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Characterization of Lipase from *Bacillus subtilis* Isolated from Oil Contaminated Soil

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Abstract An extracellular lipase from *Bacillus subtilis* isolated from oil polluted soil was partially purified and characterized in this study. The enzyme was purified to 19.36 fold and the molecular weight was estimated. The effect of temperature, time, metal ion and pH was also determined. The molecular weight of the enzyme was estimated to be 48.63 kDa by SDS-PAGE. The optimum temperature was 60°C, while the enzyme exhibited appreciable thermostability retaining 70% of activity at 70°C for 1h. The lipase was most active in the pH range of 7-9 with an optimum activity at pH 8.0. The enzyme activity declined in the presence of Al^{3+} and Fe^{2+}, while Na^{+} stimulated the activity. Olive oil was found to be the preferred substrate. The maximum velocity V_{max} and K_m of the lipase during the hydrolysis of olive oil were 39.45 µmol/min/ml and 20.01 mM respectively.

Keywords: *Lipase, olive oil, Bacillus subtilis, thermostability*

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1. Introduction

Enzymes had been used for a long period of time in different forms; as extracts derive from vegetable, animal organs or as extracts of microbes. Lipases are acyl hydrolases and water-soluble enzymes that play a key role in fat digestion by cleaving long-chain triglycerides into polar lipids [1]. Lipases are ubiquitous enzymes of considerable physiological significance in industrial potential. Lipases (Triacylglycerol acylhydrolases EC 3.1.1.3) are a class of hydrolase which catalyse the hydrolysis of triglycerides to glycerol and free fatty acids over an oil-water interface and reversing the reaction in non-aqueous media [2,3,4,5]. Lipases catalyze a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis [6]. Moreover, some lipases are also involved in the turnover of membrane lipids and lipid-anchored proteins, in altering cell membrane composition in order to change cell membrane functions or to adapt cell membrane to environmental changes, in cell signalling, in the controlled destruction of intracellular vacuoles, in cytolysis [7,8], in detoxification of biocides and polluting agents [9], or in pathogenesis, acting as virulence factors [10].

Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, and decaying food [8], compost heaps, coal tips, and hot springs [7]. Bacterial lipases are mostly released outside of the cell called extracellular enzyme. Bacterial lipases are influenced by nutritional and physico-chemical factors; such as temperature, pH, nitrogen and carbon sources, presence of lipids, inorganic salts, stirring conditions, dissolved oxygen concentration [11]. The major factor for the expression of lipase enzyme is carbon source. Lipases generally are produced in the presence of lipid source such as oil, triacylglycerols, fatty acids, hydrolyzable esters, tweens and glycerols, in addition to carbon sources. Also the type of nitrogen source is also influence the production of lipases.

Lipases are widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics, and pharmaceuticals [12]. Lipase can be used to accelerate the degradation of fatty waste and polyurethane [7].

This research was design to partially purify and characterize the lipase produced by *Bacillus subtilis* isolated from oil contaminated soil as well as examine the stability of the lipase under different physical and chemical conditions such as heat stability, pH, temperature and heavy metals.

2. Materials and Methods

2.1. Source of Stock Culture

The bacterial strain, *Bacillus subtilis* used in this study was isolated from oil contaminated soil. Stock culture of this organism was maintained on nutrient agar in the Microbiology Laboratory. Sub cultures were made from stock culture to fresh slants and incubated for 24 h at 37°C

to preserve the viability of the organism for the purpose of the research.

2.2. Qualitative Test for Lipase Production Using Tributyrin Agar

The test organism was screened for lipolytic activity on agar plates containing tributyrin (1%, w/v) agar. Lipase production is indicated by the formation of clear zones around the colonies grown on tributyrin-containing agar plates.

2.3. Lipase Production (Using Minimum Salt Medium)

Enzyme production medium used was composed as follow: $MnSO_4$ 0.002g/L; $FeSO_4.7H_2O$ 0.02g/L; KCl 0.2g/L; $MgSO_4.7H_2O$ 0.2g/L; K_2HS0_4 0.9g/L; $ZnSO_4$ 0.002g/L; olive oil 10g/L. Overnight cultures were suspended in 5ml of sterile deionised water and used as the inoculum for pre culture to obtain an initial cell density to adjust the turbidity to 0.5 McFarland standard. Submerged microbial cultures were incubated in 500 ml Erlenmeyer flasks containing 100 ml of liquid medium on a rotary shaker (150 rpm) and incubated at 30°C. After 24 hours of incubation, the culture was centrifuged at 4,000 rpm for 10 min at 4°C and the cell free culture supernatant was used as the source of extracellular enzyme.

2.4. Enzyme Assay

Lipolytic activity was determined by colorimetric method based on the activity in cleavage of p-nitrophenylpalmitate (p-NPP) at pH 8.0 [7]. The reaction mixture contained 180 µl of solution A (0:062 g of p-NPP in 10 ml of 2-propanol, sonicated for 2 min before use), 1620 µl of solution B (0.4% triton X-100 and 0.1% gum arabic in 50 mMTris-HCl, pH 8.0) and 200 µl of properly diluted enzyme sample. The product was detected at 410 nm wavelength after incubation for 15 min at 37°C. Under this condition, the molar extinction coefficient of p-nitrophenol (p-NP) released from p-NPP was 15000 M^{-1}. One unit of lipase activity was defined as 1 mmol of p-nitrophenol (p-NP) released per minute by 1 ml of enzyme.

2.5. Protein Determination

Protein concentration was determined using the Lowry method. Reagent A: 2% $NaCO_3$ in 0.1 N NaOH; Reagent B: 0.5% $CuSO_4.5H_2O$ in 1% Na or K tartarate; Reagent C: 100ml of Reagent A + 2ml of reagent B and Reagent E: 1:2 dilution of John's reagent water. Graded concentrations of Bovine Serum Albumin (BSA) in tubes were prepared. Then 0.3 ml of each concentration was measured into test tubes. 3 ml of reagent C was added, mixed and left for 10 min. Then 0.3ml of reagent E was added, mixed and left for 30 min. The optical density was read at 600nm. The graph of OD versus concentration of BSA was obtained and standard curve of BSA. The same was done for unknown substance and the protein concentrations from the standard curve are read off and obtained by multiplying with dilution factor. All readings were obtained in triplicates.

