

Isolation, Identification and Evaluation of Lipase Producing Bacteria for Biodegradation of Lipid-Rich Waste

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Abstract

Lipase catalyzes the hydrolytic degradation of fats and oils. The present research work focused on the isolation, screening of lipase producing bacteria for the degradation of lipid-rich waste from oil mills and dairy. Significant lipase producing strains were screened on tributyrin agar media based on clear zone appearance around the bacterial colonies. Four colonies LPB 2, LPB 4 and LPB 7 and LPB 11 exhibited 8.2 mm, 12.1 mm, 10.7 mm 7.6 mm respectively and identified as *Bacillus* strains (LPB2, LPB4 and LPB7) and *Pseudomonas* strain (LPB11). The degradation efficiency of lipase producing bacteria was estimated in terms of reducing BOD and lipid content of oil mill and dairy waste. Among them, LPB 4 showed maximum efficiency for waste degradation and lower the value of BOD by 42.9% and 47.2% for oil mill waste and dairy waste respectively. LPB4 could also reduce the lipid content by 53.3% and 51.3% for oil mill waste and dairy waste respectively. Lipase was partially purified from all four strains (LPB2, LPB4, LPB7 and LPB11). LPB4 Lipase showed maximum lipase activity in comparison to other three lipases. Maximum rate of degradation of waste was measured for LPB4 Lipase by enzyme kinetics. The V_{max} and K_m of LPB4 Lipase were determined by Lineweaver-burk plot by using oil mill waste and dairy waste as substrate and compared with pure substrate (p-NPP). The K_m value of lipase was 90.9, 23.8 and 19.6 mg for p-NPP, oil mill waste and dairy waste respectively. The V_{max} value of lipase was 24.39, 4.76 and 5.88 mg/min for p-NPP, oil mill waste and dairy waste respectively. The present study suggests that *Bacillus* strain (LPB4) is a good candidate for waste removal.

Keywords: Lipase, V_{max} , K_m , BOD, Lipid Content.

Introduction

Lipids are the esters of fatty acid and glycerol. Oil mills, dairy effluent and municipal waste contains high concentration of lipids (Lefebvre et al., 1998; Wakelin and Forster, 1997). Lipid form oily films on aqueous surfaces causing disruption to oxygen diffusion and promote clogging by emulsification with organic matter. High lipid content in waste affects the biological waste treatment process because it floats on water surfaces and forms a layer that minimizes the rate of oxygen transfer during the aerobic process (Becker et al., 1999). Lipase catalyzes the esterification, inter-esterification, alcoholysis, and aminolysis process (Jaeger and Eggert, 2002; Pandey et al., 1999), thus can hydrolyze lipids into fatty acids and glycerol. Lipase is used in many industrial processes like pharmaceutical (as diagnostic tool), detergent (to remove oil), dairy (for hydrolysis of milk fat), and leather (to remove lipid from the skin), (Das et al., 2016; Ferreira-Dias et al., 2013; Veerapagu et al., 2013). Lipase can be obtained from plants, animals and microorganisms (Sharma et al., 2017). For application in industries lipase produced from microorganisms are preferred because that can be produced in high quantity and many types of lipase suitable for the different process are produced by microorganisms (Akoh et al., 1996). Microbial lipase is more stable and safer than plant and animal-derived lipase (Sharma et al., 2017), and is clean and eco-friendly. Many bacterial strains are known to produce lipase and are prevalent in the ecosystem. Lipase producing Bacteria can be isolated from soil and waste food items containing oil. *Bacillus subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *Serratia marsescens*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were most widely reported for lipase production (Chandra et al., 2020; Prasad and Manjunath, 2011). In the present study identification of potential lipase producing bacteria is performed and its applicability in bioremediation of lipid waste was checked. Since lipase can transform lipid in many ways due to high stability in temperature

and solvents and its broad substrate specificity.

Materials and Methods

Isolation of Lipase Producing Bacteria

To isolate lipase producing Bacteria oily soil from waste dumping sites of Bilaspur City (22.0624° N, 82.1563° E) was collected. The sample was serially diluted and Lipase producing bacterial strains were screened on Tributyrin Agar Media (TAM) consisted of 5.0 g/L peptone, 3.0 g/L yeast extract, 10.0 g/L tributyrin and 15.0 g/L Agar at pH of 7.5. Colonies showing clear zone were picked up and purified on TAM agar plate.

