Isolation Of Lipolytic Bacteria From Waste Contaminated Soil: A Study With Regard To Process Optimization For Lipase

Kalpana Sagar, Yasir Bashir, Mayur M Phukan, B. K. Konwar

Abstract: Production of valuable metabolites by micro-organisms has always been one of the main areas of intense scientific research over the years. In this regard, the present study was undertaken to isolate lipase producing bacteria from waste contaminated site and subsequent optimization of culture parameters (temperature, pH, incubation time, agitation speed, substrate specificity) for extra-cellular lipase production. Effect of carbon source, nitrogen source and metals ions were further assessed to determine the maximum lipase activity. Selection of bacterial isolates was achieved by culture in Tributyrin agar (a selective media for lipase producing bacteria). Out of 18 bacterial isolates, 2 isolates (TU-L1 and TU-L2) were found to be prominent with regard to lipolytic activity. The optimal temperature, pH and incubation time conditions for TU-L1 and TU-L2 were 45ºC, 8.0, 18h; and 37ºC, 7.0 and 24h respectively. The present study warrants the feasibility of isolation of industrially important microbes from waste contaminated sites.

Keywords: Bacteria, Carbon source, Extra-cellular, Kitchen waste, Lipase, Oil remnants, Triacylglycerol

1 Introduction

Hydrolytic enzymes like lipases furnish the greatest share in the industrial enzyme market. Lipases (Triacylglycerol, EC 3.1.1.3) are a major group of biocatalysts that catalyze the hydrolysis of triacylglycerol to glycerol and fatty acids [1]. In wake of recent advancements in microbiology & biotechnology, lipases have emerged as key enzymes owing to their multifaceted properties which find use in a wide array of industrial applications [2, 3]. Lipase have been isolated and purified from fungi, yeast, bacteria, plant and animal sources but bacterial lipases are more economical and stable [4]. Bacterial lipases are extensively used in food and dairy industry, cheese ripening, flavour enhancement [5], detergent industry [6], textile industry [7], for synthesis of biodegradable polymers or compounds [8], different transesterification reactions [9], cosmetic industry [10], in pulp and paper industry [11], in synthesis of biodiesel [12], and in pharmaceutical industries [13]. The present investigation was carried out to isolate lipase producing bacteria from waste contaminated (basically kitchen waste dumping) sites. It is arguable to expect the presence of lipase producing bacteria in such sites since numerous lipid remnants from cooking and non-cooking processes are directly dumped in these sites. The oil remnants in these sites comprises of long chain triacylglycerols which are natural substrates of lipases. Subsequent optimization of culture parameters for lipase production has further been addressed.

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2 Materials and Methods

2.1 Study site

The study site for the current investigation was the domestic waste dumping site [26.7008°N and 92.8303°E] of Tezpur University, Assam, India.

2.2 Sample collection

Replicate soil samples were collected from a depth of 5-10 cm with the help of a sterile spatula and stored in sterile plastic bags. Following collection, the soil samples were immediately transferred to the laboratory for examination and subsequent analysis..

2.3 Isolation and screening of Lipase producing bacterial strains

A total of eighteen bacterial isolates were isolated from the study site by serial dilution. For serial dilution, 1 gm of soil sample was dissolved in 10 ml of sterile distilled water in a 50 ml Erlenmeyer flask, and agitated at 120 rpm for 30 min at 37°C on a rotary shaker. The sample (aqueous slurry) was serially diluted up to 10^{-6} dilution using 0.8% saline. 100 µl of each dilution was spread on tributyrin agar plates containing 0.5% (w/v) peptone, 0.3 % (w/v) yeast extract, 1% tributyrin and 2% agar by spread plate technique. The plates were incubated at 37°C for 24-72 hours following which the lipolytic activity was determined (visual observation by the formation of zone of hydrolysis around the bacterial colonies). Out of eighteen isolates, two accessions viz., TU-L1 and TU-L2 were found prominent with regard to lipolytic activity. The criteria for selection of the above accessions were on the basis of utilization of different substrates like tributyrin, olive oil, and anionic detergents (tween-20 and tween 80).

