ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Improvement Lipase Thermal Stability Using Immobilization Techniques.

Tahani T. Al-rehaili[1,2], Enas N. Danial[1,2,3]*, and Najla O. Ayaz[1,2].

[1]Biochemistry Department, Faculty of Science- King Abdulaziz University, Jeddah, Saudi Arabia.
[2]Applied Biochemistry Department, Faculty of Science, University of Jeddah, Jeddah, Saudi Arabia.
[3]Chemistry of Natural and Microbial Products, National Research Centre, Cairo, Egypt.

ABSTRACT

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids. Lipases occur widely in nature, but only microbial lipases are commercially significant. Many applications of lipases include specialty organic syntheses, hydrolysis of fats and oils, modification of fats, flavor enhancement in food processing, resolution of racemic mixtures, and chemical analyses. The aim of this study is to evaluate the biological importance of free and immobilized lipase enzyme. The support material used for lipase immobilization was sodium alginate with concentration 2%. Mostly, the immobilization of lipase in alginate gel exhibit more stability forheating than the free enzyme. The effect of metal ions Mg^{2+}, Hg^{2+}, Cu^{2+}, Na^{+} andCa^{2+}at a concentration 2 mol/ml used in the study showed inhibitory effect on free more than the immobilized lipase enzyme. These results highlighted the biochemical and technical advantage benefit of immobilized lipase over the free enzyme.

Keywords: lipase; kinetic studies; thermal stability; inhibitor and immobilized.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Lipases(EC 3.1.1.3) have several applications including hydrolysis of oils and fats, resolution of racemic mixtures, organic syntheses, and modification of fats, chemical analyses, and flavor enhancement food processing [1]. Lipases also transesterifytriacylglycerols or synthesize ester bonds in low aqueous media [4-6].Industrial Potential applications of lipases comprise pharmaceutical and the medical area (digestive and medicines enzymes for diagnosis), food additives (modify of aromas), wastwatertreatment (removal and decomposition of oleaginous substances), detergents (fats hydrolysis), fine chemicals (synthetic esters) and leather (removal of fat from animal skin) [5,6].

Lipases are widely extracted from animals, plants and microorganisms. Most of the enzymes uses in industries are of microbial origin. It catalyzes the hydrolysis of esters for long chain aliphatic acids from glycerol at lipid and water [7]. Several microorganisms are produced lipases by, viz., eucarya, fungi, actinomycetes, yeast, bacteria, archea, etc. Microbial produced lipases include: Bacillus, Penicillium, Staphylococcus, Geotrichum, Pseudomonas and Aspergillus[8].

However, the major contributions of microbial lipases are in the detergent formulations. The large reason in lipases for steadily growing interest is because of their enantio-selective, region-selective and chemo-selective nature [9]. Microorganisms with potentials to produce lipases can be found in multiple habitats, including wastes of dairy industries and soils contaminated by oils, vegetable oils, deteriorated food, and seeds [1]. The addition of by-products as substrates for lipase production is to rise the value and lower the cost [10].

Productions of extracellular microbial lipases are influenced by cultivation parameters such as pH, temperature, dissolved oxygen and medium composition. Lipase activity was also affected by carbon source, since lipases are inducible enzymes. These enzymes are produced in the presence of any lipid such as oil or other inducer, viz., fatty acids and triacylglycerides. Several studies have showed that hydrolysable esters, bile salts, tweens and glycerol is a suitable carbon source for production of microbial lipases [11]. Furthermore, essential micronutrients and nitrogen sources should be added in fermentation medium.

Thermo stable lipases from microbial are only commercially significant for their potential use in industries, hydrolysis of oils and fats, enhancement flavor and modification of fats in food processing [12,13], chemical analyses and resolution of racemic mixtures [14]. The major requirement for thermal stability of lipase is to perform the reaction at higher temperatures, substrate solubility and may be useful for increase the conversion rates, and reducing the contamination of microorganism.

However, little is known about production of heat-stable lipases [15]. Mesophilic bacteria or fungi that produce most of thermostable lipases are commercially available. Production of thermostable lipases from thermophilic strains is of importance in industrial processes due to the valuable role of lipases in the enzyme market [16,17].

The immobilization of enzymes has proven particularly valuable, because it has allowed enzymes to be easily reused multiple times for the same reaction with longer half-lives and less degradation and has provided a straightforward method of controlling reaction rate as well as reaction start and stop time. It has also helped to prevent the contamination of the substrate with enzyme/ protein or other compounds, which decreases purification costs. These benefits of immobilized enzymes have made them highly applicable to a range of evolving biotechnologies[18].