Identification of isolated strains

Bacterial strains forming a clear zone around the colony were selected and pure cultures were preserved (agar slant with 1% olive oil) for further experimental work. Morphological and Biochemical characteristics of bacterial strains were determined and identified as described in Bergey's Manual of Determinative Bacteriology (Malook, 2018).

Assessment of waste degradation

For assessment of waste degradation capabilities of isolated strains, Oil mill wastes were collected from Sirgitti area District Bilaspur and dairy wastes were collected from Gokulnagar Bilaspur. Oil mill and dairy waste (25.0 ml each, separately) were homogenized with distilled water (25.0 ml). 50.0 ml of homogenized waste was inoculated with lipase producing bacterial strains and incubated at 40°C for 15 days. During the incubation period, after every 48 hours sample was withdrawn and estimated its BOD and lipid content.

Determination of BOD

BOD was determined by the method described by Helrich (1990) with slight modification (Chemists. and Helrich, 1990; Jouanneau et al., 2013). Diluted samples (5.0 ml sample in 95 ml distilled water) were taken in a clean BOD bottle. 2.0 ml MnSO₄ and 2.0 ml alkaline iodine-sodium azide solution was added. BOD bottles were capped by stoppers and air bubbles were removed by inverting bottles. After precipitation, 2.0 ml of H₂SO₄ was added, mixed well and incubated for 5 days. Then the starch indicator was added to 2.0 ml sample, titrated with Na₂S₂O₃ until blue color disappeared and the volume of Na₂S₂O₃ was used to calculate BOD of the sample.

Determination of lipid content

Lipid content was estimated by the partition-gravimetric method as described by Kirschman and Pomeroy (1949) (Kirschman and Pomeroy, 1949). The sample was acidified (pH 2.0) with dilute HCl and lipid was repeatedly extracted with 1,1,2-trichloro- trifluoroethane (freon) until the aqueous phase with no oil layer appeared. The solvent was evaporated at 70°C and residual lipid was weighted.

Partial Purification of Lipase

To purify lipase from the isolated strains submerged fermentation method was used. The strains were grown in media containing 3.0 gm/L yeast extract, 3.0 gm/L sucrose, 0.1 gm/L CaSO₄, 0.5 gm/L KH₂PO₄, 0.1 gm/L MgSO₄ · 7H₂O, with 10% dairy or oil waste as substrate and incubated at 40°C for 20 days. The broth was collected at different interval and bacterial cells were removed by centrifugation. The supernatant obtained was concentrated using 10-80 % ammonium sulphate. Fractioned samples were subjected to dialysis for partial purification. Protein concentration was measured spectrophotometrically by Lowry method (LOWRY et al., 1951).

Lipase assay

Lipase activity was checked by spectrophotometric method as described by Ghori et al. (2011) (Ghori et al., 2011) with modification (Ghori et al., 2011). For lipase assay, 5.0 mL Lipase (0.2 mg/mL protein) was mixed with 45.0 mL phosphate buffer (pH 8.0) and incubated at 37°C for 15 min followed by addition of 25 ml of substrate (8 mM, p-nitrophenyl laurate; pNPL in isopropanol). The reaction mixture was incubated at 37°C for 15 min. after incubation the reaction was stopped by adding 0.5 mL HCl (3.0 M). The reaction mixture was centrifuged at 10000 rpm for 20 minutes and the supernatant was collected. 1.0 ml of NaOH (2.0 M) was

added to the supernatant to make the pH alkaline and absorbance was measured at 420 nm. Unit enzyme activity was calculated as the amount of enzyme required to release 1 mM of pNP from pNPL in one minute.

Effect of pH and temperature on Lipase activity

To check the effect of pH on the lipase activity, an assay was performed as described above in buffer with pH range of 4 to 11. Similarly to assess the effect of temperature on activity incubation was done at different temperatures ranging from 10°C to 70°C.

Determination of Km and Vmax of Lipase

To determine the K_m and V_{max} of lipase, lipase assay was performed at different concentrations (5 mg to 40mg) of substrate p-nitrophenyl laurate, oil mill waste and dairy waste (for waste equivalent lipid content were taken as substrate). K_m and V_{max} were determined by Lineweaver-Burk plot method described by Ghori et al. (2011) with slight modification (Ghori et al., 2011).

