Bacteria isolates were screened on Bushnell-Hass Mineral Salt medium containing 1% v/v Diesel for oil degradation. The potent isolates were identified using 16S rRNA as *Bacillus thuringiensis*. The isolates were immobilized in gelatin matrix and cultured for lipase production in a submerged medium. The crude lipase extracted was used for degradation of Diesel. Optimum degradation of Diesel 41.4% was obtained by lipase from Immobilized *Bacillus thuringiensis* and 31.6% for *Lysinibacillus sphaericus* at pH 7 and 35 in 20 days. GC-MS analysis was carried out to show the compounds degraded after 20 days. This study therefore presented the use of immobilized bacterial lipase in degradation of Diesel as a simple and effective approach.

**Keywords:** *Bacillus thuringiensis*, *Lysinibacillus sphaericus*, Immobilization, Biodegradation, Lipase

### INTRODUCTION

Lipases are serine hydrolases that catalyze both hydrolysis and synthesis of long-chain triacylglycerols. Lipases occupy a place of prominence among biocatalysts owing to their ability to catalyze a wide variety of reactions and are an important group of biotechnologically relevant enzymes and they find massive applications (Mohammed, 2013).

Lipases are carboxylic ester hydrolases currently attracting an enormous attention due to their versatile nature and widely used enzyme in biotechnological applications and owing to their unique properties (Cristian, 2005). Lipases are produced by microorganisms (bacteria and fungi), plants and animals. However, microbial lipase especially from bacteria are more useful than their plant and animal origin, since they have great variety of catalytic activities and microorganisms are easy to manipulate genetically and capable of rapid growth on inexpensive media (Sirisha, 2010; Mongkolthanaruk and Boonmahome, 2013; Veerapagu *et al.*, 2014; Jinyong, 2014). Microbial lipases are commercially most important mainly are secreted into the culture medium by many of microbial species are belong to bacteria, fungi, yeasts and actinomycetes (Babu and Rap, 2007; Abada, 2008). Lipases play a vital role in the manufacturing and services sectors for the mankind. Microbial lipases have gained special industrial attention due to their selectivity, stability and broad substrate specificity. Microbial enzymes are also more stable than their corresponding animal and plant enzymes and their production is more convenient and safer (Veerapagu *et al.*, 2013). Generally, bacterial lipases are glycoproteins but some extracellular bacterial lipases are lipoproteins (Abdul-Hamid *et al.*, 2013; Sagar *et al.*, 2013). The production of extracellular lipases from bacteria is greatly influenced by medium composition besides physicochemical factors such as temperature, pH and dissolved oxygen. The major factor for the expression of lipase activity has always been reported as the carbon source, since lipase are inducible enzymes. These enzymes are generally produced in the presence of a lipid such as oil or any other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, Tweens, glycerol and bile salts. However, nitrogen sources and essential micronutrients should also be carefully considered for growth and production optimization (Veerapagu *et al.*, 2013). Considering the important of lipase enzyme, the present study was aimed to isolate, identification and immobilize lipase producing bacteria from different sources of oil contaminant soil and study the activity of these isolates. This study therefore aimed at production of bacterial lipase from immobilized bacteria organisms and subsequent use for degradation of Diesel.

### MATERIALS AND METHODS

#### 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 samples were stored at refrigeration temperature before the experimental work.

#### 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.

#### Screening of hydrocarbon degrading bacteria

The isolated bacteria were inoculated on an enrichment medium that contains mineral salt medium (MSM) supplemented with single hydrocarbon compound as sole carbon source (1% petrol and diesel). The MSM composition was made up of basal salt medium and trace element solution. The basal medium contain (g/L): K<sub>2</sub>HPO<sub>4</sub>, 1.8; KH<sub>2</sub>PO<sub>4</sub>, 1.2; NH<sub>4</sub>Cl, 4.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2; NaCl, 0.1; yeast extract, 0.1 and FeCl<sub>2</sub>·4H<sub>2</sub>O, 0.05 and trace elements solution contain: H<sub>3</sub>BO<sub>3</sub>, 0.1; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.1; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.05 and MnSO<sub>4</sub>·H<sub>2</sub>O, 0.04 with the pH of 6.5 (Balogun and Fagade, 2010).