MATERIALS AND METHODS

Lipase producing bacteria

Among different bacterial strains isolated from soil samples contaminated with oil collected from Arabian-American Oil Company,Rabigh, Saudi Arabia, a potent strain which gave a high yield of lipase was chosen for further study. The isolated strain was routinely grown on growth medium at 45°C for 2-3 days then preserved at 4°C in nutrient agar medium.

Lipase activity assay

One ml of culture filtrate was mixed with 2 ml of 0.1 M of potassium phosphate buffer (pH 7.5), 5ml of 10% (v/v) triglyceride emulsion **(**10 % (v/v) of the triglycerides olive oil emulsified in 10 % (w/v) Arabic gum in a hot water and homogenized in a top drive homogenizer for 10 min. The mixture was incubated for 30 min at 37 ºC in a shaking water bath. At the end of incubation time 15 ml of acetone: alcohol (1:1) was added and the resulting mixture was then titrated against 0.05 N NaOH using phenolphthalein as indicator.

One unit of lipase activity was defined as the amount of enzyme that produced 1 µmole of free fatty acids per min under the standard assayed conditions. The activities were expressed in U/ml[19].

Assay of immobilized lipase

The alginate beads (17 beads) with lipase entrapped were taken in 2 ml potassium phosphate buffer (0.10 M, pH 7.5) solution and reacted with 5ml of 10% (v/v) triglyceride emulsion for 30 min. The same procedure was used to follow the reaction and the measurements were performed in the same manner as for the native enzymes studies. Here, the beads were removed before chilling the solution and 15 ml of acetone: alcohol (1:1) was added to stop the reaction [19].

Preparation of immobilized lipase enzyme

Lipase enzyme was mixed with 2% Sodium alginate then the mixture was Dripping into 2%,$CaCl_2$ solution (w/v) from a constant.

Figure 6: Optical images of alginate beads immobilized by lipase.

Effect of different pH on lipase enzyme

The free and immobilized enzymes were incubated for 30 min at various pHs(5,6,7, 8, 9,10), lipase activity was determined.

Optimum temperature of lipase enzyme

The free and immobilized enzymes were incubated for 30 min at various temperatures (25, 37, 45, 55, 65̊C), lipase activity was determined as described before.

Thermal stability of lipase enzyme

The effect of thermal stability on lipase enzyme was monitored by incubating the enzyme in different time period(0, 15, 30, 45, 60, 75, 90and 105 min) at different incubation temperature (25, 40, 50, 70̊C), lipase activity was determined as described before.

ISSN: 0975-8585

Effect of inhibitor on lipase enzyme

Inions and cations with concentration of 2 mol/ml were added to Potassium phosphate buffer to study the effect of these ions and cations on free and immobilized lipase.

Effect of different enzyme concentration on lipase enzyme

The free and immobilized lipase were incubated for 30 min at different enzyme concentrations (0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2), lipase activity was measured as described before.

Effect of different substrate concentration on lipase enzyme

The free and immobilized lipase were incubated for 30 min at various different substrate concentrations (5, 10, 15, 20, 25, 30, 35, 40, 45)mM, lipase activity was measured as described before.

RESULTS AND DISCUSSION

Optimum pH

The effect of pH on the activity of free enzymes and immobilized enzymes were incubated for 30 min at 37 ^{o}CIn the pH range 5- 10 as shown in Figure 1, the alginate beads encapsulated with enzyme exhibited a comparable stability. The effect of different pHs on free and the immobilized lipase were very close as they were at pH 6.Within a certain pH range, most proteins were stable. As the proteins contacted with a very high pH media, the ionic groups within the protein molecule would produce a strong electrostatic repulsion, which made the protein molecule stretching, degeneration and destruction of the enzyme active center possibly occurred, resulting in the decrease in enzymatic activity. Generally, after immobilization, the lipase enzyme molecules in the alginate pore was restricted, and the stretching of the enzyme molecules was blocked. Thus, the pH stability of the immobilized enzyme expected to be higher than that of the free enzyme [20].