Mutagenic studies

For mutagenic studies the strains were grown in presence of chemical mutagens. Ultraviolet (UV), N-methyl-N'-nitro-N-nitrosoguanidine (NTG), N-methyl-N-nitrosourea (NMU) and Nitrous acid (HNO_2) (M. Faisal, 2013; Sidorkina et al., 1997) were used as mutagenic agent. The Lethality of Mutagens on *Bacillus* strain. 1, *Bacillus* strain. 2, *Bacillus* strain. 3 and *Pseudomonas* strain were evaluated. The each strain was grown and the 24 h old culture was centrifuged at $5,000 \times g$ for 5.0 min. The cells were washed twice using 5% sodium chloride. Washed cells were transferred to a fresh medium consisted mutagens viz., UV, NTG, NMU and HNO_2 at different concentration and incubated at 120 rpm for 24 h. Samples were withdrawn and washed with 5% sodium chloride. UV light exposure was given by UV lamp (Philips UVC lamp, 15W) for 10 min (Lin and Wang, 2001). NTG and NMU were used at a concentration of 0.5–1.0 mg/ml for 30 min at pH between 6.0–8.0 (Bharat Bhushan Chattoo and Sinha, 1974). HNO_2 was used at a concentration of 0.05M for 10 min .

The survivability of cells was calculated with formula:

$$\text{Survivability} = \frac{\text{The ratio of viable count of the sample}}{\text{The viable count of untreated control suspension}}$$

Results and discussion

To isolate lipase producing bacteria muddy soil of municipal waste was collected, diluted and plated on TAM plate. Total of nineteen different bacterial strains were obtained (named LPB1 to LPB19) from the municipal waste and eight bacterial strains formed a clear zone on TAM plate. Out of the eight strains, four strains LPB 2, LPB 4 (Figure 1), LPB 7 and LPB 11 showed large clear zones of 8.2 ± 0.73 mm, 12.1 ± 0.82 mm, 10.7 ± 0.64 mm 7.6 ± 0.59 mm respectively. The four strains were subjected to biochemical analysis. Three strains LPB 2, LPB 4, LPB 7 had characteristics similar to the *Bacillus* strains and named *Bacillus* strain 1, *Bacillus* strain 2 and *Bacillus* strain 3 respectively, LPB11 had characteristics of *Pseudomonas* strain (**Table No. 1**).



Figure: 1: Lipolytic activity of LPB4 on Trybutyrin agar plate. LPB4 was streaked onto the TAM plate and incubated. Clear zone around bacteria showed the lipolytic activity of bacteria.

Table 1: Biochemical Characterization of isolated strains

Characteristics	LPB2	LPB4	LPB 7	LPB11
Colony color	Whitish to light brown	White	Cream to yellowish	Cream
Colony size(mm)	3-4	2-3	0.8-2	0.7-2.5
Shape	Irregular	Irregular	Circular to Irregular	Irregular
Margin	Undulate to fimbriate	Undulate	Entire	Entire
Elevation	Umbonate	Raised	Raised	Convex
Gram stain	+	+	+	-
Spore	+	+	+	-
Motility	+	+	+	+
Indole	+	-	+	-
Voges-Proskauer	-	+	+	-
Methyl Red	-	-	-	-
Nitrate Reductase	-	+	+	+
Gelatin Liquefaction	+	+		+
Citrate utilization	-	-	+	+
Catalase	-	+	+	-

Oxidase	+	+	+	+
Carbohydrate Fermentation (Acid) - Glucose	+	+	+	+
Carbohydrate Fermentation (Gas) - Glucose	-	+	-	-
Urea Hydrolysis	-	-	+	-