#### Biochemical tests of the isolates

Different types of biochemical tests were done such as Gram's staining, Indole test, Methyl red test, VP test, Citrate utilization test, Urease test, Catalase, Oxidase and Starch hydrolysis (Cheesbrough, 2006).

#### Molecular characterization of isolates

##### Dna extraction

Molecular characterization of isolates was carried out by extracting DNA (1 ml) of bacterial isolate using the method of (Keramas *et al.*, 2004). Sterile distilled water was added into the eppendorf tubes. The bacterial isolates were added and mixed by vortexing. Centrifugation was carried out at 10,000 rpm for 5 min at 4 °C. The supernatant was discarded. 200 µl of sterile distilled water was added and vortex to homogenize the pellets. The tubes containing the homogenized pellets were boiled at 100 °C for 10 minutes. After boiling, the tubes were vortexed again and centrifuged at 10,000 rpm for 5 minutes. The supernatant were transferred into another pre-labeled eppendorf tube by gentle aspiration using a micropipette.

##### Polymerase chain reaction

Fragments of the gene of interest, the 16S ribosomal gene, were amplified using standard PCR protocol and the universal primer. The PCR reaction mixture (20 µl) consisting of 4 µl PCR master mix (Solis Biotec), 0.5 µl of each primer, 14.1 µl nuclease free water and 1.5 µl template DNA (Keramas *et al.*, 2004).

### Agarose gel electrophoresis

Agarose powder of (1.5 g) was added to 150 mls of 0.5x TAE buffer and dissolved by boiling using microwave oven. The mixture was allowed to cool to about 60 °C. Ethidium bromide (10 ml) was added and mixed by swirling gently it was then poured into electrophoresis tank with the comb in place to obtain a gel thickness of about 4-5 mm. The tank was filled with 1x TAE buffer. Thereafter the comb was removed. 10 µl of sample were mixed with 1µl of the 10x loading dye. The samples were carefully loaded into the wells created by the combs. The electrodes were connected to the power pack in such a way that the negative terminal was at the end where the sample was loaded. The electrophoresis was allowed to run at 60- 100 V until the loading dye migrates about three- quarter of the electrodes. Electrodes were turned off and disconnected. The gel was observed on a UV- trans-illuminator (Keramas et al., 2004).

### Screening of the isolates for lipase activity

Lipolytic microorganisms were screened by qualitative plate assay method of Singh et al., 2006. Bacterial strains were grown on nutrient medium substrate containing Tween-80 agar plates and incubated at 37°C for 24 to 48 hours and zone were observed.

### Immobilization of *Bacillus thuringiensis* and *Lysinibacillus sphaericus* within a gelatin matrix

Immobilization of bacteria isolate was done within gelatin matrix using a modified method of Osho et al. (2001). Aqueous solution of gelatin (7-10.0% w/v) was cross linked by adding ethanolic formaldehyde to give a final formaldehyde concentration of 2% (v/v) and incubated for 20 min at 37 °C. *Bacillus sp* were added to the cross-linked gelatin under vigorous stirring at 35 °C. The mixture was poured into a burette and added drop-wise 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 24 h. They were washed with sterile distilled water and stored in the refrigerator at 4 °C. For optimal bead size determination, an aqueous solution of gelatin (8%w/v) with 1ml of the cell culture was entrapped at 35°C 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 (2 ml/min). The gel bead were hardened by suspending in the cross linking agent for 24 h. They were washed with sterile distilled water and stored in the refrigerator at 4 °C prepared.

### Lipase production

Lipase production was carried out in a submerged medium containing peptone 0.2(% w/v);  $\text{NH}_4\text{H}_2\text{PO}_4$  0.1; NaCl 0.25;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.04;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.04; olive oil 2.0 (v/v); pH 7.0; 1-2 drops Tween 20 as emulsifier. Overnight cultures were suspended in 5ml of sterile deionised water and used as the inoculum. Submerged microbial cultures were incubated in 500 ml Erlenmeyer flasks containing 100 ml of liquid medium on a rotary shaker (150 rpm) at 36°C. After 24 hours of incubation, the culture was centrifuged at 10,000 rpm for 20 min at 4°C and the cell free culture supernatant fluid was used as the sources of extracellular enzyme. The lipase activity in the supernatant was determined by the colorimetric method.