2.6. Partial Purification of Lipase from *Bacillus subtilis*

2.6.1. Ammonium Sulphate Precipitation

The crude enzyme was precipitated by adding ammonium sulphate to 60% saturation. The mixture was centrifuged at 10,000 rpm for 15 min at 4°C. The precipitates were then re-suspended in 50 mMTris-HCl, pH 8.0 and dialyzed against the same buffer overnight at 4°C with three buffer changes. Dialyzed enzyme solution was concentrated with 4M sucrose solution to get concentrated enzyme free from salt and metal ions.

2.6.2. Gel filtration Chromatography (Using Sephadex G-100)

Dialysate containing lipase activity was concentrated and applied on a sephadex G-100 column (1.5 cm diameter x 75 cm length) pre-equilibrated with 50 mMTris-HCl, pH 8.0. 5 ml fractions were collected at a flow rate of 20 ml/h at room temperature. The protein content of fractions was determined by measuring optical density at 280 nm. The protein-containing fractions were assayed for lipase activity. Fractions containing lipase activity were pooled and concentrated for further analysis [13].

2.7. Optimization of Lipase Production (Characterization of the Partially Purified Assay)

2.7.1. Effect of Temperature on Lipase Activity and Stability

The enzyme stability at different temperatures was studied by incubating the enzyme in 50 mMTris-HCl, pH 8.0 at different temperatures for 2h, followed by the activity estimation at 37°C.

2.7.2. Effect of pH on Lipase Activity and Stability

The effect of pH on enzyme activity was determined by incubating the enzyme with p-nitrophenyl acetate substrate, prepared in different buffers in the pH range 3 to 9. The buffers used are sodium acetate (pH 3-5), sodium phosphate (pH 6 - 7) and Tris-HCl (pH 9).

2.7.3. Effect of Metal Ions on Lipase Activity

The effect of metal ions was determined by estimation of the activity in presence of 10 mM solution of metal salts. The enzyme was incubated in presence of metal ions for 15 min followed by estimation of activity by p-Nitrophenol liberation [14].

2.7.4. Lipase Optimization by Substrates

By using different substrate sources such as olive oil, groundnut oil, palm oil, and soy bean oil, their effects on lipase production by the *Bacillus subtilis* was assessed at optimum pH and temperature of 7.4 and 37°C respectively.

3. Results

Bacillus subtilis used in the research showed cream colour on nutrient agar, Gram positive rods, spore formers,

positive to catalase, citrate, methyl red and Voges-Proskauer. The organism grew on Tributyrin agar producing a zone of hydrolysis (9.8 mm). In minimum medium containing olive oil as the substrate, the activity of the crude lipase was 38.52 Units/ml.

3.1. Effect of Time on of Lipase Production

B. subitilis grew in basal medium containing 1% olive oil as the sole carbon source (Figure 1). Production of lipase increased with period of incubation achieving a maximum in the 22nd hour.

3.2. Purification of Lipase of *Bacillus subtilis*

The elution profile of *B. subtilis* lipase on Sephadex G-100 chromatography column revealed a single peak of lipase activity (Figure 2). The specific activity of the lipase increased from 0.94 to 18.12 units/mg protein during the process of purification. The ammonium sulphate precipitated fraction had 1.2 fold purity, while the gel filtration resulted into approximate 20 fold purity.

3.3. Effect of Temperature on Lipase Activity and Stability

There was an increase in lipase activity of *B. subtilis* as the temperature increase beyond the optimum led to decline in the enzyme activity (Figure 3). At 60°C the enzyme maintained 80% relative activity for 120 minutes similar treatment at 80°C and 90°C led to 80% and complete loss of activity after 120 minutes respectively (Figure 4).

3.4. Effect of pH on Lipase Activity and Stability

The optimum pH for the activity of the lipase was found to be 8.0 (Figure 5). While the enzyme were stable between pH 7.0 and 8.0 (Figure 6).

3.5. Effect of Metal ions on Lipase Activity

Among the metal ions tested, only NaCl stimulated the lipase activity, KCl had no effect on the activity of the lipase while Al_2Cl_3 had the highest inhibitory effect (Figure 7).

3.6. Effect of Substrates on Lipase Activity

The effects of different carbon (lipid) sources used for lipase production by *Bacillus subtilis* are presented in Figure 8. The highest lipase activity (45 umol/min/ml) was observed when olive oil served as the carbon source.