2.4 Morphological and biochemical characterization of lipase producing bacterial strains

The morphological and biochemical characterization of lipase producing bacterial isolates was done in accordance with the Bergey's manual of systematic bacteriology.

2.5 Lipase production and Isolation

The liquid culture medium (Tryptic soy broth) containing (g/L) pancreatic casein, 17; enzymatic digest soybean, 3; NaCl, 5;

dipotassium phosphate, 2.5; glucose, 2.5; 1% olive oil; pH 7.5 was used for lipase production. For the production of lipase, an Erlenmeyer flask (250 ml) containing 100 ml of medium was inoculated with an aliquot of approximately 1% of the preculture prepared in LB broth (g/L); Tryptone, 10; Yeast extract, 5: NaCl, 10; pH 7.5. The inoculated flasks were then incubated for a period of 16 hours at 37ºC with constant shaking at 200 rpm. Cell-free supernatant was recovered by centrifugation at 5,000 rpm for 15 min at 4ºC and the clear supernatant was used to determine the lipolytic activity by universal titrimetric method [14, 15].

2.6 Enzyme Assay

Lipase activity was measured by titrimetric method using olive oil as substrate. 10% olive oil (v/v) was emulsified with 5% gum Arabic (w/v) in 50 mM sodium phosphate buffer pH 7.0. 100 µl of cell free culture supernatant was added to the emulsion and incubated for 15 minutes at 37ºC. The reaction was stopped and fatty acids were extracted by addition of 1.0 ml of acetone: ethanol solution (1:1). The amounts of fatty acids liberated were estimated by titrating with 0.05M NaOH until pH 10.5 using phenolphthalein as an indicator [16].

2.7 Optimization of lipase production

The lipase production was determined after culture in tryptic soy broth from 6 to 72 h at 28ºC, 30ºC, 37ºC, 40ºC, 45ºC, 50ºC, 55ºC, 60ºC. The lipase production was then evaluated over a wide range of pH ranging from 3 to 10.5. The pH of the medium was adjusted prior to autoclaving. Further, the changes in lipase production in response to the following carbon sources (1% w/v) were evaluated: glucose, fructose, xylose, lactose, sucrose, mannitol and starch. Olive oil, Soybean oil, tributyrin, and anionic detergents (Tween-80 and Tween-100) were used as lipase inducers at a concentration of 0.5% (v/v). The positive control for the assay comprised of a medium with no lipase inducer.

2.8 Optimization of Media Parameters for Profound Enzyme Activity

2.8.1 Effect of Incubation Period on Lipase Activity

TU-L1 and TU-L2 were cultured in media containing yeast extract, peptone and NaCl. 1% Tributyrin and olive oil were added into culture media as a substrate for TU-L1 and TU-L2 respectively. Bacterial cultures were incubated at 37ºC in an orbital shaker with an agitation speed of 180 rpm. The culture broth was harvested after 6 hour intervals by centrifugation at 8000xg for 20 min and 4ºC. The supernatant was used as crude enzyme solution and was assayed for enzyme activity.

2.8.2 Effect of Temperature on Lipase Activity

TU-L1 and TU-L2 were cultured at temperatures ranging from 20ºC to 45ºC to select the optimum temperature for maximum enzyme production by keeping the remaining parameters constant.

2.8.3 Effect of pH on Lipase Activity

The optimum pH for TU-L1 and TU-L2 for maximum enzyme production was optimized by varying the pH of the tributyrin broth from 5 to 11 whereas the other parameters were unaltered.

2.8.4 Effect of Metal Ions on Lipase Activity

To evaluate the effect of various divalent metal ions on enzyme activity, Hg2+, Fe2+, Zn2+, Cu2+, Ni2+, Mn2+, Ca2+, Co2+ and Mg2+ were added to the reaction mixture at a final concentration of 1mM.