### Determination of lipase biodegradation of diesel

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

### Optimization studies on degradation

Optimization studies of the enzymes on degradation of hydrocarbons was done, effect of temperature (20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C), effect of pH (5, 5.5, 6, 6.5, 7, 7.5, 8) and degradation time (5 days, 10 days, 15 days, 20 days). Degradation was carried out using the method described previously in section 2.7.

### 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)

$$\text{Percentage of Diesel oil degraded} = \frac{\text{Weight of Diesel oil degraded}}{\text{Weight of Diesel oil present originally}} \times 100$$

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

### 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).

## RESULTS AND DISCUSSIONS

**Table 1** Biochemical tests of the isolates

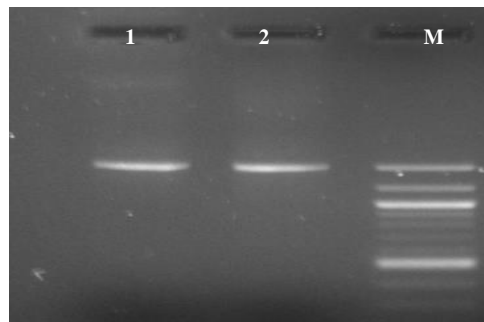
Code	Gram staining	Catalase	Oxidase	Indole	Motility	Methyl Red	Voges P	Citrate	Urease	Starch hydrolysis	Probable organisms
1	GPB	+	-	-	+	-	+	-	-	+	<i>Bacillus thuringiensis</i>
2	GNB	+	+	+	-	-	+	+	+	-	<i>Klebsiella oxytoca</i>
6	GPC	+	+	-	-	-	-	+	+	NA	<i>Staphylococcus aureus</i>
7	GPB	+	-	-	+	-	-	-	+	NA	<i>Corynebacterium striatum</i>
11	GPB	+	-	-	+	-	+	-	-	+	<i>Lysinibacillus sphaericus</i>

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

**Table 2** Primary Screening and enzyme activity of the isolates

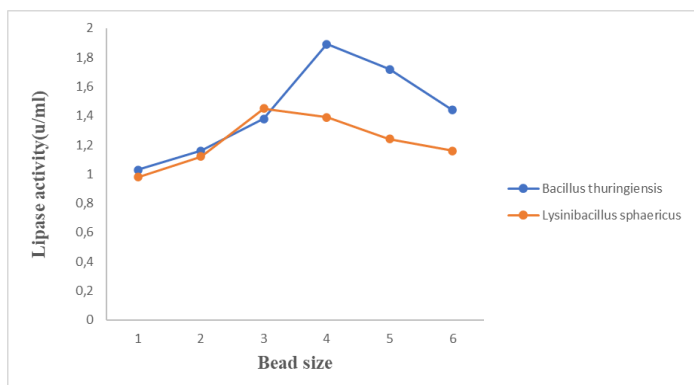
Isolates	Zone of hydrolysis	Enzyme activity(u/ml)
1	5.67±1.45 <sup>cd</sup>	2.63±0.04 <sup>e</sup>
3	4.68±0.20 <sup>cd</sup>	1.53±0.04 <sup>d</sup>
7	1.82±0.11 <sup>a</sup>	1.65±0.03 <sup>c</sup>
10	5.36±0.52 <sup>cd</sup>	1.73±0.04 <sup>d</sup>
11	6.40±0.46 <sup>d</sup>	1.96±0.07 <sup>d</sup>

