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Molecular Identification, Production and Optimization of Lipase from Oil Contaminated Soil using Submerged Fermentation

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Abstract

The most important products obtained for human needs are enzymes, through microbial sources. Hydrolytic enzymes occupied major industrial share such as esterases, lipases, amylases and proteases. Applications of these enzymes are varied in medical, cosmetics, food, dairy, pharmaceutical, leather, detergents, bioremediations and paper industries. So, lipases are gaining more attention now a day. Lipases hydrolyse fats into monoglycerides, diglycerides, free fatty acids and glycerol. *Bacillus* and *Stenotrophomonas*, are found to be the efficient lipase producers. A total of 48 different lipolytic organisms were isolated from soil samples. Organisms producing lipases were selected on the basis of clear zone formation in tributyrin agar medium. Maximum enzyme yield was obtained at 40˚C with 16 U/ml in *Bacillus* and 14 U/ml in *Stenotrophomonas* sp., at a pH of 7 in 3 days. The optimum parameters were studied. The yield of lipase enzyme was high while using peptone as a nitrogen source in *Stenotrophomonas* sp., where as in presence of $CaCl_2$, *Bacillus* sp., produced more lipase enzyme. However the presence of glucose as carbon source also yielded a much closer value to be highest yield. The lipase producing strains were subjected to gram staining and biochemical characterization following Bergey's manual of systematic bacteriology. 16SrRNA studies and physiological characterization reveals that *Bacillus* sp., *Stenotrophomonas* sp., *Halomonas* sp., *Serratia* sp., and *Enterobacter* sp., are the efficient producers. The sequences were deposited in Genbank.

Keywords: Enzymes, Lipase, *Bacillus, Stenotrophomonas*, Optimization, Applications

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INTRODUCTION

Lipase produced from bacteria is an important enzyme in food, detergents and pharmaceutical industries [Abu *et al.*, 2005]. These are very important in environmental managements [Aravindan *et al.*, 2007]. All other organisms including humans, animals, and plants depend upon them, without microorganisms no life can exist on earth [Bajpai and Tyagi, 2007]. Microorganisms are capable of tolerating wide range of conditions, so they are great interest as small biofactories. Hydrolases include lipases, proteases, pectinases and carbohydrases [Fujii *et al.*, 1986]. Lipases are fat splitting enzymes which require excess water to catalyze the formation of glycerol and fatty acids from long-chain triacylglycerol's by hydrolysis [Jaeger *et al.*, 1994, Pallavi *et al.,* 2010 and Ram Reddy & Pallavi, 2012]. Lipases produced by bacteria are used in dairy industries to hydrolyze milk fat, cheese ripening and flavor enhancement. Lipases are also used as additives in detergent industry. [Basketter *et al.*, 2008, Falch, 1991]. Mostly microbial lipases are extracellular and production is influenced by media composition with various physico chemical factors such as pH, temperature and dissolved oxygen. [Gulati *et al.,* 2005, Gilman, 1998]. Optimization of culture medium achieved high productivity. [Podar and Reysenbach, 2006].

MATERIALS AND METHODS

Collection of Sample

Samples were collected from oil contaminated soils around Salem, Coimbatore, Vellore and Chennai. Collected Samples were taken to the laboratory and refrigerated at 4°C**.**

Isolation and Identification of Bacteria producing lipase

Soil samples were serially diluted and 0.1ml from 10^{-4} and 10^{-5} dilutions were plated on nutrient agar. Identification of organisms was done based on morphological and biochemical characters following Bergey's manual of Systemic Bacteriology [Sneath, 1986]. Organism producing lipase were identified by formation of clear zone around the colony in tributyrin agar, after 48 – 72 hrs of incubation at 37°C. Clear zone producing colonies were picked and stored on nutrient agar slants for further studies. [Mobarak – Qamsaari *et al.*, 2011].

Submerged Fermentation

Medium for production of enzyme consists of peptone 0.2, $NH_4H_2PO_4$ 0.1, $NaCl_2$ 0.25, Olive oil 2 (V/V), $Cacl_2 2H_2O$ 0.04, $MgSo_4$ $7H_2O$ 0.04, pH 7, 1-2 drops of Tween 80 as emulsifier for 100ml distilled water. 100ml of inoculated media were kept in rotary shaker at 150 rpm for 24hrs. After inoculation, the broth culture was centrifuged at 10,000 rpm for 10min and placed at 4°C. The cell fitrate is the source of extracellular enzyme. [Aliyu *et al.,* 2011].