To estimate the waste degradation capability of the strains biochemical parameters of the waste were measured. The pH of Oil mill waste and dairy waste was 6.5 ± 0.3 and 6.9 ± 0.4 respectively. The initial estimated BOD of oil mill waste and dairy waste was 3724.32 and 2341.38 mg/L respectively (**Table No. 2**). After treating the oil mill waste and dairy waste with Bacterial strains the value of BOD got reduced significantly. By treating the oil mill waste with *Bacillus* strain 1, *Bacillus* strain 2, *Bacillus* strain 3, and *Pseudomonas* strain the value of BOD reduced to 3123.38, 2124.32, 2987.22 and 2465.42 mg/L respectively (**Table No. 2, Figure 2a**) from its initial value. Similarly, by treating the dairy waste with *Bacillus* strain 1, *Bacillus* strain 2, *Bacillus* strain 3, and *Pseudomonas* strain the value of BOD reduced to 2023.45, 1234.32, 1865.21 and 1554.42 mg/L (**Table No. 2, Figure 2a**) respectively from its initial value. After treating the waste samples with bacterial strains BOD of waste samples got reduced significantly. By treating the waste sample with *Bacillus* strain 1, *Bacillus* strain 2, *Bacillus* strain 3 and *Pseudomonas* strain, BOD of oil mill waste reduced by 16.04%, 42.9%, 19.7% and 37.73% respectively (Table No. 2, Figure 2b). Similarly, BOD of dairy waste got reduced to 13.54%, 47.26%, 20.3% and 33.58% respectively (Table No. 2, Figure 2b). Data obtained suggest that *Bacillus* strain 2 have maximum waste degrading capabilities followed by *Pseudomonas* strain (Figure 2b).

Table No. 2. BOD reduction by bacterial isolates.

S. No.	Bacterial Strains	BOD (mg/L)		BOD reduction (mg/L)		BOD reduction percentage (%)	
		Oil mill	Dairy waste	Oil mill	Dairy waste	Oil mill	Dairy waste
1	No strain	3720.32	2340.38	--	--	--	--
2	<i>Bacillus</i> strain. 1	3123.38	2023.45	596.94	316.93	16.04	13.54
3	<i>Bacillus</i> strain. 2	2124.32	1234.32	1596	1106.06	42.90	47.26
4	<i>Bacillus</i> strain. 3	2987.22	1865.21	733.1	475.17	19.70	20.30
5	<i>Pseudomonas</i> strain.	2465.42	1554.42	1254.9	785.96	33.73	33.58

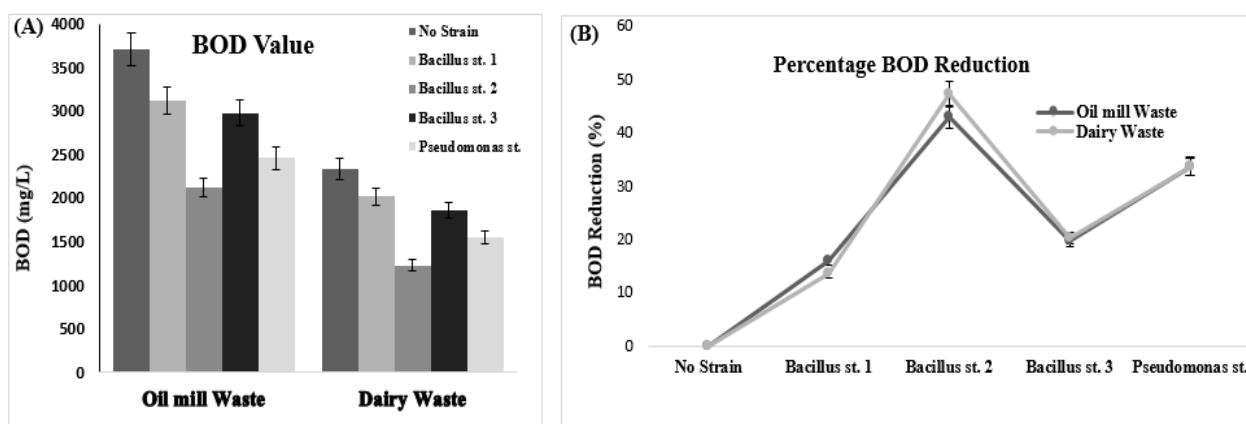


Figure 2: Comparative BOD reduction capabilities of isolated strains. (A) Dairy waste and oil mill waste were treated with isolated strains and BOD was estimated. (B) Percentage reduction of BOD was measured from the untreated sample.

