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Biodegradation of premium motor spirit (PMS) by lipase from *Bacillus thuringiensis* and *Lysinibacillus sphaericus*

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Abstract

This study reported production of lipase by *Bacillus thuringiensis* and *Lysinibacillus sphaericus*. Bacteria isolates were screened on Bushnell-Hass Mineral Salt medium containing 1% PMS for oil degradation. Two potent isolates were identified using 16S rRNA as *Bacillus thuringiensis* and *Lysinibacillus sphaericus*. They were cultured for lipase production in a submerged medium. The crude lipases extracted were used for degradation of PMS. Optimum degradation of PMS 44.5% and 37.4% were obtained by lipase from *Bacillus thuringiensis* and *Lysinibacillus sphaericus* respectively at pH 7 and 35 in 20 days. This study therefore presented the use of bacterial lipase in degradation of PMS as a simple and effective approach.

Keywords: *Bacillus* spp., biodegradation, lipase, PMS

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Introduction

Lipases (EC 3.1.1.3; triacylglycerol acylhydrolases) are hydrolases which catalyze the hydrolysis of carboxylic ester bonds to liberate glycerol and free fatty acids (Dhima and Chapadgaonkar, 2013). Lipases are ubiquitous in microbial world and are mainly produced by bacteria, fungi and actinomycetes. The extracellular bacterial lipases are of considerable importance commercially as their bulk production and recovery is much easier than from fungi and actinomyces (Bhosale et al., 2012).

Lipases occur widely in nature, but only microbial lipases are commercially significant. They can catalyze reactions of insoluble substrates at the lipid-water interface, preserving their catalytic activity in organic solvents (Gupta et al., 2004). Different bacterial species especially *Bacillus* sp were applied in bioremediation of crude oil and clean soil from this oil, the lipase produced from this bacterial plays a crucial role in biodegradation of crude oil (Chuks et al., 2008).

Many microorganisms are known as potential producers of extracellular lipases, including bacteria, yeast, and fungi (Abada, 2008). Most common bacterial lipases are reportedly obtained from *Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis, Bacillus coagulans, Bacillus stearothermophilus*, and *Bacillus alcalophilus* (Bhosale et al., 2012). In addition, *Pseudomonas sp., Pseudomonas aeruginosa, Burkholderia multivorans, Burkholderia cepacia,* and *Staphylococcus caseolyticus* are also reported as bacterial lipase producers (Ertugrul et al., 2007). Most commercially important lipase-producing fungi belonged to the genera *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., Geotrichum sp., *Mucor* sp., and *Rhizomucor* sp.

Lipase-producing microorganisms have been found in different habitats such as industrial wastes, vegetable oil processing factories, dairy plants, and soil contaminated with oil and oilseeds among others (Sharma et al., 2001). Microbial lipases have high biotechnological applications (Chirajyoti et al., 2006). It becomes important biocatalyst in various industrial sectors such as dairy and food industries for cheese ripening flavor enhancement and hydrolysis of milk fat,

and lipolysis of cream and butter fat (Feng et al., 2010).

Other applications include paper, pharmaceutical, cosmetics, detergent, leather, single cell protein production of fine chemicals, waste water treatment, bakery products, and biofuels industries (Bhavani et al., 2012).

The direct use of microorganisms in bioremediation of oil polluted sites may be technically difficult, hence the need to focus on microbial enzymes is considered as alternative. This study therefore aimed at production of bacterial lipase and subsequent use for degradation of petrol.

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 sample was 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_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).

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 ℃. 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 ℃ 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 Biodyne)), 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 60ºC. Ethodium 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 gown on nutrient medium substrate containing Tween-80 agar plates and incubated at 37°C for 24 to 48 hours and zone were observed.

Lipase Production

Lipase production was carried out in a submerged medium containing peptone 0.2(%w/v); $NH_4H_2PO_4$ 0.1; NaCl 0.25; $MgSO_4.7H_2O$ 0.04; $CaCl_2._2H_2O$ 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 4ºC 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 PMS

The degrading activities of each enzyme were obtained 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 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 ºC, 25ºC, 30ºC, 35ºC, 40ºC, 45ºC, 50ºC), effect of pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) 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)

$$\frac{\text{Percentage of PMS oil degraded}}{\text{Weight of PMS oil degraded} \times 100} =$$

Weight of PMS oil present originally

Where, the weight of PMS oil degraded (= original weight of PMS oil – weight of residual PMS 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 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* spp. This agrees with earlier reports by Kumar et al. (2012) which stated that *Bacillus* sp. *Pseudomonas* sp. *Micrococcus, Aeromonas* spp. are among bacteria found in oil-contaminated areas.