Enzyme Assay

Lipase activity was demonstrated by using spectrophotometrically at 30°C by using p-nitrophenol palmitate (pNPP) as a substrate [Winkler and Stuckmann, 1979]. Reaction mixture contains 300µl of lipase solution with 700µl *p*NPP solution. Preparation of *p*NPP by addition of solution A (0.001 g *p*NPP in 1ml isopropanal) in solution B (0.01 g gum arabic, 0.02 g Sodium deoxycholate, 50µl Triton X-100 and 9 ml of 50 mM Tris-HCl buffer, pH 8). Measured the absorbance at 410 nm by first 2 min of reaction. One unit was defined as the amount of enzyme liberated 1µmol of *p*NP per minute under laboratory conditions [Karadzic *et al.*, 2006, Rathi *et al.*, 2001, Tyagi *et al.,* 2002, Lubna *et al.*, 2015].

Optimization of lipase production

Production of lipase was carried out at different pH (4-8), temperature (30°- 70°C) and incubation period (3-7 days) [Kumar *et al.*, 2012]. Various metal ions such as calcium chloride, sodium chloride, magnesium sulphate, ferrous sulphate and zinc chloride, various carbon sources such as maltose, mannitol, lactose, sucrose and glucose, Nitrogen sources such as yeast extract, peptone, casein, albumin and urea are separately added to a final concentration of 1% (w/v) to media and fermented. After incubation enzyme activity was observed as described above. [Gupta *et al.*, 2004, Kim *et al.*, 1996, Gao *et al.,* 2000, Chen *et al.*, 1998].

Oil Source and Surfactants

Production of lipase was enriched by addition of various lipid sources such as coconut oil, groundnut oil, olive oil, castor oil and neem oil and surfactants such as Tween 20, Tween 40,

Table 1. Biochemical characterization of the isolates

S. No	Strains	Gram staining	Motility test	Indole test	MR test	VP test	Citrate test	Catalase test	Oxidase test	Urease test	Carbohydrate Fermentation test
1	*Bacillus sp.,*	+	+	–	–	+	+	+	–	–	+
2	*Pseudomonas sp.,*	-	+	-	–	–	+	+	+	–	– –
3	*Staphylococcus sp.,*	+	–	–	+	+	+	+	–	+	+
4	*Streptomyces sp.,*	+	-	-	-	+	+	-	+	+	–
5	*Klebsiella sp.,*	-	-	-	-	+	+	+	-	+	+
6	*Proteus sp.,*	-	+	-	+	–	+	+	-	+	–
7	*Serratia sp.,*	-	+	-	-	+	+	+	-	+	–
8	*Lactobacillus sp.,*	+	-	-	+	-	-	-	+	-	– +
9	*Streptococcus sp.,*	+	-	-	-	-	-	-	-	-	+
10	*Aeromonas sp.,*	-	+	+	-	+	+	+	-	-	–
11	*Micrococcus sp.,*	+	-	-	-	-	-	+	+	+	–
12	*Arthrobacter sp.,*	+	-	-	-	-	-	+	-	--	–
13	*Escherichia coli*	-	+	+	+	-	-	+	-	-	– +
14	*Halomonas sp.,*	-	+	+	-	+	-	+	+	-	+
15	*Enterobacter sp.,*	-	+	-	-	+	+	+	-	-	–
16	*Stenotrophomonas sp.,*	-	+	-	-	-	+	+	-	-	–

Tween 60, Tween 80, Tween 100, Triton X – 100, SDS) were added separately and tested at a concentration of 1% in production medium.

Identification and Molecular characterization of the isolates

The lipase producing strains were subjected to gram staining and biochemical characterization using standard methods. 16S rRNA was done to confirm the organisms using 27F and 1492R. Studies and physiological characterization reveals that *Bacillus* sp., *Stenotrophomonas* sp., *Halomonas* sp., *Serratia* sp., and *Enterobacter* sp., are the efficient producers. The sequences were deposited in Genbank.

RESULTS AND DISCUSSION

Isolation and Identification of Lipolytic Microorganisms

Forty eight microorganisms were isolated from soil samples based on the formation of clear zones on tributyrin agar. (Fig. 1). Sixteen organisms were selected for further study selected based on wider zones on tributyrin agar. The results were given in the Table 1.