The initial estimated lipid content of oil mill waste and dairy waste was 15726.86, and 14840.61 mg/L (Table No. 3) respectively. After treating the oil mill waste with *Bacillus* strain 1, *Bacillus* strain 2, *Bacillus* strain 3, and *Pseudomonas* strain the value of lipid content reduced to 11234.1, 7340.14, 9876.32 and 8124.78 mg/L (Table No. 3, Figure 3a) respectively from its initial value. Similarly, by treating the dairy waste with *Bacillus* strain 1, *Bacillus* strain 2, *Bacillus* strain 3, and *Pseudomonas* strain the value of lipid content reduced to 9872.74, 7216.12, 9124.25 and 7981.23 mg/L (Table No. 3, Figure 3a) respectively from its initial value. After treating the oil mill waste with *Bacillus* strain 1, *Bacillus* strain 2, *Bacillus* strain 3, and *Pseudomonas* strain the value of lipid content reduced by 28.54%, 53.31%, 37.17% and 48.32% respectively (Table No. 3, Figure 3b). Value of lipid content of dairy waste after treatment got reduced by 33.47%, 51.37%, 38.51% and 46.22% respectively (Table No. 3, Figure 3b).. Similar to BOD reducing capability the lipid content degrading capability was maximum in *Bacillus* strain 2 followed by *Pseudomonas* strain (Figure 3b). Therefore *Bacillus* strain 2 and *Pseudomonas* strain are good candidates against lipid waste.

Table No. 3. Lipid Content reduction by bacterial isolates.

S. No.	Bacterial Strains	Lipid Content (mg/L)		Lipid Content reduction (mg/L)		Lipid Content reduction percentage (%)	
		Oil mill	Dairy waste	Oil mill	Dairy waste	Oil mill	Dairy waste
1	No strain	15720.86	14840.61	--	--	--	--
2	<i>Bacillus</i> strain. 1	11234.1	9872.74	4486.78	4967.86	28.54	33.47
3	<i>Bacillus</i> strain. 2	7340.14	7216.12	8380.76	7624.48	53.31	51.37
4	<i>Bacillus</i> strain. 3	9876.32	9124.25	5844.58	5716.35	37.17	38.51
5	<i>Pseudomonas</i> strain.	8124.78	7981.23	7596.12	6859.37	48.32	46.22