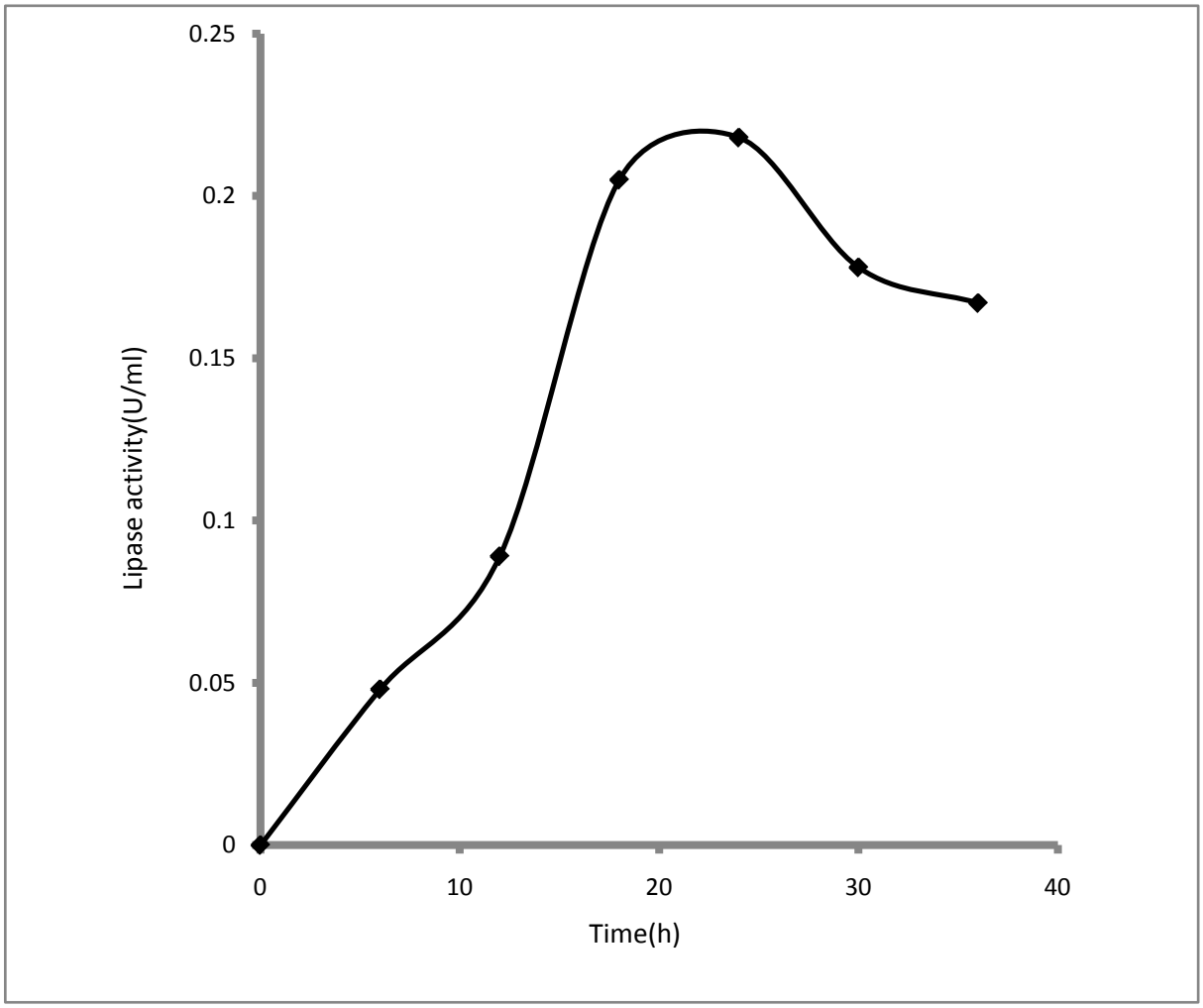

Figure 1. Time course of lipase production by *B.subtilis*

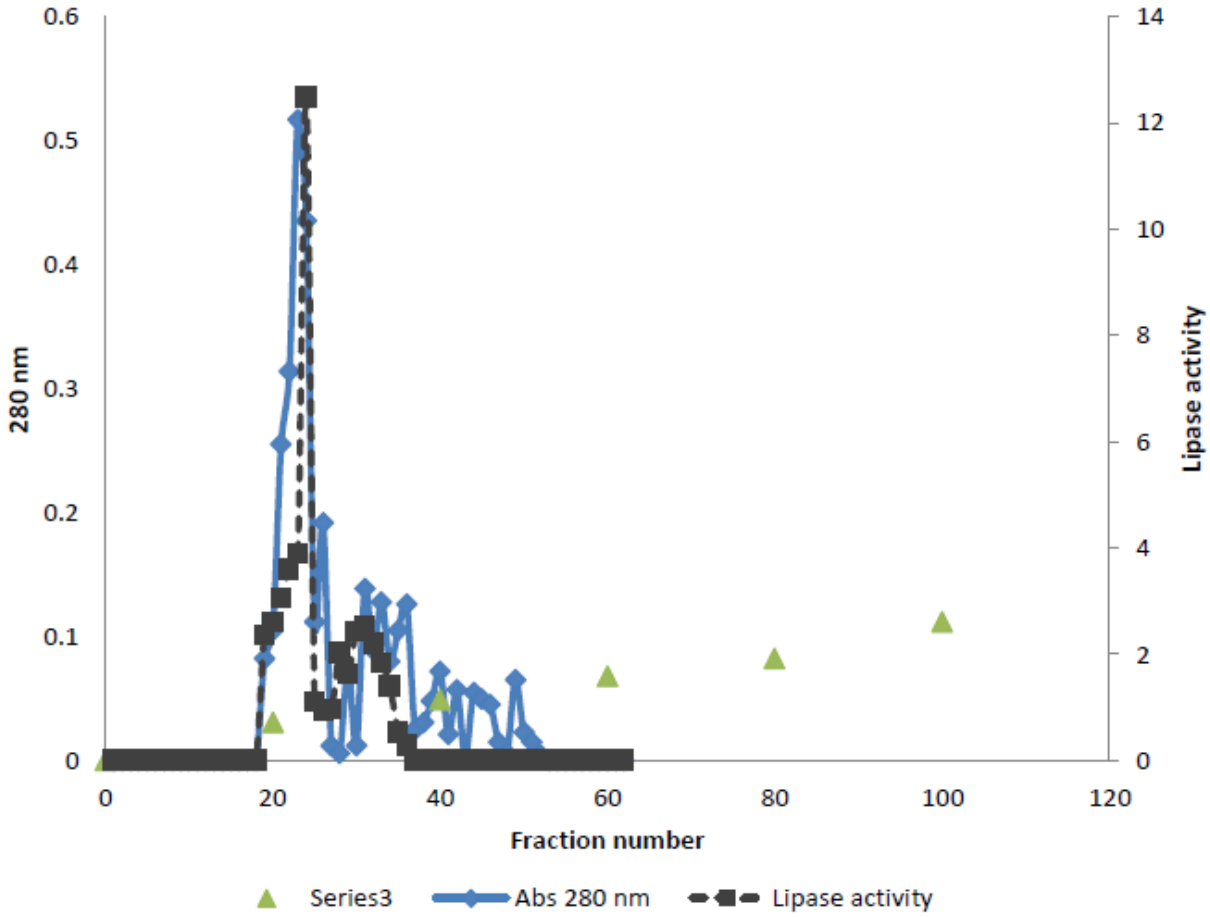

Figure 2. Elution profile of lipase produced by *Bacillus subtilis* on Gel filtration chromatography