Enzyme assay

Among 48 organisms, the selected 16 organisms were subjected to production of lipase.

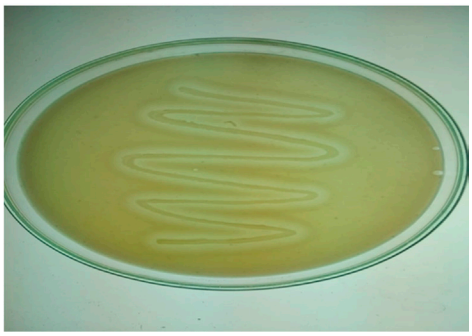

Fig. 1. Plate showing zone around isolated colonies

The enzyme activities of all the isolates are shown in table 2. Among 16 isolates, 5 isolates (*Bacillus sp., Halomonas sp., Enterobacter sp., Serratia sp., Stenotrophomonas* sp.,) were able to produce lipase above 11U/ml when incubated for 72 Hrs at pH 7 at 37°C. On the basis of production rate, out of 16 organisms, 5 isolates were selected for further studies.

Optimization of enzyme production

Effect of temperatures and pH on enzyme production

Production of lipase at different temperatures (20 - 60°C) was observed for 72 hrs. Lipase activity was maximum at 40°C in all the isolates. The enzyme activities at different temperatures are shown in Fig. 2. Effect of pH on enzyme activity was shown in Fig. 3. Maximum production of lipase was obtained at pH 7 for

Table 2. Lipase enzyme production in submerged fermentation (SSF) by selected Bacterial species

S. No.	Strains	Lipase Activity (U/ml) SSF
1.	*Bacillus sp.,*	14.03±0.75
2.	*Staphylococcus sp.,*	9.03 ±0.850
3.	*Escherichia coli*	4.86 ±0.808
4.	*Micrococcus sp.,*	7.96 ± 0.85
5.	*Streptomyces sp.,*	07 ±1
6.	*Klebsiella sp.,*	3.93 ±0.901
7.	*Serratia sp.,*	13 ±1
8.	*Proteus sp.,*	10 ±1
9.	*Pseudomonas sp.,*	9.833±1.25
10.	*Enterobacter sp.,*	12 ±1
11.	*Aeromonas sp.,*	07 ±1
12.	*Pseudomonas sp.,*	10.83 ±0.763
13.	*Micrococcus sp.,*	05 ±1
14.	*Halomonas sp.,*	12 ±1
15.	*Stenotrophomonas sp.,*	14.83 ±0.763
16.	*Streptococcus sp.,*	9.01 ±0.6

Table 3. Effect of Incubation periods on lipase production

Incubation periods	*Bacillus sp.,* (U/ml)	*Serratia sp.,* (U/ml)	*Stenotrophomonas sp.,* (U/ml)	*Halomonas sp.,* (U/ml)	*Enterobacter sp., (*U/ml)
12hrs	13.06±0.3	9.3±0.9	8.16±0.5	10.16±0.5	9.1±0.65
18hrs	14.93±0.7	11.9±0.4	13.93±0.4	12.83±0.4	11.96±0.45
24hrs	17.96±0.7	13.8±0.7	17.1±0.6	13.96±0.5	13.93±0.7
48hrs	14.86±0.8	14.2±0.5	14.9±0.4	13±0.5	13.06±0.5
60hrs	10.2±0.5	9±0.7	8.2±0.6	11.96±0.4	8.93±0.7

Serratia sp. and *Stenotrophomonas* sp., whereas for *Bacillus* and *Halomonas* sp. at pH 8.

Effect of Incubation Periods

Production of lipase by isolated organisms after incubation at different time periods ranging from 12 – 72 hrs were assayed. *Bacillus* sp., shows maximum production of enzyme at 24 hrs with 18U/ml. *Serratia* sp., produce 14U/ml of enzyme at 24 - 48Hrs. *Stenotrophomonas* sp., produce 17U/ml at 24hrs. *Halomonas sp.,* produce maximum lipase activity at 24hrs with 14U/ml. *Enterobacter sp.,* produce maximum activity of 14U/ml at 24 hrs. Table 3 Maximum [Mahanta *et al.*, 2008] production of lipase by *Bacillus stearothermophilus* occurred at 24hr of incubation. Decrease in production of enzyme may be due to interaction of enzyme with other compounds in medium or due to reduction of nutrients in batch cultures.