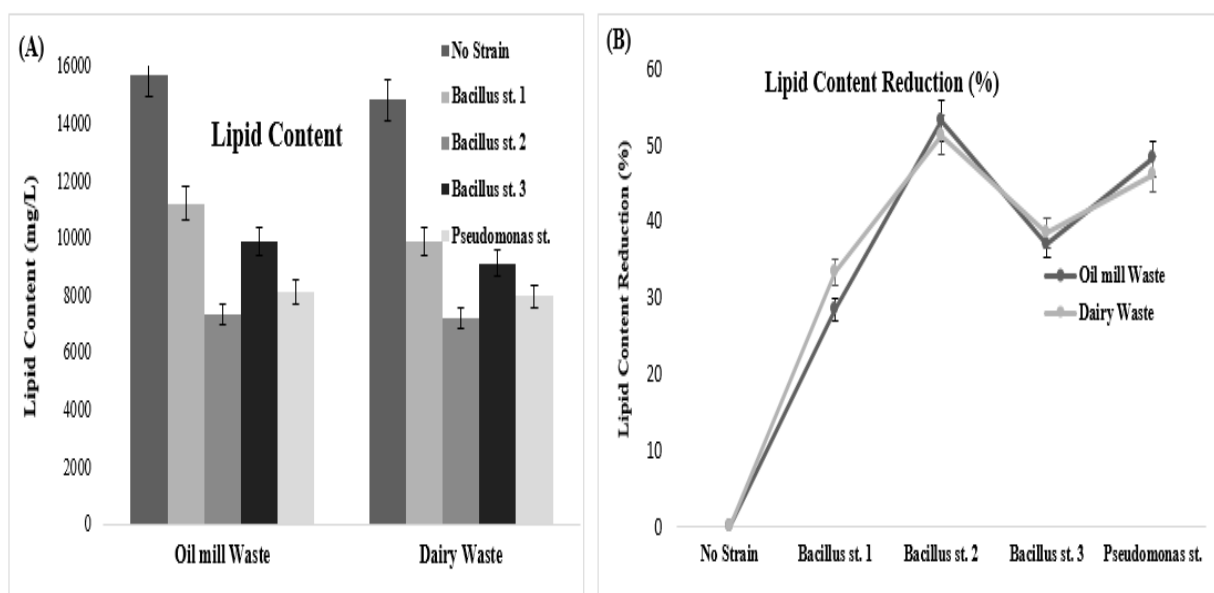


Figure 3: Comparative lipid content reduction capabilities of isolated strains. (A) Dairy waste and oil mill waste were treated with isolated strains and lipid content was estimated. **(B)** Percentage reduction of lipid contents were measured from the untreated sample

To check the lipase activity, partial purification was performed and lipase activity was determined of all four strains by the method described above. Maximum lipase activity was found in the protein of *Bacillus* strain 2 followed by *Pseudomonas* strain. Lipase activity of *Bacillus* strain 1, *Bacillus* strain 2, *Bacillus* strain 3, and *Pseudomonas* strain was 8.2, 14.1, 8.7 and 11.6 U (mg/min) respectively (Table No. 4). Lipase (Partially purified) obtained from *Bacillus* strain 2 showed maximum lipase activity, 21.5% higher than the nearest Lipase (Partially purified) isolated from *Pseudomonas* strain. Therefore, further study was performed for Lipase (Partially purified) isolated from *Bacillus* strain 2.

Table 4: Relative lipase activity of partially purified lipases

S.No.	Bacterial isolates	Identified as	Qualitative Lipase activity (mm)
1	LPB 2	<i>Bacillus</i> strain. 1	8.2 ±0.73
2	LPB 4	<i>Bacillus</i> strain. 2	14.1 ±0.82
3	LPB 7	<i>Bacillus</i> strain. 3	8.7 ±0.64
4	LPB 11	<i>Pseudomonas</i> strain.	11.6 ±0.59

Partially purified protein of *Bacillus* strain 2 showed maximum lipase activity in comparison to other strain. *Bacillus* strain 2 showed activity in pH 4.0-11.0, maximum activity was observed at pH 8.0 and 9.0 (**Figure 4**). Similarly, the activity in different temperature was checked, maximum activity was observed at 40°C (**Figure 5**).

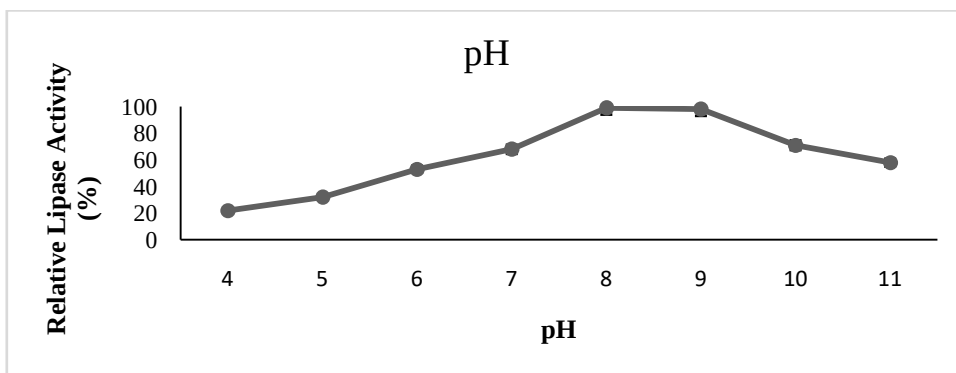


Figure 4: Determination of optimum pH of LPB4 Lipase

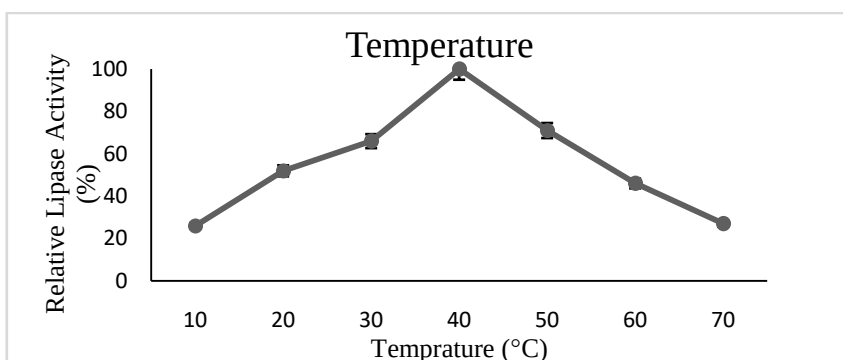


Figure 5: Determination of optimum temperature of LPB4 Lipase.