The biochemical properties of the various bacterial isolates was shown in Table 1. Dominant bacterial cultures were inoculated in mineral salts broth (MSB) medium with 1% PMS 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 utilized 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. Their nucleotide sequences were 97% and 99% identical to *Lysinibacillus sphaericus* strain 2362 and *Bacillus thuringiensis* strain VITSJ-01 respectively.

In the screening medium, *Bacillus thuringensis* shows maximum lipase production which produced 2.6 u/ml followed by *Lysinibacillus sphaericus* which produced 1.96 u/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). 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 PMS varied after incubation at 20 days.

Table 3 presented degradation of PMS with free lipase of *Bacillus thuringiensis* and *Lysinibacillus sphaericus*. Lipase from *Bacillus thuringiensis* degraded 44.5% PMS while free lipase from *Lysinibacillus sphaericus* degraded 37.4% PMS. The result of pH on degradation of PMS showed that degradation increased progressively with increase in pH from 5-8.5 reaching a maximum at 7 for PMS degradation from lipase of *Bacillus thuringiensis* and *Lysinibacillus sphaericus* (figure 1). A comprehensive review of all bacterial lipase done by 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 PMS showed that degradation of PMS increased progressively with increase in temperature from 20oC reaching a maximum at 35oC for lipase of *Bacillus thuringiensis* and 40 ºC for *Lysinibacillus sphaericus* (Figure 2).

An optimum temperature of 40ºC for phenantheren degradation was reported by Stringfellow and Aitken (1994). Effect of degradation time on lipase activity of the crude enzyme on degradation of PMS 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 PMS as presented in Table 4 showed 97.8% and 99.3% reduction in Hexadecane by lipases from *Bacillus thuringiensis* and *Lysinibacillus sphaericus* respectively. Maximum degradation of PMS from lipase of *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_{12}-C_{30} n-alkanes in 5 days.

Conclusion

In this study, lipase from *Bacillus thurigiensis* shows a better degradation of PMS than lipase from *Lysinibacillus sphaericus*. It can be concluded that enzymatic degradation of PMS using bacterial lipase is an effective and eco-friendly biotechnological approach.

Table 1: Biochemical tests of the isolates

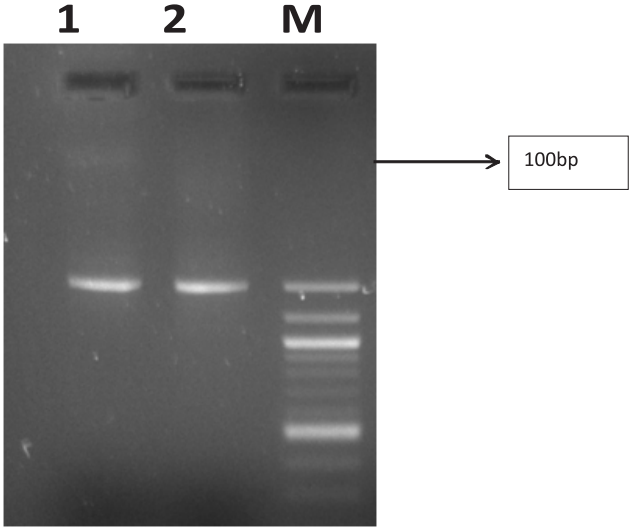
Code		Catalase	Oxidase	Indole	Motility	Methyl Red	Voges P	Citrate	Urease	Starch hydrolysis	Probable organisms
1	GPB	+	-	-	+	-	+	-	-	+	*Bacillus thuringiensis*
2	GNB	+	+	+	-	-	+	+	+	-	*Klebsiella oxytoca*
6	GPC	+	+	-	-	-	-	+	+	NA	*Staphylococcus aureus*
7	GPB	+	-	-	+	-	-	-	+	NA	*Corynebacterium striatum*
11	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 (mm)	Enzyme activity(U/ml)
1	5.67 ± 1.45^{cd}	2.63 ± 0.04^{e}
3	4.68 ± 0.20^{cd}	1.53 ± 0.04^{d}
7	1.82 ± 0.11^{a}	1.65 ± 0.03^{c}
10	5.36 ± 0.52^{cd}	1.73 ± 0.04^{d}
11	6.40 ± 0.46^{d}	1.96 ± 0.07^{d}

Key:- indicates negative reaction



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*

Plate 1: Gel electrophoresis of the isolates

Table 3: Biodegradation of PMS by lipase from *Bacillus thuringiensis* and *Lysinibacillus sphaericus*