2.8.5 Effect of Nitrogen Sources as Lipase Inducers

Different nitrogen sources like yeast extract, soya bean, tryptone, and peptone were added to the broth at varying concentrations to see the effect of nitrogen source on enzyme activity. Remaining parameters were unaltered.

2.8.6 Effect of Carbon Sources as Lipase Inducers

TU-L1 and TU-L2 were cultured in tributyrin broth supplemented with different carbon sources like sucrose, xylose, fructose, mannitol, starch, and glucose to the culture media at varying concentrations to see the effect of carbon source on enzyme activity. Remaining parameters were unaltered.

2.8.7 Effect of Substrate on Lipase Activity

To evaluate the effect of various substrates on lipase activity, 1% of different substrates like tween-20, tween-80, tributyrin, olive oil, sunflower oil, mustard oil, groundnut oil and soybean oil were added to the reaction mixture.

3. Results and Discussions

3.1 Screening and Morphological Identification of Lipolytic Isolates

A total of 18 morphologically distinct bacterial strains were isolated from waste-contaminated soil of Tezpur University, Assam, India. Waste contaminated soil in this study basically refers to soil from kitchen waste dumping sites. Kitchen wastes usually comprise of a significant proportion of oil remnants, and as such it is quiet arguable to expect a wide range of bacterial strains in these sites which may utilize oil remnants (lipids) as the sole carbon source. The isolated strains were screened for extracellular lipase using Tributyrin agar media. Two of the isolates (TU-L1 and TU-L2) produced larger clear zone (23 mm and 21 mm respectively) than the others, indicating higher lipase activity. These two isolates were identified based on morphological and biochemical characterization (Table 1). Both the bacterial strains were Gram positive and coccoid in shape. In accordance with the Bergey's manual of systematic bacteriology, the isolates were likely to be belonging to genus *Staphylococcus*.

3.2 Optimization of Culture Conditions

TU-L1 showed maximum enzyme activity at 45ºC and pH 8.0 whereas TU-L2 showed maximum enzyme activity at 37ºC and pH 7.0 (Fig 1). Both the enzymes showed stability over a pH range of 6.0-11.0 (Fig 2). The maximum enzyme activity was observed at 18 hour and 24 hour of incubation time for TU-L1 and TU-L2 respectively. The enzyme activity gradually decreased after 18 hour of incubation time for TU-L1 and 24 hour of incubation time for TU-L2 (Fig 3). Lipases active and stable in alkaline media are very attracting, for example, lipase produced by Acinetobacter radioresistens has an optimum pH of 10.0 and it was stable over a pH range of 6.0-10.0; this enzyme has a great potential for application in the detergent industry [17,18]. TU-L2 also showed an optimum pH of 8.0 (alkaline) and was stable over a wide pH range, so we can

infer it may possibly have applications in the detergent industry.

Test	TU-L1	TU-L2
Colony Morphology		
Configuration	Circular	Circular
Margin Elevation	Entire	Entire
Surface	Smooth	Smooth
Density	Opaque	Opaque
Pigments	Cream	White
Gram reaction	Positive	Positive
Shape	Cocci	Cocci
Size	Small	Medium
Arrangement	Single	Single or in pairs
Biochemical Tests		
Citrate Utilization	+	+
Catalase	+	+
Gelatin liquefaction	+	+
Nitrate reduction	+	+
Oxidase	-	-
Indole	+	+
Methyl red	-	-
Voges-Proskauer	-	+
Urease	-	-
Casein Hydrolysis	+	+
Starch Hydrolysis	+	+
Cellulose Hydrolysis	-	+
Tween-80 Hydrolysis	+	+
H_2S production	+	+
Motility Test	Motile	Motile
Sugar Fermentation		
Glucose	+	+
Sucrose	+	+
Lactose	+	+
Xylose	+	+
Fructose	+	+
Maltose	+	+
Galactose	+	+
Mannitol	+	+