After getting the optimum pH and temperature V_{max} and K_m were determined by using p-NPP and lipid content equivalent of oil mill waste and dairy waste. V_{max} and K_m of lipase obtained from *Bacillus* strain 2 were calculated by the Lineweaver-Burk plot method. The lipase activity was measured at optimal pH and temperature by using p-NPP and lipid equivalent of oil mill waste and dairy waste. The K_m value of lipase was 90.9, 23.8 and 19.6mg for p-NPP, oil mill waste and dairy waste respectively (Table 5, Figure 6a, 6b, 6c). The V_{max} value of lipase was 24.39, 4.76 and 5.88 mg min^{-1} for p-NPP, oil mill waste and dairy waste respectively (Table 5, Figure 6a, 6b, 6c). V_{max} and K_m both got reduced when wastes were used as substrate, suggest that the waste contains lipase inhibitor. The value of V_{max} and K_m got reduced by 73.8% and 80.4% respectively for oil mill waste and 78.4% and 75.8% for dairy waste. The reduction in V_{max} and K_m suggests the presence of an Un-competitive inhibitor in waste samples.

Table 5: V_{max} and K_m of LPB4 lipase for defferent substarte

Substrate	V_{max}	K_m
p-NPP	24.39	90.9
Dairy waste (Lipid Equivalent)	4.76	23.80
Oil mill (Lipid Equivalent)	5.88	19.60