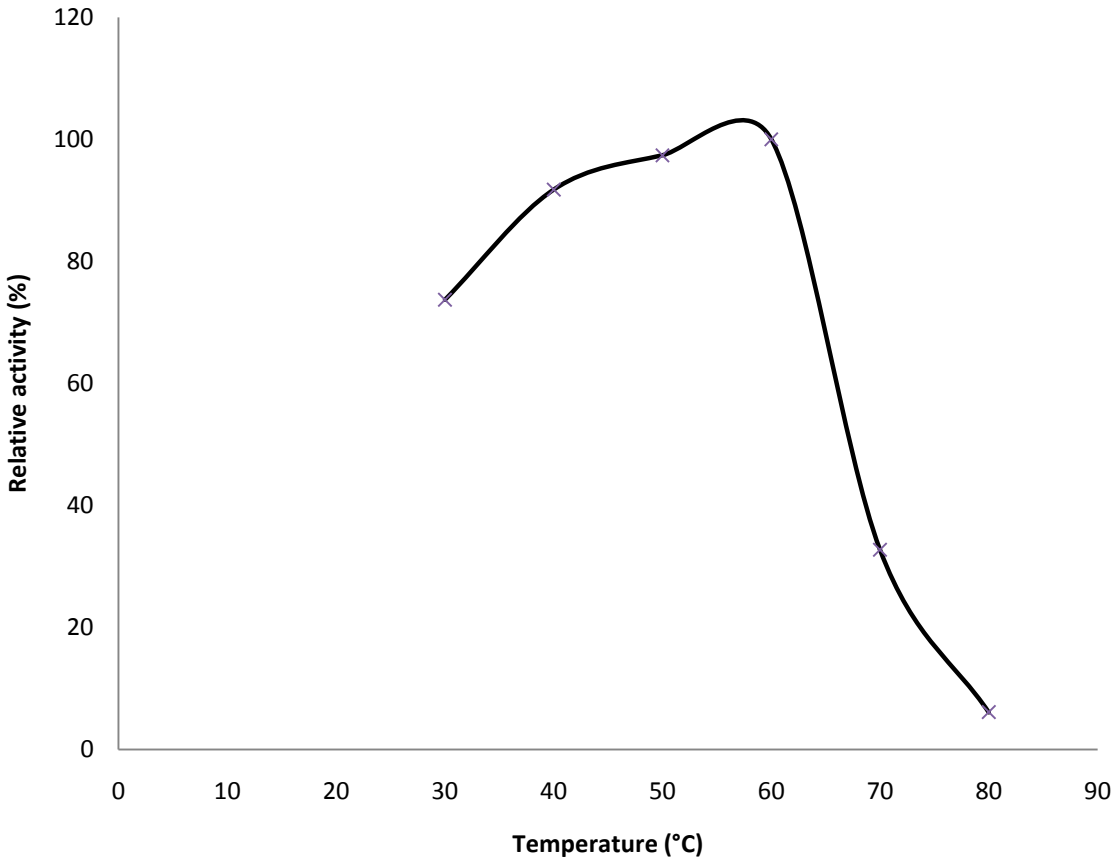

Figure 3. Effect of temperature on the activity of partially purified lipase from *Bacillus subtilis*