Legend: - indicates negative reaction

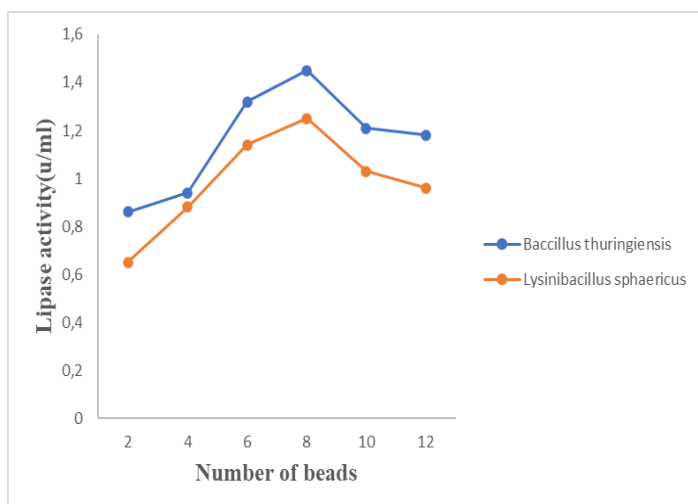


**Plate 1:** Gel electrophoresis of DNA extraction of bacteria isolates  
M- 100bp marker

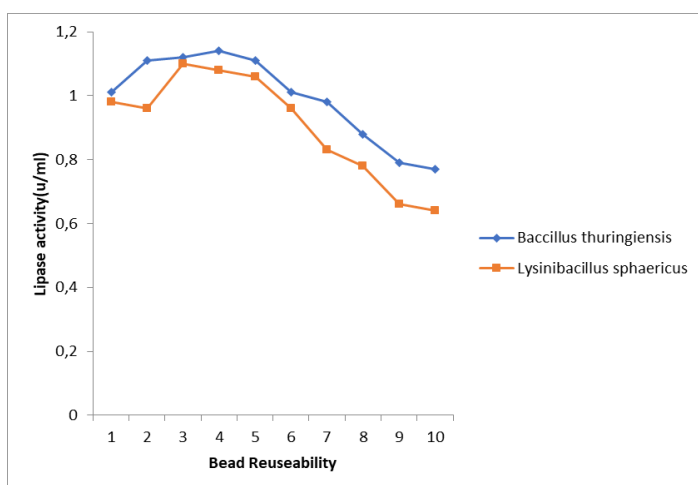
1- Sample 1- Sequencing result showed it to be *Bacillus thuringiensis*  
2- Sample 2- Sequencing result showed it to be *Lysinibacillus sphaericus*



**Figure 4** Effect of bead size on lipase activity by immobilized *Bacillus thuringiensis* and *Lysinibacillus sphaericus*



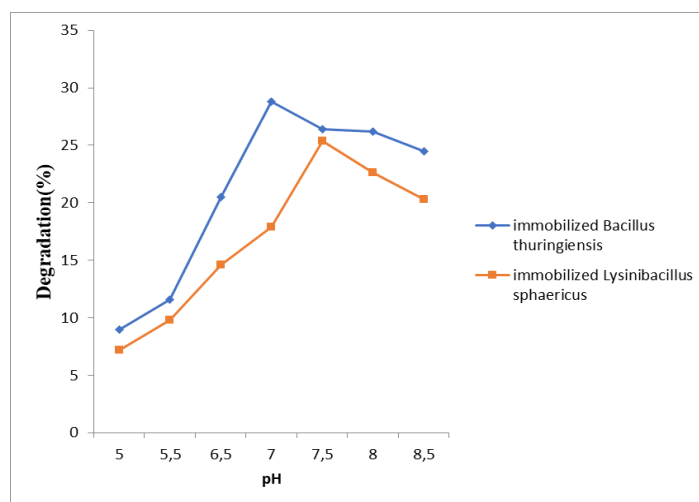
**Figure 5** Effect of number of beads on lipase activity by immobilized *Bacillus thuringiensis* and *Lysinibacillus sphaericus*



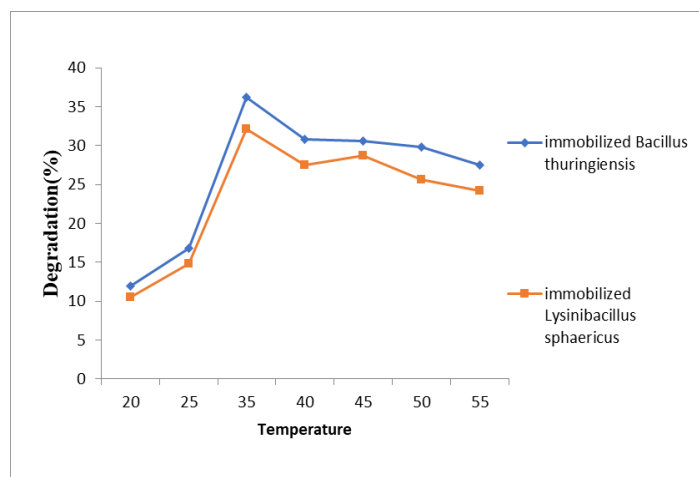
**Figure 6** Bead reusability on lipase activity by immobilized *Bacillus thuringiensis* and *Lysinibacillus sphaericus*