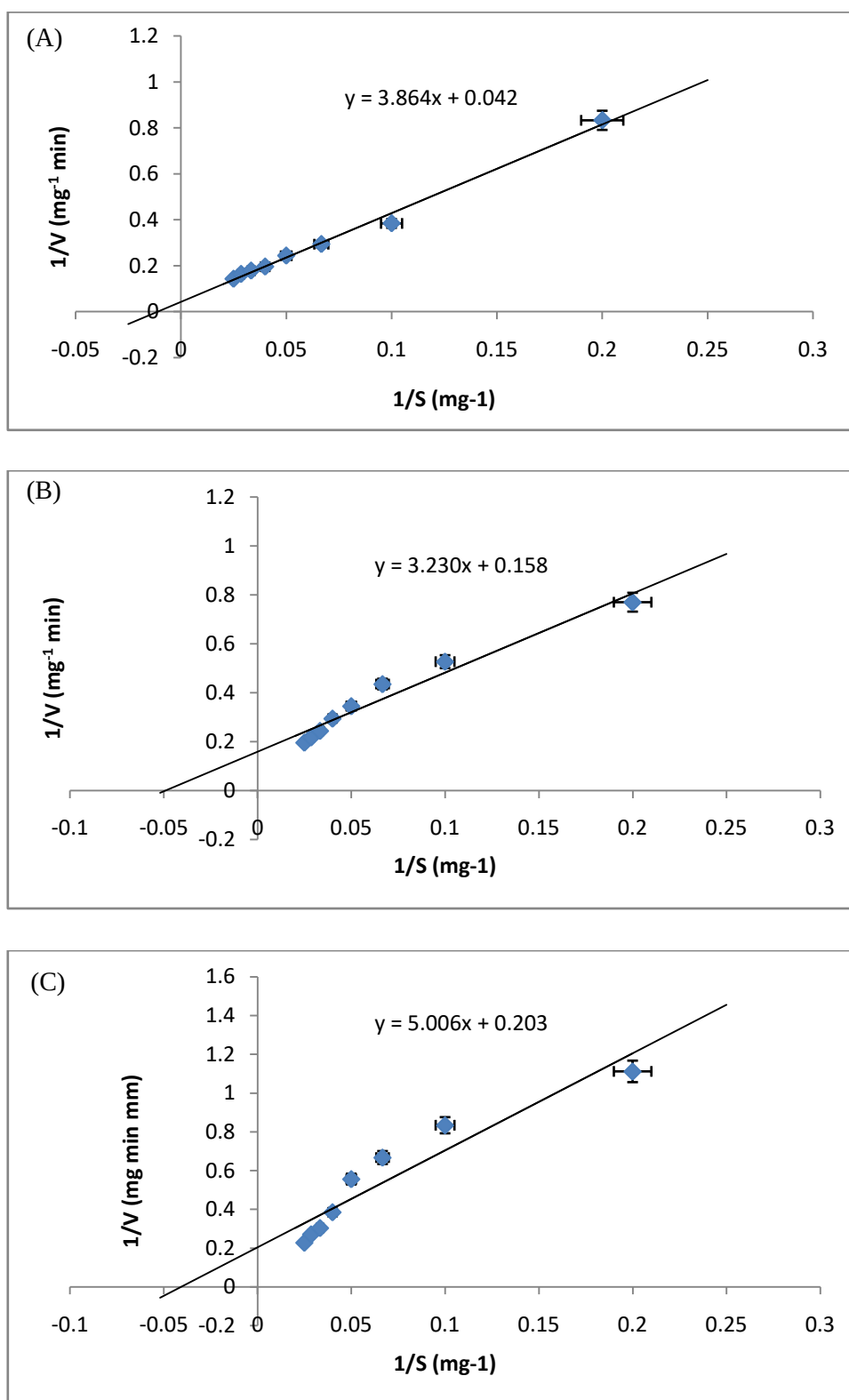


Figure 6: Estimation of Vmax and Km of LPB4 Lipase.(A) By using p-NPP as Substrate (B) By

using oil mill waste (Lipid content) as substrate (C) By using Dairy waste (Lipid equivalent) as substrate.

Lipase activity was assessed of different lipase in presence of mutagens. Lipase activity was highest in LPB4 lipase I presence of mutagens in comparison to lipases isolated from other microorganism(**Table 6**). After treatment of mutagens the lipase activity got increased suggesting positive effect of mutagen on lipase activity.

Table 6: Lipase activity of different lipases in presence of mutagen

S.No.	Bacterial isolates	Identified as	Qualitative Lipase activity (mm)				
			Natural/Wild strain	Treatment with Mutagens			
				UV	NTG	NMU	HNO ₂
1	LPB 2	<i>Bacillus</i> strain. 1	8.2 ±0.73	10.3±0.88	11.1±0.78	9.8±0.64	9.4±0.69
2	LPB 4	<i>Bacillus</i> strain. 2	14.1 ±0.82	18.3±1.12	15.4±1.07	16.7±1.09	14.9±1.01
3	LPB 7	<i>Bacillus</i> strain. 3	8.7 ±0.76	13.8±0.93	11.5±0.81	14.2±0.98	12.3±0.89
4	LPB 11	<i>Pseudomonas</i> strain.	11.6 ±0.59	16.9±1.04	15.2±1.02	14.5±78	12.1±0.98

Conclusion

Microorganisms, involved in the degradation process, have to go through a variety of enzymatic pathways (El-Borai et al., 2016). Enzymes act as biocatalysts and facilitate the degradation of pollutants. *Bacillus* and *Pseudomonas* sp. have been widely reported for oil degradation(Fagbemi and Sanusi, 2016; Phulpoto et al., 2016; Sukumar and Nirmala P, 2016). Microbes are most often utilize the pollutants as a nutritional source to generate energy for their growth and reproduction. The efficiency of biodegradation relies on the characteristics and concentration of pollutants as well as its availability to microbes (El Fantroussi and Agathos, 2005). Efficient biodegradation has been affected by the availability of pollutants to microbes and environment parameters (i.e. temperature, pH, oxygen concentration and nutrient sources). In present study different lipase producing microorganisms were isolated and characterized by biochemical analysis as *Bacillus* strains and *Pseudomonas* strains. Lipase activity was checked of the partial purified proteins isolated from the strains. The waste degrading capability of *Bacillus* strain 2 was checked for lipid degradation and BOD reduction. The strain was found efficient in waste degradation capability. This is for the first time any such comparison for waste degrading rate has been performed and the data suggest that Lipase (Partially purified) has great capability of waste degradation and therefore the isolated strain *Bacillus* strain 2 will be greatly helpful in waste management.

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