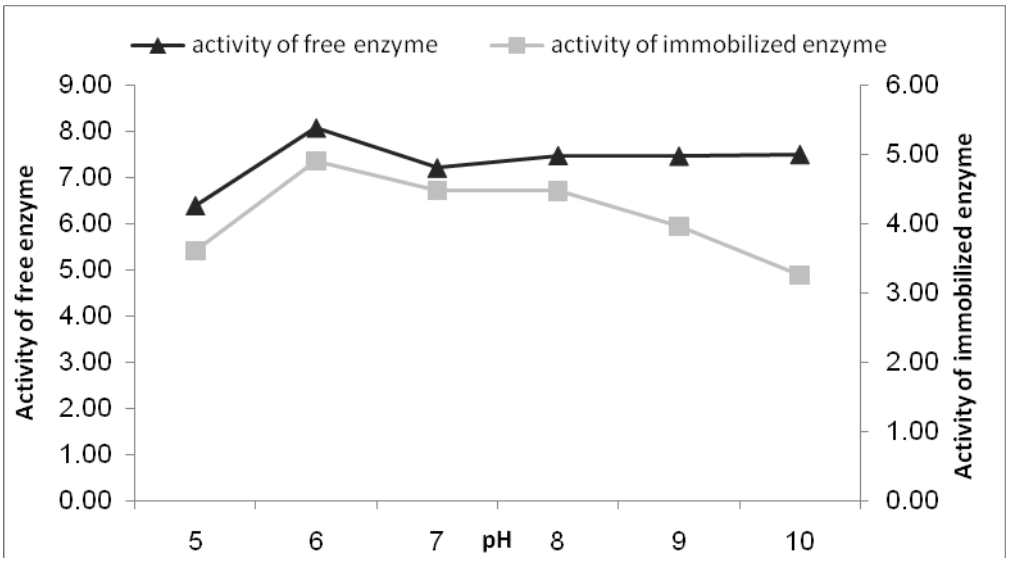

Figure 1: pH profile of free and immobilized lipase.

Optimum temperature

The effect of temperature on the activity of free enzymes and immobilized enzymes was investigated by using olive oil as a substrate at pH 6.0 in the temperature range of 25-65 °C as shown in Figure 2. The activity of the lipase increased with the increase of the operating temperature up to 50°C. The maximum activity of both free and immobilized enzymes appeared at 37°C. However, above 50°C, the enzyme activity of the free enzyme was significantly decreased with the increasing temperature, while the enzyme activity of the

ISSN: 0975-8585

immobilized enzymes slightly decreased.Comparable perceptions upon the immobilization oflipasedifferent enzyme have been accounted for byAwang[21].

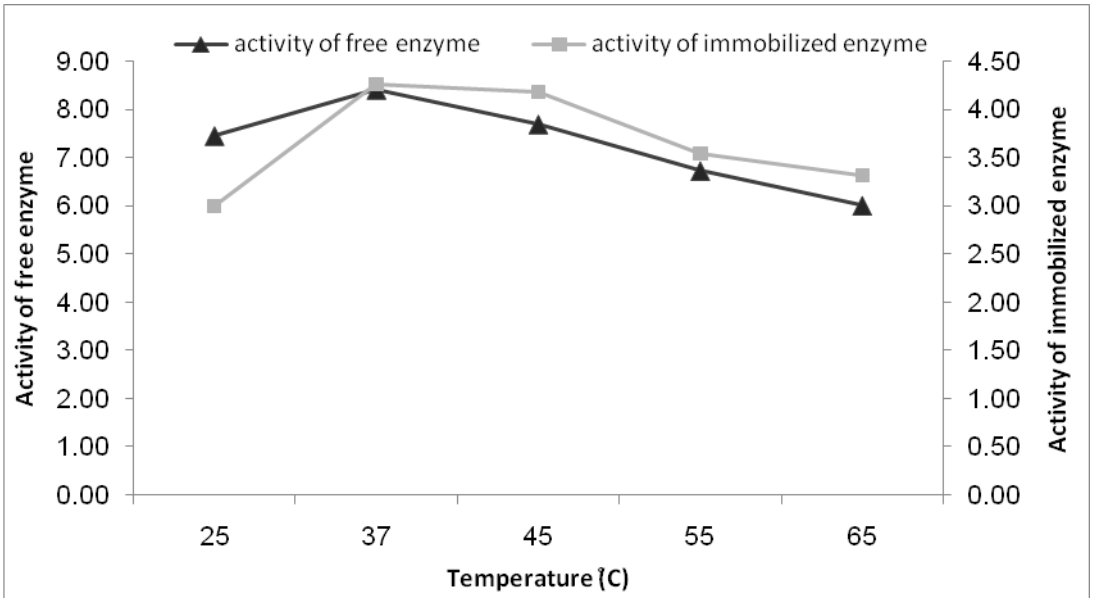

Figure 2: Optimum temperature profile of free and immobilized lipase.