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

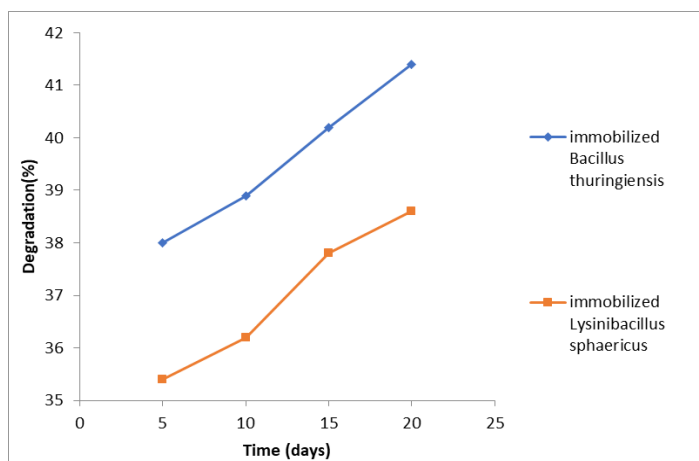
Test enzyme + Hydrocarbons	<i>Bacillus thuringiensis</i>		<i>Lysinibacillus sphaericus</i>	
	OD	Oil consumption (%)	OD	Oil consumption (%)
Free lipase + Diesel	0.52	38.2	0.32	26.8
Immobilized Lipase + Diesel	0.52	41.4	0.44	38.6
Diesel with no enzyme(Control)	1.82	0.0	1.82	0.0



**Figure 8** Effect of pH on degradation of Diesel using lipase from immobilized cells of *Bacillus thuringiensis* and *Lysinibacillus sphaericus*



**Figure 10:** Effect of temperature on degradation of Diesel using lipase from immobilized cells of *Bacillus thuringiensis* and *Lysinibacillus sphaericus*

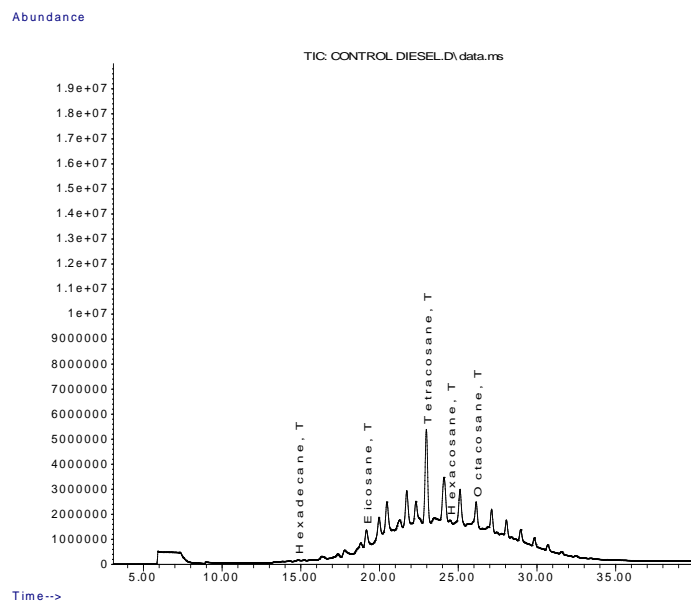


**Figure 11** Effect of degradation time on degradation of Diesel using free lipase and lipase from immobilized cells of *Bacillus thuringiensis* and *Lysinibacillus sphaericus*

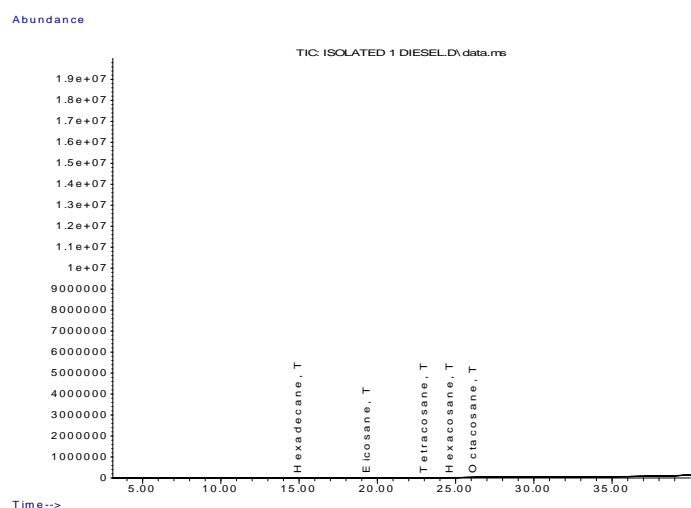
**Table 4** GC-MS results on degradation of Diesel by lipase produced from immobilized cells of *Bacillus thuringiensis* and *Lysinibacillus sphaericus*