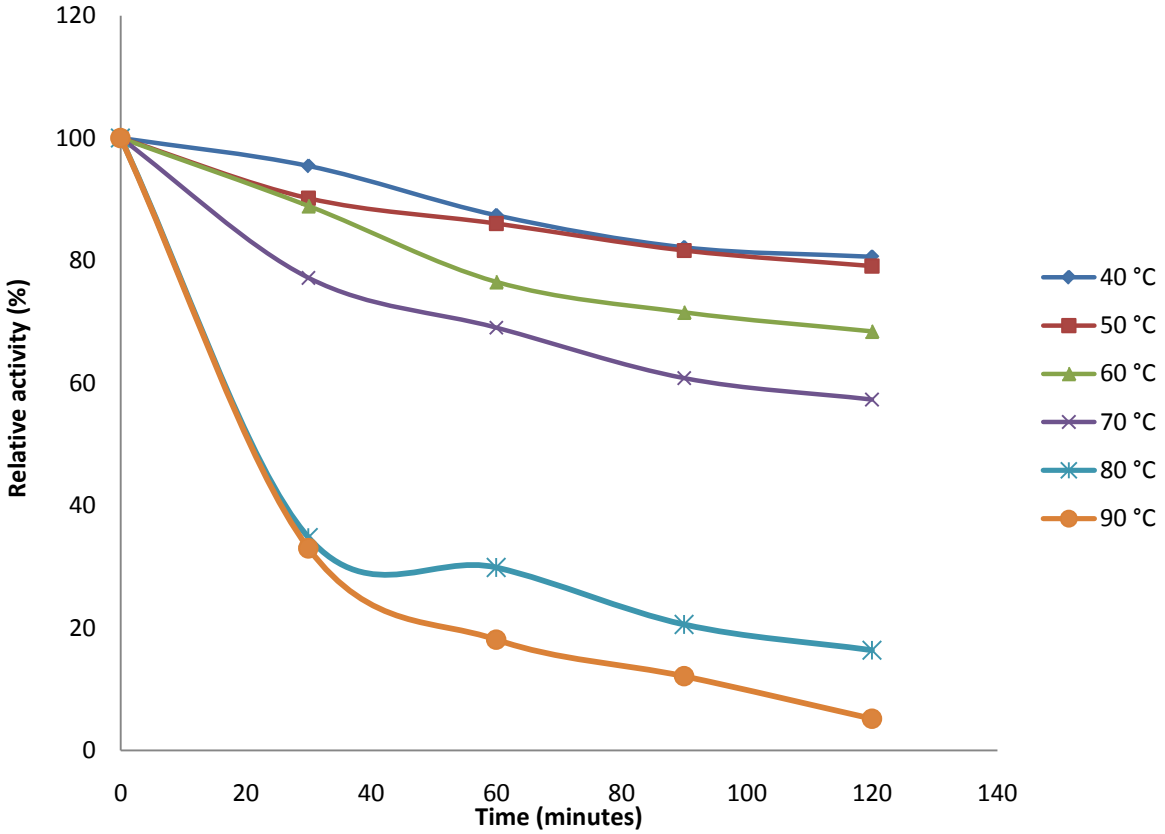

Figure 4. Thermostability of lipase activity of *Bacillus subtilis*

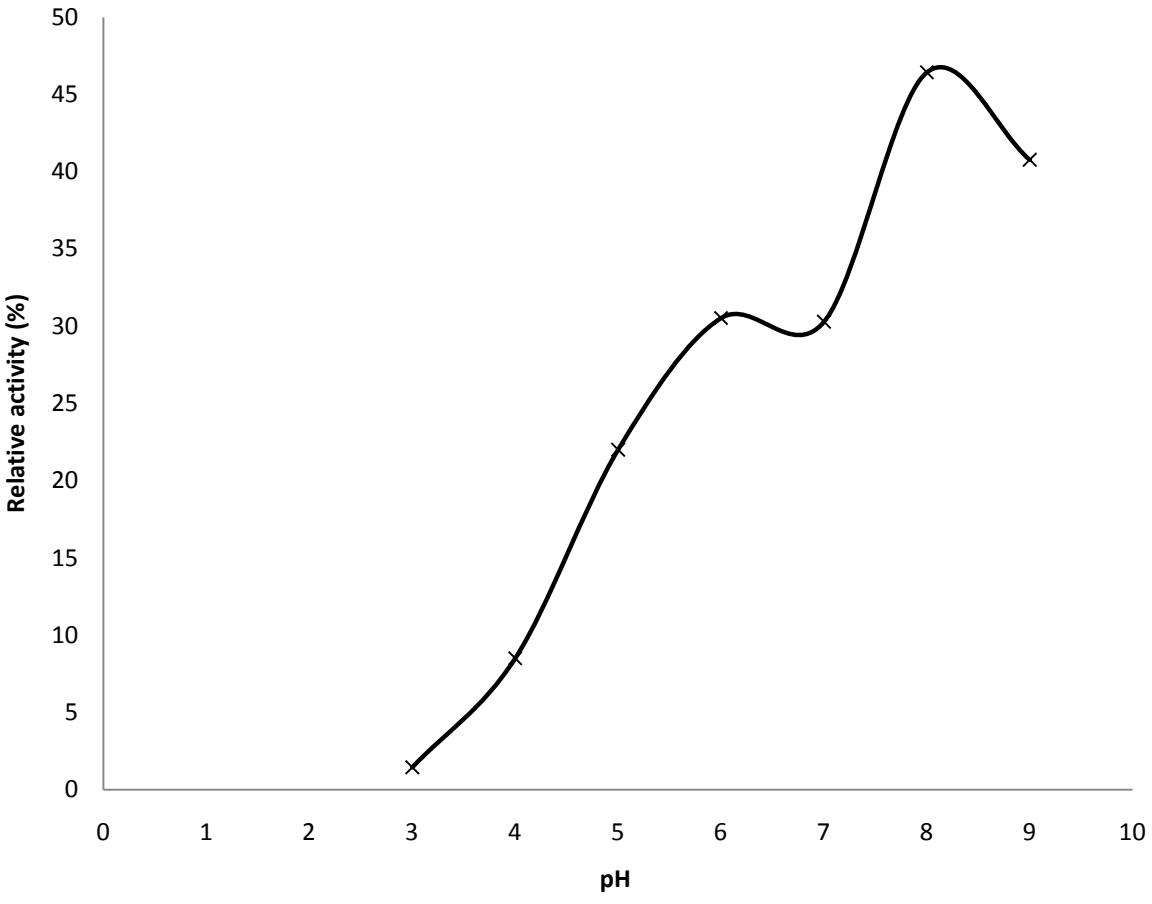

Figure 5. Effect of pH on the activity of partially purified lipase from *Bacillus subtilis*