Table 1: Morphological and Biochemical Characterization of TU-L1 and TU-L2

3.3 Effect of Culture Variable on Enzymatic Activity

The major factor for the expression of lipase activity has always been carbon, since lipases are inducible enzymes. Beside carbon source, the type of nitrogen source in the medium also influences the lipase titers in production broth [19]. Generally, microorganisms provide high yields of lipase when organic nitrogen sources are used, such as peptone and yeast extract, which have been used for lipase production by various thermophilic *Bacillus sp*. and various *Pseudomonads* [20,21]. For selection of optimum carbon and nitrogen sources for maximum enzyme activity, different concentrations of various carbon and nitrogen sources were supplemented with Tributyrin broth media. TU-L1 showed maximum enzyme activity when Tributyrin broth media was supplemented with 0.5% sucrose as carbon source whereas TU-L2 showed maximum enzyme activity when Tributyrin broth media was supplemented with 0.5% lactose as carbon source (Fig 4). The enzymes from both the isolates showed maximum activity when 1% peptone was added as the nitrogen source (Fig 5).

3.4 Effect of Metal Ions on Enzyme Activity

TU-L1 showed maximum enzyme activity in presence of Cu^{2+} whereas TU-L2 showed maximum enzyme activity in presence of Cu^{2+} as well as Mg^{2+}. Previously, it has been demonstrated that the activity of *staphylococcal* lipases may depend on the presence of Ca^{2+} ions [22] whereas in our study, Ca^{2+} was found to have a less significant effect on enzymatic activity as compared to Cu^{2+}. Other metals such as Zn^{2+}, Co^{2+} and Hg^{2+} etc. had no significant effect on enzyme activity in our study (Fig 6).

3.5 Effect of Substrate on Enzyme Activity

TU-L1 exhibited maximum enzyme activity when the media was supplemented with 1% Tributyrin while TU-L2 exhibited maximum enzyme activity when the media was supplemented with 1% olive oil. Apart from these, the other substrates also showed positive effect on the enzymatic activity but it was less significant and in case of tween-20, the effect was the least (Fig 7).