	Compound	R.T(min)	Area	PPM	Area (%)
Control	Hexadecane	14.828	136545	21.78	100
	Eicosane	19.171	1487708	151.23	100
	Tetracosane	22.977	7541703	685.45	100
	Hexacosane	24.487	63934	5.54	100
	Octacosane	26.152	2108528	172.87	100
<i>Bacillus thuringiensis</i>	Hexadecane	14.811	84	0.01	0.06
	Eicosane	19.194	222	0.02	0.01
	Tetracosane	22.862	232	0.02	0.003
	Hexacosane	24.499	208	0.02	0.33
	Octacosane	26.992	350	0.03	0.01
<i>Lysinibacillus sphaericus</i>	Hexadecane	14.788	609	0.10	0.45
	Eicosane	19.103	656421	66.73	44
	Tetracosane	22.862	3685954	335.01	49
	Hexacosane	24.395	28777	2.49	45
	Octacosane	26.055	1026102	84.13	48

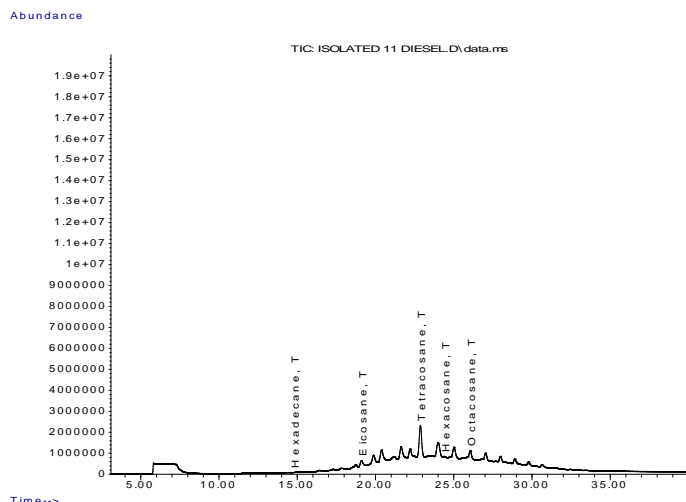
**Key:** R.T- Retention time, PPM- Part per million