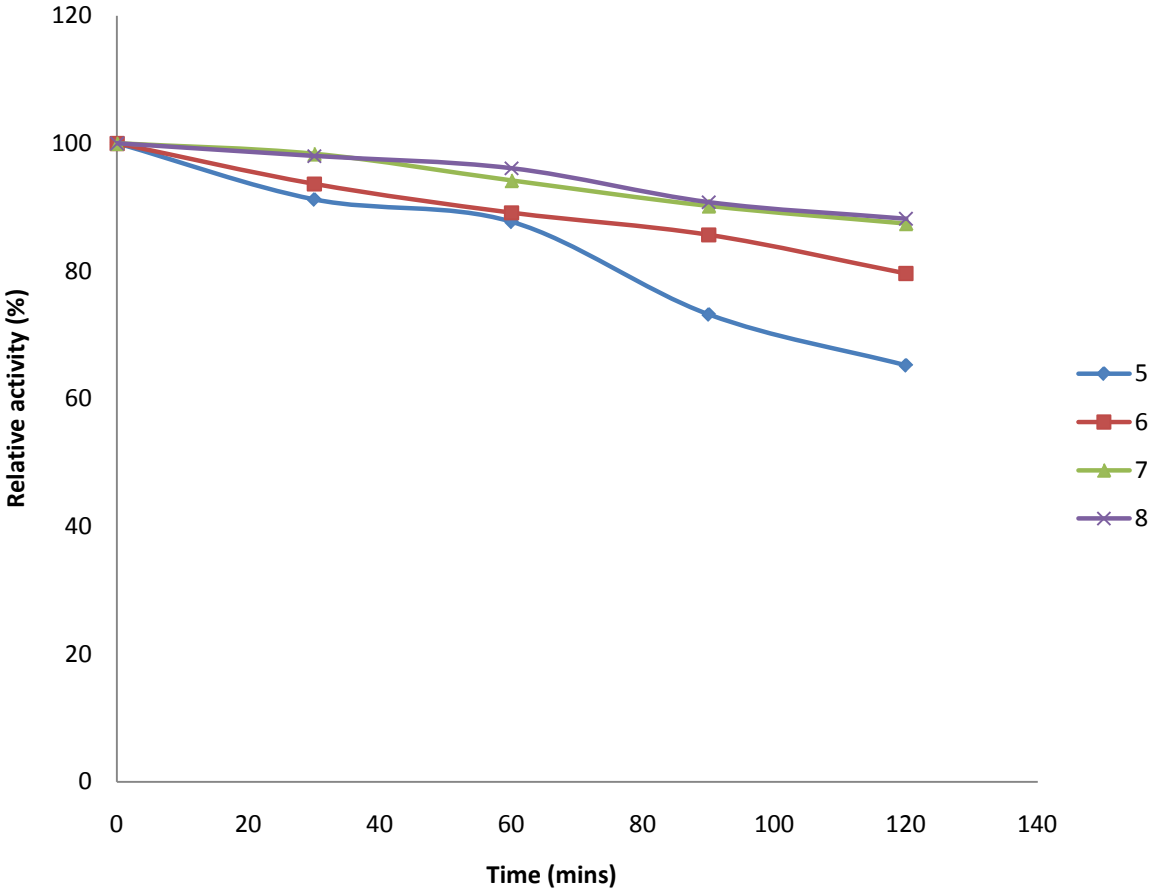

Figure 6. pH stability of lipase of *Bacillus subtilis*

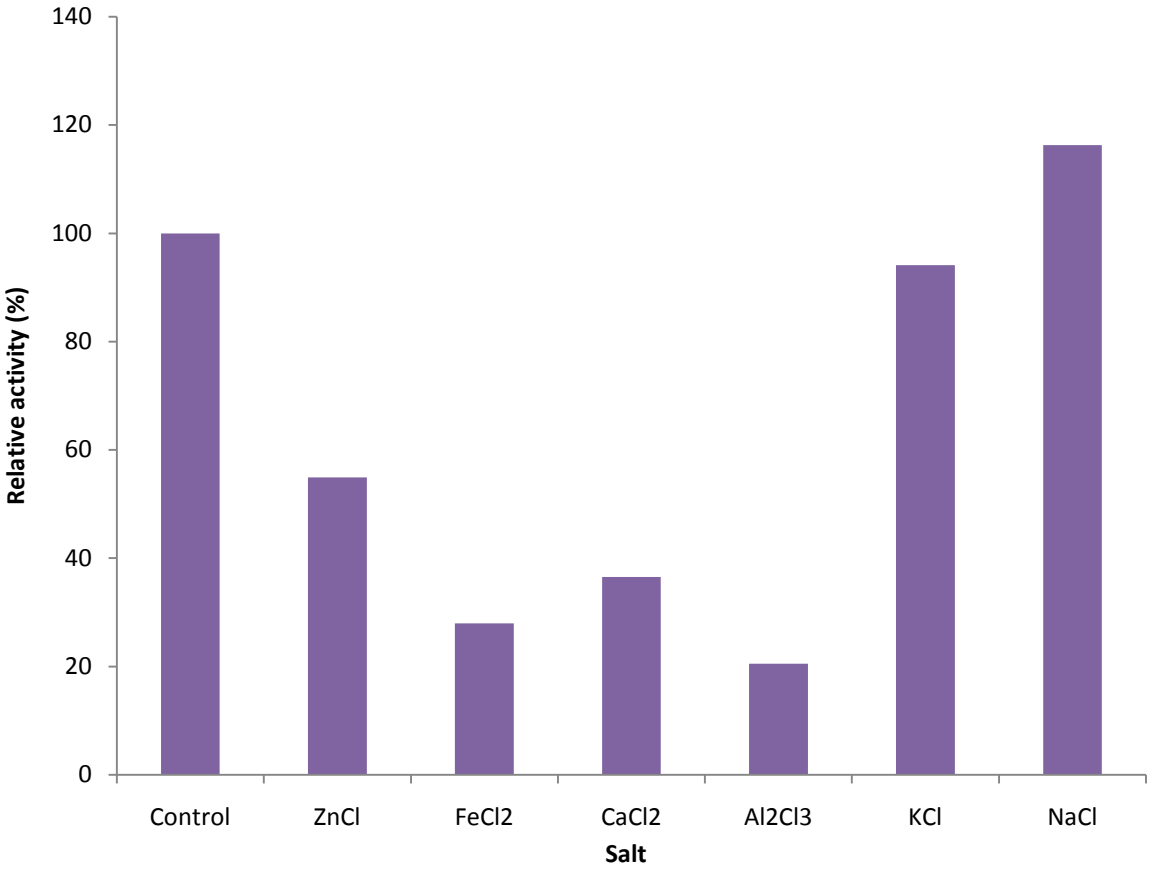

Figure 7. Effect of salts on the activity of partially purified lipase from *Bacillus subtilis*