Effect of various carbon, nitrogen sources and metal ions

Various carbon sources such as glucose, sucrose, mannitol, lactose and maltose are used in media for production of enzyme. The effect of different carbon sources are shown in Fig 4. Glucose as a carbon sources was found to be the optimum for *Bacillus* sp., *Serratia* sp., and *Stenotrophomonas* sp., while mannitol was found to be the best carbon sources for *Halomonas* sp.,and *Enterobacter.* Ghaima *et al.*, (2014) had reported that with maltose as the carbon source, *B. cereus* had the highest activity however in our study high yield was obtained with glucose in *B. cereus.*

Various nitrogen sources such as peptone, yeast extract, casein, Albumin and urea are assayed for maximum production of lipase. The effect of nitrogen sources are shown in Fig. 5. Peptone is

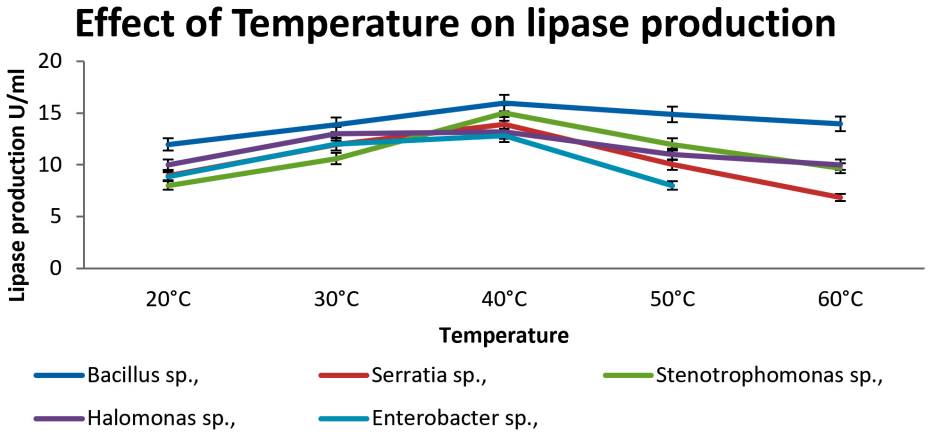

Fig. 2. Effect of Temperature on lipase producing strains

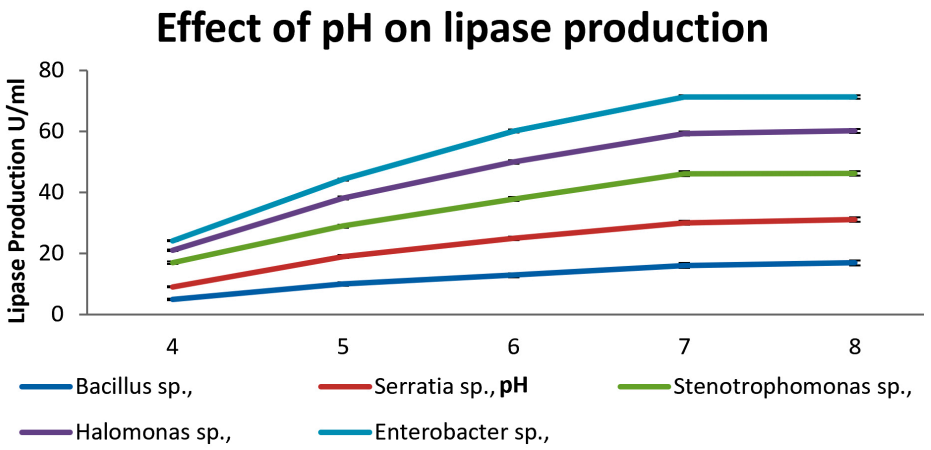

Fig. 3. Effect of pH on lipase producing strains

known as common inducer for lipase. The releasing of NH_4+ ions from peptone influenced its efficacy for higher enzyme activity because NH_4 stimulates the growth and increases enzyme production rate [Kumar *et al.,* 2012]. Peptone was found to be best nitrogen source for the isolates except *Enterobacter* sp., which produced maximum lipase with casein.