	Bacillus thuringiensis		*Lysinbacillus sphaericus*	
Test enzyme + Hydrocarbons	OD	Oil consumption (%)	OD	Oil consumption (%)
Free lipase + PMS	0.44	44.5	0.47	37.4
PMS with no enzyme(Control)	1.98	0.0	1.98	0.0

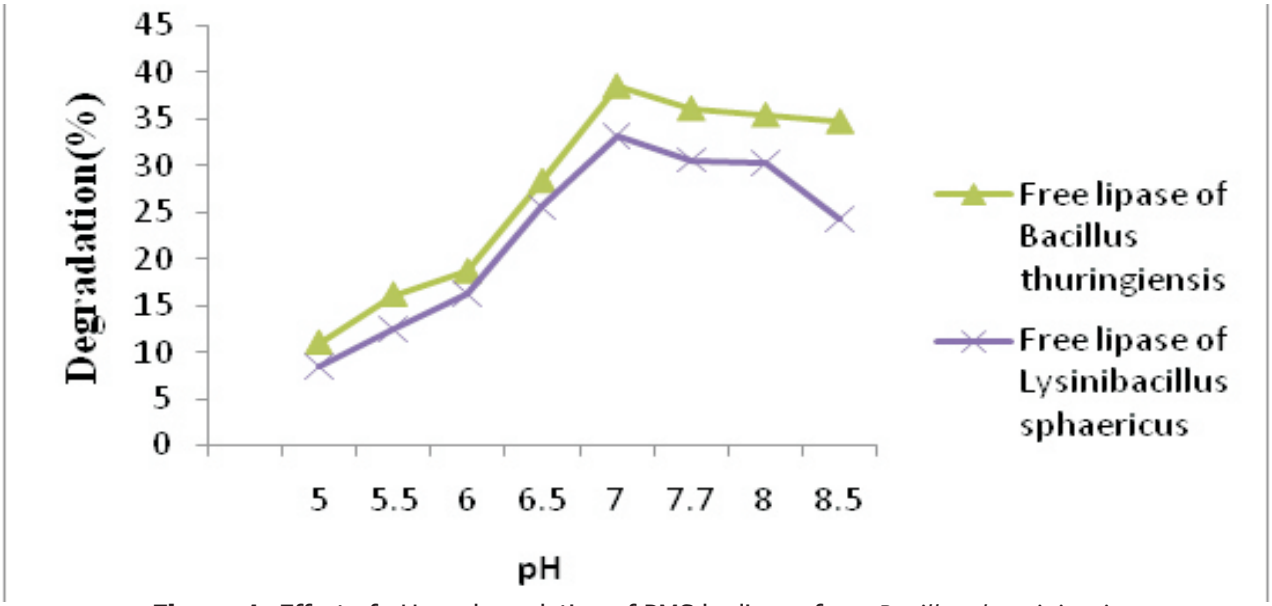


Figure 1: Effect of pH on degradation of PMS by lipase from *Bacillus thunrigiensis and Lysinibacillus sphaericus*