**Figure 12** GC-MS of Diesel without enzyme as the control



**Figure 13** GC-MS result of Diesel degraded by lipase produced from immobilized *Bacillus thuringiensis*



**Figure 14** GC-MS result of diesel degraded by lipase from immobilized *Lysinibacillus sphaericus*

## DISCUSSION

Many bacterial species are present in the soil normally and adapting to the soil conditions and almost the bacteria that degrade the oil present in the soil contaminated with oil, which help in cleaning the soil from oil products (Cesarini et al., 2014). Prominent among the bacteria found in the oil contaminated soil is *Bacillus sp.*, this agrees with earlier reports by Kumar et al., (2012) stated that *Bacillus sp.*, *Pseudomonas sp.*, *Micrococcus*, *Aeromonas sp.* are among bacteria found in oil contaminated areas. The biochemical of the various bacterial isolates was shown in Table 1. Dominant bacterial cultures were inoculated in mineral salts broth (MSB) medium with 1% Diesel as a carbon source to determine their biodegradative ability; all the isolates were able to utilize the crude oil as their carbon source. This corresponds to the findings of Latha and Kalaivani (2012) where the isolates were able to utilize hydrocarbons- as their carbon source. A total of 5 bacterial colonies were selected and isolated. Only two out of five 5 isolates showed good clear zone in the olive agar medium with phenol red as indicator. Plate 1 shows gel electrophoresis of DNA extraction of bacteria isolates using 100bp marker. The nucleotide sequence was 99% identical to *Bacillus thuringiensis* strain VITSJ-01 and *Lysinibacillus sphaericus* strain 2362. In the screening medium, *Bacillus thuringiensis* shows maximum lipase production which produced 2.6 U/mL and *Lysinibacillus sphaericus* at 1.96U/ml was selected for further research while others showed less than 1.96 U/mL, (table 2). It was reported that maximum lipase production was at 72 hours for *Bacillus coagulans* (PrasanthKumar et al., 2007). *Bacillus thuringiensis* and *Lysinibacillus sphaericus* were immobilized on solid surfaces using gelatin matrix. Effect of varying bead size (1, 2, 3, 4, 5 and 6) on lipase production by immobilized bacteria cell is shown in Figure 1. Lipase activity increased gradually and further increased in bead size beyond 4 lead to decrease in lipase activity (Figure 4). Maximum lipase activity was achieved with immobilized *B. thuringiensis* (1.89U/ml) and *Lysinibacillus sphaericus* (1.45U/ml). Effect of varying bead number (5, 10, 15, 20 and 30) on lipase production by immobilized -bacteria cell is shown in Figure 1. Lipase activity increased gradually and further increased in bead number beyond 8 lead to decrease in lipase activity (Figure 5). Maximum lipase activity was achieved with immobilized *B. thuringiensis* (1.45 U/ml) and *Lysinibacillus sphaericus* (1.25 U/ml). Effect of varying bead reuseability on lipase production by immobilized bacteria cell is shown in Figure 2. Lipase activity increased gradually and further increased in bead reuseability beyond 4 lead to decrease in lipase activity (Figure 6). Maximum lipase activity was achieved with immobilized *B. thuringiensis* (1.14 U/ml) and *Lysinibacillus sphaericus* (1.11 U/ml). Contrary, Hung and coworkers, 2003; Won and coworkers, 2005) found that the immobilized lipase

of *C. rugosa* entrapped in Ca-alginate gel beads retained 72% after three uses, also found that the repeated use for immobilized *C. rugosa* lipase on chitosan retained 74% after 10 reuses. Similarly Yi and coworkers (2009) showed that the activity of immobilized lipase of *C. rugosa* on alanine chitosan beads retained 77% of the initial activity after 10 times of reuse.

Bacterial strain isolated in this study was identified among hydrocarbon degrading microorganisms for crude oil. The results obtained clearly showed that lipase produced from this microorganism had biodegradable abilities and values of degraded Diesel varied after incubation at 20 days. Table 3 presented degradation of Diesel with immobilized lipase of *Bacillus thuringiensis* and *Lysinibacillus sphaericus*. Lipase from Immobilized *Bacillus thuringiensis* degraded 41.4% Diesel and *Lysinibacillus sphaericus* 38.6% Diesel. The result of pH on degradation of Diesel showed that degradation increased progressively with increase in pH from 5-8.5 reaching a maximum at 7 for Diesel degradation from lipase of immobilized *Bacillus thuringiensis* and *Lysinibacillus sphaericus* (figure 1). Gupta et al., (2004), states that maximum activity of lipases at pH values higher than 7 has been observed in many cases. Effect of temperature on lipase activity of the crude enzyme on degradation of Diesel showed that degradation of Diesel increased progressively with increase in temperature from 20 °C reaching a maximum at 35 °C for lipase of *Bacillus thuringiensis* and *Lysinibacillus sphaericus* (figure 2). An optimum temperature of 40 °C for phenanthrene degradation was reported by Stringfellow and Aitken (1994). Effect of degradation time on lipase activity of the crude enzyme on degradation of Diesel was presented in figure 3, an increase in degradation was observed along with the increase in time from 5 to 20 days. Siddiqui et al., (2001) reported the percentage degradation for individual *n*-alkanes increased with time and in the range of 43.5 - 53.9% on 7th day. The result of the GC-MS analysis of Diesel as presented in Table 4 showed 99.94% and 99.45% reduction in Hexadecane by lipases from Immobilized *Bacillus thuringiensis* and *Lysinibacillus sphaericus*. Maximum degradation of Diesel from lipase of Immobilized *Bacillus thuringiensis* and *Lysinibacillus sphaericus* was at 20 days. In contrary, Verma et al., (2006) tested the ability of *Bacillus sp.* SV9 to degrade *n*-alkanes fraction of oily sludge and reported that *Bacillus sp.* SV9 was able to degrade 88.9±1.24% of C<sub>12</sub>-C<sub>30</sub> *n*-alkanes in 5 days.

## CONCLUSION

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

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