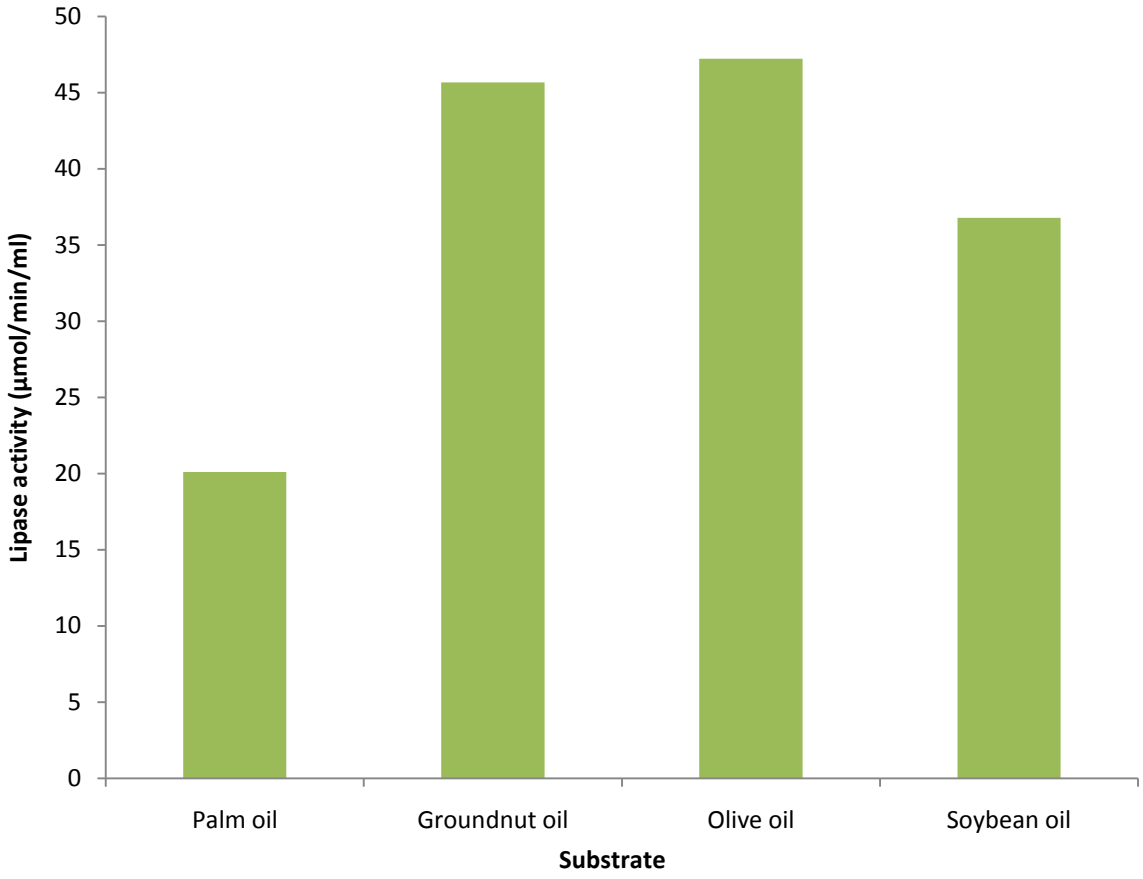

Figure 8. Effect of substrates on the activity of partially purified lipase from *Bacillus subtilis*

4. Discussion

4.1. Partial Purification Step

The plates contained a lipid substrate and lipolysis was observed as clear halos or opaque zones around the culture as recommended by [15]. In the present study, the maximum zone of hydrolysis (9.8 mm) and specific lipase activity (38.52 U/mg) noticed for crude lipase from *Bacillus subtilis*. Precipitation of enzymes was carried out by ammonium sulphate since it was highly soluble in water, cheap and had no deleterious effect on structure of protein. In this research work, the precipitate lipase by ammonium sulphate, experiment was conducted at 20%, 40%, and 60%, saturation of ammonium sulphate salt. Results revealed that 40% saturation was proved to be effective for maximum specific activity ranging from 38.52 Units/ml of crude lipase to a specific activity of 27.70 Units/ml with purification of approximately 20-fold.

4.2. Effect of Temperature on Enzyme Activity and Stability

Lipase produced by *B. subtilis* showed maximum activity at a temperature of 60°C (Figure 3). Lipase was stable, retaining more than 50% of its activity over the range of temperatures studied (25 to 80°C). [16] reported a lipase produced by *B. subtilis* that had maximum activity at 60°C, with 72% stability when incubated at 50°C for 1 h. [17] who reported a lipase activity of *B.megaterium* observed maximum activity at 55°C and was stable from 30 to 60°C.

4.3. Effect of pH on Enzyme Activity and Stability

Lipase activity was maximal at pH 8.0 and high in the range of pH 7.0 to 11.0. The lipase remained stable at a pH range of 7.0 to 11.0 after incubation for 1 h at 30°C, with a residual activity remaining above 50% for pH 7.0, 8.0 and 9.0, respectively (Figure 5). However, drastic deactivation of the lipase was observed at pH 6.0 and 10.0. These results are in agreement with [18] who reported the production of a lipase by *B. Stearothermophilus* MC 7 that had a maximum activity at pH 8.0, but showed inhibition at pH 6.0 and was stable over the range of 6.5 to 8.0. [19] also reported the effect of pH on the activity of lipase enzyme from *Bacillus subtilis* showed that pH is an important factor in enzyme activity and the maximum pH was 8.0.

4.4. Effect of Metal ions and Substrates on Lipase Activity and Stability

On the effect of varied concentration of calcium chloride ($CaCl_2$), potassium chloride (KCl), sodium chloride (NaCl), zinc chloride ($ZnCl_2$), iron chloride ($FeCl_2$), and aluminium chloride (Al_2Cl_3), [20] reported that the presence of metal ions in the substrate often stimulate the production of lipase.

The substrate specificity of lipase is important for their application in analytical and industrial purposes. The profile of the different substrates (palm oil, olive oil, soybean oil, and groundnut oil) was tested. The result of

the study showed that *Bacillus subtilis showed* most preference for olive oil as the best substrate. [21] reported *Bacillus* lipase with most preference to olive oil as substrate (100%) and then groundnut oil (95%). Similar hydrolytic activity in groundnut oil and olive oil substrates was reported by [22,23].

5. Conclusion

Lipase enzyme is much in demand because it is an important hydrolytic enzyme with innumerable applications in industries and biotechnological processes. A more economic process is required for producing lipase enzyme with the use of very cheap and easily accessible raw material.