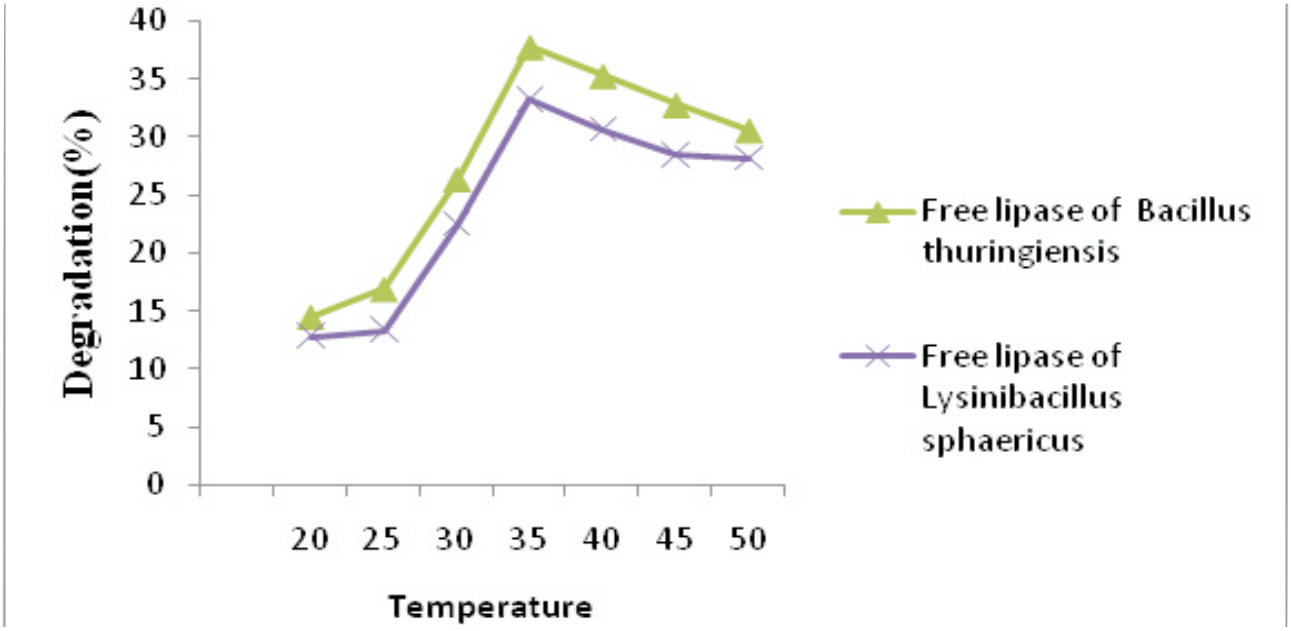


Figure 2: Effect of temperature on degradation of PMS by lipase from *Bacillus thunrigiensis and Lysinibacillus sphaericus*

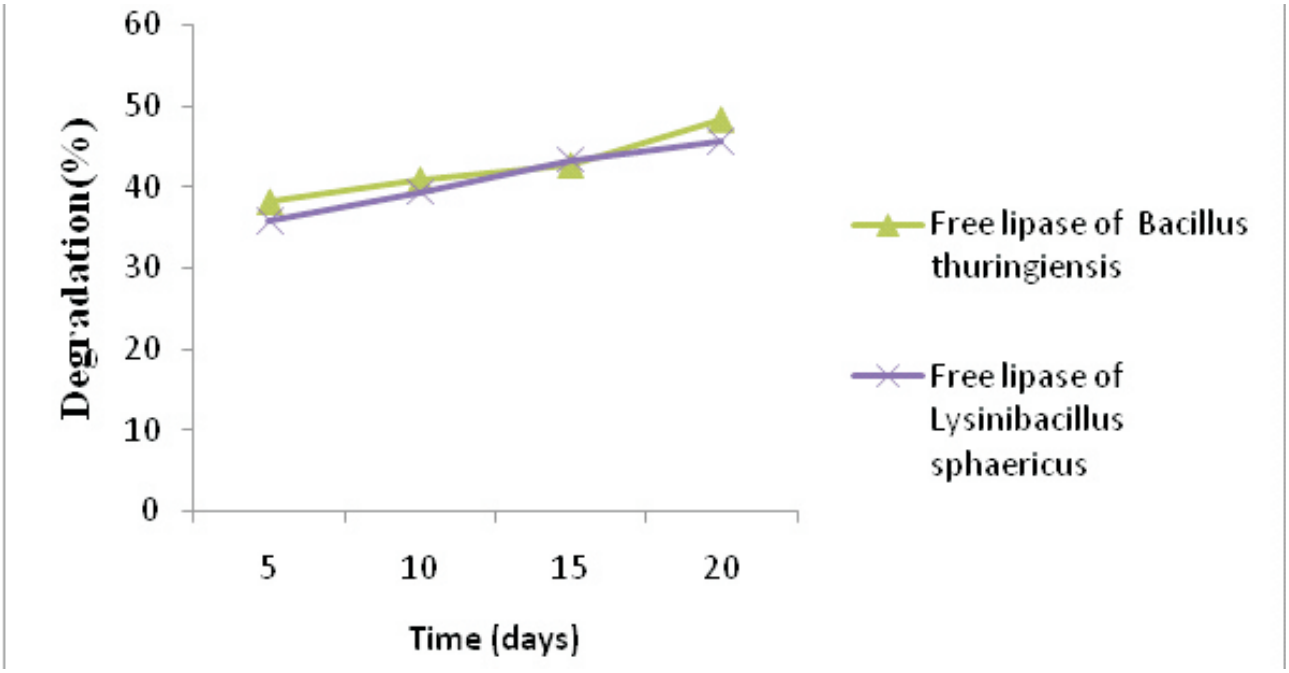


Figure 3: Effect of time on degradation of PMS by lipase from *Bacillus thunrigiensis and Lysinibacillus sphaericus.*

Table 4: GC-MS results on degradation of PMS

Compound	PMS	*Bacillus thurigiensis* lipase (ppm)	*Lysinibacillus sphaericus* lipase(ppm)
Hexadecane	6.71	0.03	0.14
Eicosane	0.08	0.01	0.07
Tetracosane	0.03	0.03	0.03
Hexacosane	0.02	0.03	0.03
Octacosane	0.04	0.00	0.14

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