Various metal ions such as $MgSO_4$, $CaCl_2$, $FeSO_4$, $NaCl_2$ and $ZnCl_2$ are assayed for production of lipase.$CaCl_2$ enhanced the production of lipase by the organisms. The effects of different ions are shown in table 4. Extracellular lipase produced by *Bacillus* sp., yield high activity of lipase in presence of Ca^{2+} which is in agreement with several previous reports. [Jayaraman, 1981, Bajpai and Tyagi, 2007]. Table 8

Influence of oils and surfactants

Lipases are induced mostly in presence of oils in culture medium. Maximum lipase production was seen in presence of olive oil (29U/ml) in *Bacillus* sp., *Stenotrophomonas* sp.,

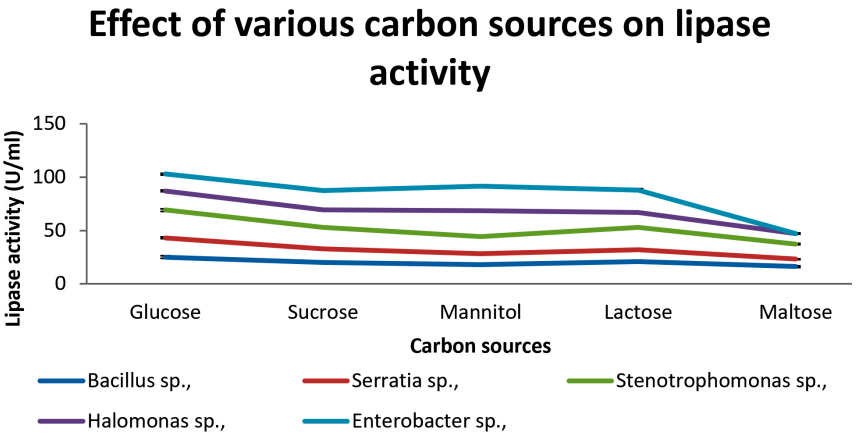

Fig. 4. Effect of Various carbon sources from lipase production

Table 4. Effect of Metal ions on lipase production

Metal ions	*Bacillus sp.*, (U/ml)	*Serratia sp.*, (U/ml)	*Stenotrophomonas sp.*, (U/ml)	*Halomonas sp.*, (U/ml)	*Enterobacter sp.*, (U/ml)
$MgSo_4$	24.9±0.36	19.9±0.45	20.1±0.36	18.03±0.35	18.9±0.36
$CaCl_2$	27±0.5	19.1±0.61	28.1±0.55	19.9±0.4	22.13±0.15
$FeSo_4$	17.96±0.35	13±0.5	18.03±0.45	13±0.5	17.9±0.4
$NaCl_2$	16.06±0.6	15±0.5	19±0.5	16±0.5	19.8±0.32
$ZnCl_2$	16±0.5	9.9±0.5	16.03±0.5	12±0.5	20.03±0.25

Table 5. Influence of surfactants on lipase production

Surfectants	*Bacillus sp.,* (U/ml)	*Serratia sp.,* (U/ml)	*Stenotrophomonas sp.,* (U/ml)	*Halomonas sp.,* (U/ml)	*Enterobacter sp.,* (U/ml)
Tween 20	14.03±0.45	8.16±0.37	13.03±0.15	10±0.3	9.03±0.35
Tween 40	10.2±0.52	11.93±0.4	10±0.2	8.1±0.5	9.9±0.35
Tween 60	19.16±0.56	16±0.5	19.06±0.3	13.06±0.3	10±0.2
Tween 80	30±0.2	23.03±0.45	25±0.5	19.06±0.4	20.06±0.3
Tween 100	21.13±0.3	20.06±0.3	18.1±0.55	14.03±0.45	14.03±0.45
Triton X-100	16±0.5	16.06±0.5	10.1±0.45	13.03±0.45	16.03±0.45
SDS	12.06±0.3	14.9±0.3	11.03±0.15	11.9±0.35	13±0.4