This study has shown that lipase producing bacteria can be easily isolated from palm oil contaminated soil within our immediate locality. Further studies will be directed towards improving the ease with which enzymes could be isolated from microbes using very cheap and available raw material.

Conflict of Interests

The authors declare they have no conflict of interests.

References

[1] Reis, P.; Holmberg, K.; Watzke, H.; Leser, M. E. and Miller, R. (2008). Lipases at interfaces: A review. *Advances in Colloid and Interface Science*.

[2] Park, H.; Lee, K.; Chi, Y. and Jeong, S. (2005). Effects of methanol on the catalytic properties of porcine pancreatic lipase. *Journal of Microbiology and Biotechnology;* 15(2), 296.

[3] Gupta, N.; Shai, V. and Gupta, R. (2007). Alkaline lipase from a novel strain Burkholderiamultivorans: Statistical medium optimization and production in a bioreactor. *Process Biochemistry*; 42(2), 518.

[4] Grbavcic, S. Z., Dimitrijevic-Brankovic, S. I., Bezbradica, D. I., Siler-Marinkovic, S. S. and Knezevi, Z. D. (2007). Effect of fermentation conditions on lipase production by *Candidautilis*. *Journal of the Serbian Chemical Society;* 72(8-9), 757.

[5] Franken, L. P. G., Marcon, N. S., Treichel, H., Oliveira, D., Freire, D. M. G. and Dariva, C. (2009). Effect of treatment with compressed propane on lipases hydrolytic activity.*Foodand Bioprocess Technology*; 10:0087-0095.

[6] Joseph, B., Ramteke, P. W. and Thomas, G. (2008). Cold active microbial lipases: Some hot issues and recent developments. *Biotechnology Advances;* 26: 457-470.

[7] Schmid, R. and Verger, R. (1998). Lipases: Interfacial enzymes with attractive applications. *Angew. Chemical International Edition*; 37: 1608-1633.

[8] Titball, R. W. (1998). Bacterial phospholipases. Symp. Ser. Soc. *Applied Microbiology*; 27:127S-137S.

[9] Margesin, R.; Labbé, D.; Schinner, F.; Greer, C. W. and Whyte, L. G. (2003). Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. *Applied Environmental Microbiology*; 69: 3085-3092.

[10] Jaeger, K.E. and Eggert, T. (2004). Enantioselective biocatalysis optimized bydirected evolution. *Current Opinion Biotechnology*; 15: 305-313.

[11] Rosenau, F. and Jaeger, K. E. (2000). Bacterial lipases from *Pseudomonas*: Regulation of gene expression and mechanisms of secretion. *Biochimie;* 82:1023-1032.

[12] Kahveci, D., Guo, Z., Ozcelik, B. and Xu, X. (2009). Lipase-catalyzedglycerolysis in ionic liquids directed towards diglyceride synthesis. *Process Biochemistry*; 44: 1358-1365.

[13] Shah, S., Sharma, S. and Gupta, M. N. (2004).Biodiesel preparation by lipase catalysed transesterification of Jatropha oil. *Energy Fuels*; 18: 154-9.

[14] Kumar, S., Kikon, K., Upadhyay, A., Kanwar, S. S.and Gupta, R. (2005). Production, purification and characterization of lipase from thermophilic and alkaliphilic *Bacillus coagulans* BTS-3.*Protein Expression and Purification*; 41: 38-44.

[15] Nawani, N., Khurana, J. and Kaur, J. (2006). A thermostable lipolytic enzyme from a thermophilic *Bacillus* species. Purification and characterization. *Molecular Cellular Biochemistry*; 290: 17-22.

[16] Olusesan, A. T., Azura, L. K., Forghani, B., Abu Bakar, F., Mohamed, A. K., Radu, S., Abdul Manap, M. Y. and Saari, N. (2011). Purification, characterization and thermal inactivation kinetics of a non regioselective thermostable lipase from a genotypically identified extremophilic *Bacillus subtilis* NS N. *Biotechnology*.

[17] Lima, V., Krieger, N., Mitchell, D., Baratti, J., De Filippis, I., and Fontana, J. (2004). Evaluation of the potential for use in biocatalysis of a lipase from a wild strain of *Bacillusmegaterium*. *Journal of Molecular Catalyst and Biological Enzyme*; 31: 53-61.

[18] Kambourova, M., Kirilova, N.; Mandeva, R. and Derekova, A. (2003). Purification and properties of thermostable lipase from a thermophilic *Bacillus stearothermophilus* MC. *Journal of Molecular Catalyst and Biological Enzymes*; 7(22): 307-313.

[19] Singh, M., Kumar, S., Neha, S. and Krishnan. K. (2010). Lipase Production by *Bacillus subtilis* OCR-4 in Solid State Fermentation Using Ground Nut Oil Cakes as Substrate. *Current Research Journal of Biological Sciences* 2(4): 241-245.

[20] Prasad, M. P. and Sethi, R (2013). Comparative Studies on the production of Lipase by *Bacillus* species under various growth parameters. *Int.J.Curr. Microbiol. App. Sci*2 (11): 179-185.

[21] Praveen, D. and Sharmishtha, P. (2011). Isolation, screening and production of extracullular alkaline lipase from a newly isolated *Bacillus* species. PD-12. *Journal of Biological Sciences;* 11(5): 381-387.

[22] Kojima, Y., Kobayashi, M. and Shimizu, V. (2003). A Novel Lipase from *Pseudomonas fluorescens* HU380: Gene Cloning, Overproduction, Reneturation-Activation, Two-Step Purification, and Characterization. *Journal of Bioscience and Bioengineering*; 96(3): 242-249.

[23] Dahiya, P., Arora, P., Chand, S., Chandhury, A. and Dilbaghi, N. (2010). Characterization of an extracellular alkaline lipase from *Pseudomonas mondocina*M-37.*Journal of Basic Microbiology*. 50: 420-426.