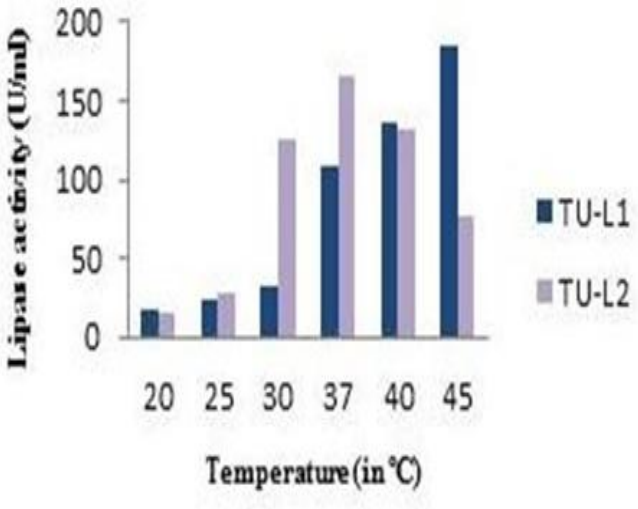

Fig 1: Effect of temperature on Enzyme activity

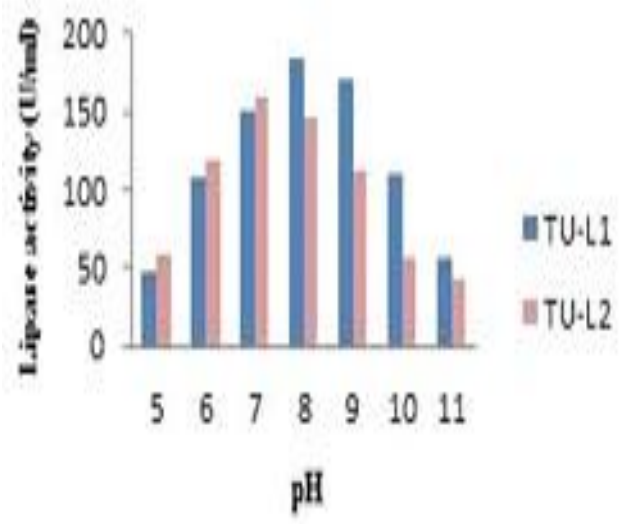

Fig 2: Effect of pH on Enzyme activity

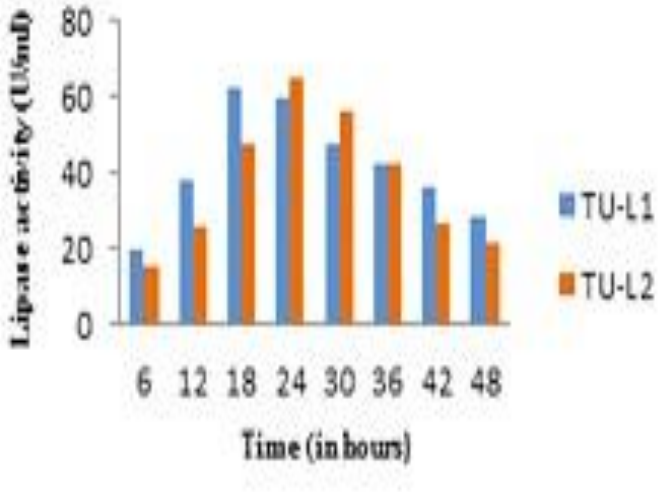

Fig 3: Effect of Incubation period on Enzyme activity

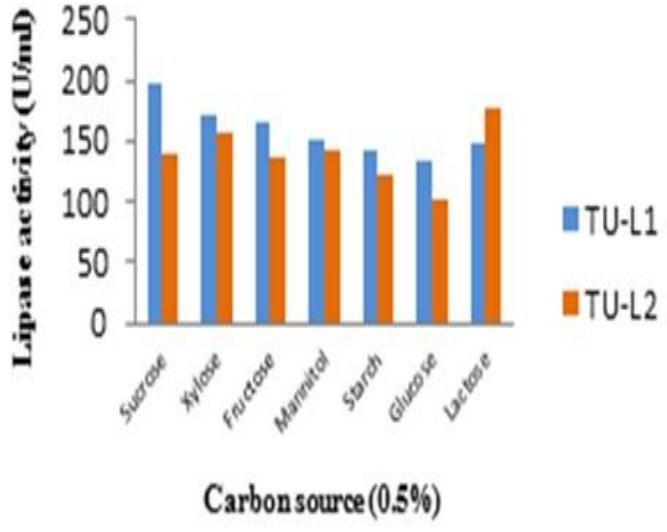

Fig 4: Effect of Carbon source on Enzyme activity

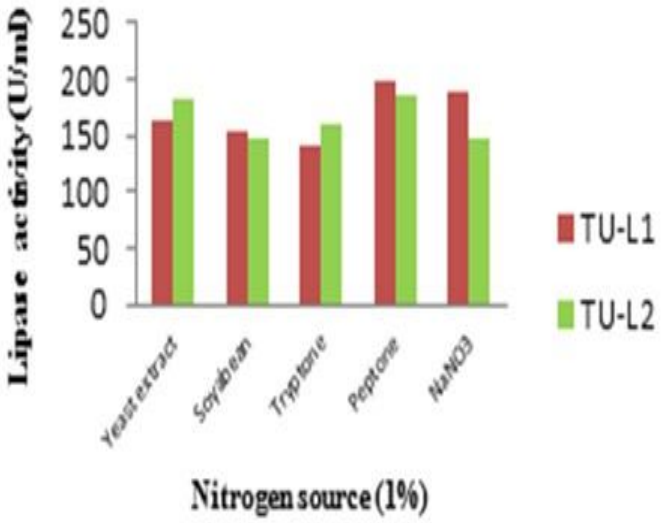

Fig 5: Effect of Nitrogen source on Enzyme activity

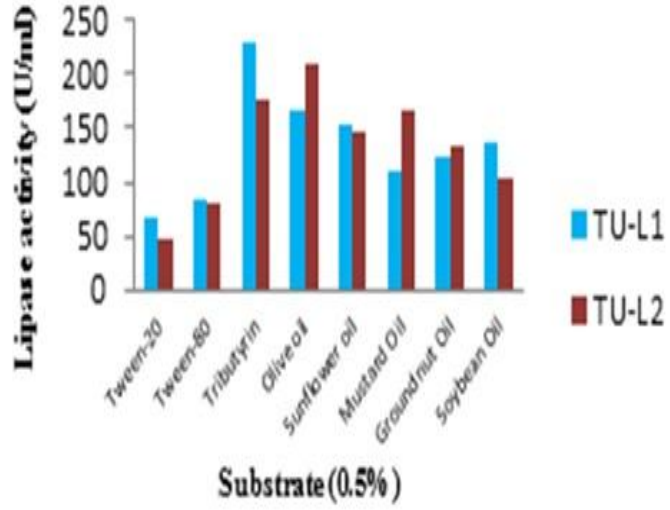

Fig 6: Effect of Substrate on Enzyme activity

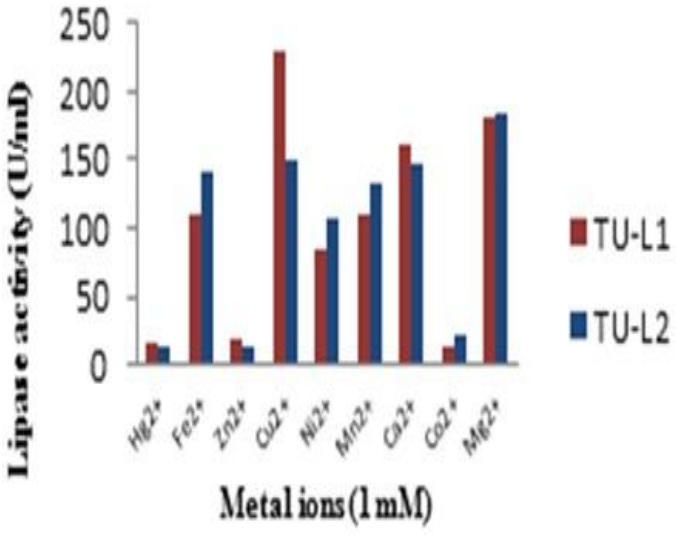

Fig 7: Effect of Metal ions on Enzyme activity

Conclusion

Experimental results suggest that various media compositions influenced enzyme (lipase) production by indigenously isolated bacterial strains TU-L1 and TU-L2. Optimization of growth parameters viz., temperature, pH, agitation (rpm), carbon and nitrogen source etc. had significant effect on lipase activity. However, the present study requires greater research capacities (further purification of the crude enzyme) for comparison with commercial lipase with regard to specific activity. The study also suggests that waste contaminated sites (dumped with kitchen wastes, which are usually comprised of numerous lipid remnants from cooking and non-cooking processes) may serve as excellent breeding grounds for the isolation of lipolytic bacteria of industrial significance.