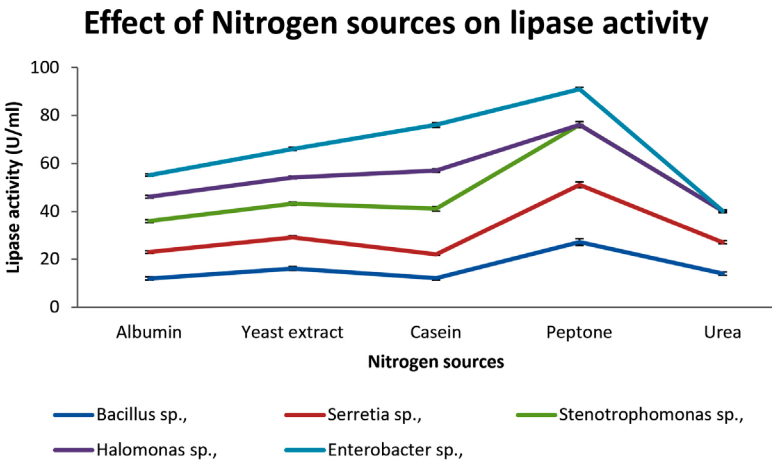

Fig. 5. Effect of various Nitrogen sources by lipase production

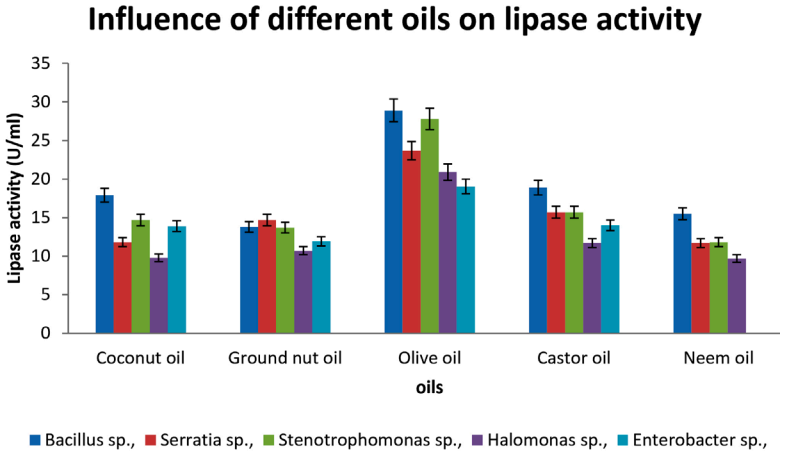

Fig. 6. Influence of different oils on lipase production

(28U/ml), *Enterobacter* sp., (19U/ml) *Halomonas* sp., (21U/ml) and *Serratia* sp., (24U/ml). Similar studies conducted on lipase production by *Bacillus* sp., showed maximum yield in olive oil [8]. Various studies revealed that olive oil is more effective to induce production of lipase, due to lipid content in olive oil, which is structurally easily digested by microorganisms [Zarevucka, 2012].

Fermentation medium enhance the secretion of proteins by alterating the cell membrane permeability, so addition of surfactants enhances production of lipase. Tween-80 showed maximum yield of lipase in *Bacillus sp*., (30 U/ml) *Stenotrophomonas sp*., (25 U/ml) *Halomonas* sp., (19 U/ml) *Serratia* sp., (23 U/ml) and in *Enterobacter* sp., (20 U/ml). The effect of surfactants on lipase production is shown in fig.6. Thus our results in accordance with Duttat and Ray, 2009 who had shown that surfactants have no or little inhibitory activity on enzyme activity.

Molecular characterization of the isolates

Based on 16S rRNA sequencing the five isolated organisms are identified as *Bacillus cereus, Serratia marcescens, Stenotrophomonas maltophila, Serratia marcescens and Enterobacter cloaca.* The accession numbers of the sequences deposited in NCBI are MNO94422, MK999992, MNO94371, MNO94423 and MK999988 respectively.

CONCLUSION

Lipase is one of the most valuable industrial enzymes. In present study, lipase producing organisms are isolated from soil sample. 48 organisms was isolated from soil sample and optimized with various physico – chemical parameters for lipase production. The enzyme produced by B. cereus was found to be stable at 60°C though its optimum is 40°C. A novel strain *Stenotrophomonas maltophila* was found to produce lipase with the simple sugar glucose. Also it is observed that these enzymes remain active at alkaline pH. Because of this, these enzymes could be promising biocatalyzing agents in industries.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

None.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication. Joyruth performed the experiments and prepared the manuscript.

LG provided technical guidance and reviewed the manuscript.

ETHICS STATEMENT

The research does not involve human or animal experimentation.

DATA AVAILABILITY

All available data studied are incorporated in the manuscript.

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