Acknowledgments

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References

[1] R. Sharma, Y. Chisti, and U. C. Banerjee, “Production, purification, characterization, and applications of lipases,” Biotechnol. Adv. **Vol**. 19, 2001

[2] S. Benjamin and A. Pandey, “Optimization of liquid media for lipase production by *Candida rugosa*,” Bioresource Technology, vol. 55, no. 2, 1996

[3] K. E. Jaeger, S. Ransac, B. W. Dijkstra, C. Colson, M. van Heuvel, and O. Misset, “Bacterial lipases,”. FEMS Microbiology Reviews, Vol. 15, 1994

[4] E. A. Snellman, E. R. Sullivan and R. R. Colwell, “Purification and properties of the extracellular lipase, *LipA*, of *Acinetobacter* sp. RAG-1,” European Journal of Biochemistry, Vol. 269, 2002

[5] E. A. Falch, “Industrial enzymes — Developments in production and application,” Biotechnol Advances, Vol. 9, 1991

[6] T. Fujii, T. Tatara, M. Minagawa, “Studies on applications of lipolytic enzyme in detergency I. Effect of lipase from *Candida cylindracea* on removal of olive oil from cotton fabric,” Journal of the American Oil Chemists’ Society, Vol. 63, 1986

[7] R. Sharma, Y. Christi and U.C. Banerjee, “Production, purification, characterization and applications of lipases,” Biotechnology Advances, Vol. 19, 2001

[8] Y. Y. Linko, M. Lamsa, X. Wu, E. Uosukainen, J. Seppala and P. Linko, “Biodegradable products by lipase biocatalysis, “ Journal of Biotechnology, Vol. 66, 1998

[9] F. Hasan, A. A. Shah and A. Hameed, “Industrial applications of microbial lipases,” Enzyme and Microbial Technology, Vol. 39, 2006

[10] E. W. Seitz, “Industrial application of microbial lipases: A review, “Journal of the American Oil Chemists’ Society, Vol. 51, 1974

[11] P. Bajpai, “Application of enzymes in the pulp and paper industry,” Biotechnology Progress, Vol. 15, 1999

[12] H. Noureddini, X. Gao and R.S. Philkana, “Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil,” Bioresource Technology, Vol. 96, 2005

[13] S. Higaki, and M. Morohashi, Propionibacterium acnes lipase in seborrheic dermatitis and other skin diseases and Unsei-in. Drugs under experimental and clinical research, Vol. 29, 2003

[14] J Cardenas, E Alvarez, M-S de Castro-Alvarez, J-M Sanchez-Montero, M Valmaseda, SW Elson, and J-V Sinisterra, “Screening and catalytic activity in organic synthesis of novel fungal and yeast lipases,” Journal of Molecular Catalysis B: Enzymatic, Vol. 14, 2001

[15] T. Godfrey, and S. West, “Introduction to Industrial Enzymology,” In: Industrial Enzymology, T. Godfrey, and S. West (Eds). 2nd Edn, Stockholm Press, New York, 1996

[16] R.G. Jensen, “Detection and determination of lipase (acylglycerol hydrolase) activity from various sources,” Lipids, Vol. 18, 1983

[17] S. J. Chen, C. Y. Cheng, T. L. Chen, “Production of an alkaline lipase by *Acinetobacter radioresistens*,” Journal of Fermentation and Bioengineering, Vol. 86, 1998

[18] A. Illanes, “Stability of biocatalysts,” Electronic Journal of Biotechnology, Vol. 2, 1999

[19] E. M. Qamsari, R. K. Kermanshahi, and Z. M. Nejad, “Isolation and identification of a novel, lipase-producing bacterium, *Pseudomonas aeruginosa* KM110,” Iranian Journal of Microbiology, Vol. 3, 2011

[20] R. Sharma, S. K. Soni, R. M. Vohra, R. S. Jolly, L. K. Gupta, J. K. Gupta, “Production of extracellular alkaline lipase from a *Bacillus* sp. RSJ1 and its application in ester hydrolysis,” Indian Journal of Microbiology, Vol. 42, 2002

[21] Sugihara, T. Tani, and Y. Tominaga, “Purification and characterization of a novel thermostable lipase from *Bacillus* sp,” Journal of Biochemistry, Vol. 109, 1991

[22] Sayari, N. Agrebi, S. Jaoua, and Y. Gargouri, “Biochemical and molecular characterization of *Staphylococcus simulans* lipase,” Biochimie, Vol. 83, 2001