Thermal Stability of lipase enzyme

One of the primary objectives of this examination was too efficient the enzyme thermal stability to be reasonable for applicable utilize. The immobilized enzyme expose a higher thermal strength over the free protein as appeared in Table (1), which demonstrated the profile of stability for free and immobilized lipase. Obviously the relative activities of the immobilized lipase were 78% while the free lipase 39% at 50ºC after 30 min for a similar incubation period. The relative activities of the immobilized and free compounds were keeping at 82 and 62% at 60°C for 30 min, respectivelyas shown in Table1. The thermal stability of immobilized lipase increased considerably as a result of immobilization in alginate gel. These results could be explained by the existence of a local environment for the immobilized enzymes, which was less damaging than bulk solution conditions. These outcomes could be clarified by the presence of the immobilized proteins, which was less harming than free proteins in solution; these results were in accordance with [22].

Table 1: Thermal stability of free and immobilized lipase

Time (min)	Temperature (°C)							
	30		40		50		70	
	Enzyme Activity (U/min/ml)		Enzyme Activity (U/min/ml)		Enzyme Activity (U/min/ml)		Enzyme Activity (U/min/ml)	
	Free	Imm	Free	Imm	Free	Imm	Free	Imm
0	100	100	100	100	100	100	100	100
15	100	100	92	100	39	58	14	12
30	100	100	83	100	35	52	13	12
45	92	100	65	95	29	52	3	10
60	86	100	63	87	28	50	2	3
75	69	95	59	81	8	47	0	0
90	68	94	56	70	13	41	0	0
105	0	0	0	0	0	0	0	0

ISSN: 0975-8585

Effect of inhibitoron lipase activity

In order to determine the effect of metallic ions on lipases activity, assays were performed at the concentrations of 2 mmol of the metal ions usedMg^{2+}, Hg^{2+}, Cu^{2+}, Na^{+} and Ca^{2+}. Figure 3showthat heavy metals inhibit lipase activity[23].

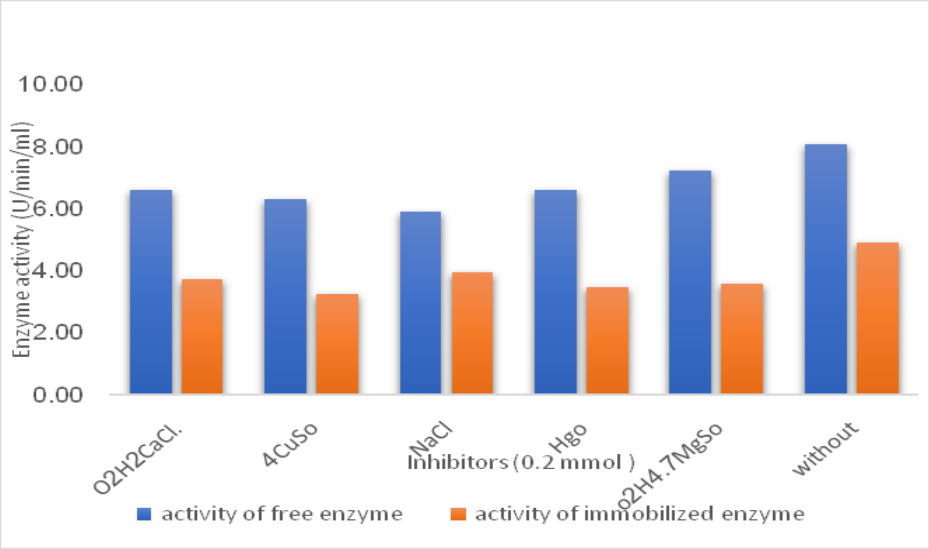

Figure 3: metal ions profile of free and immobilized lipase.

Effect of enzyme concentration on lipase activity

Lipase activity was measured at different concentration of enzyme from 0.2 ml to 2 ml. The result Figure4 showed that for rising enzyme concentration from 0.2 to 2 ml, there was a corresponding increase in the rate of reaction with the increase in the enzyme concentration from 0.2 to 2 ml.

The effect of enzyme concentration on the activity of free enzymes and immobilized enzymes was investigated by using olive oil as a substrate at pH 6.0 in the temperature 37°C, Figure 4 shows the amount of lipase in free and immobilized solution ranging from 0.2 to 2 ml.

It can be seen that both the free and immobilized enzymes activity increased with the increase of lipase provided.The maximum activity of enzymes was increased from 1 to 1.2 ml.However, the enzyme loading kept increasing until the lipase concentration reached 1.2ml[24].

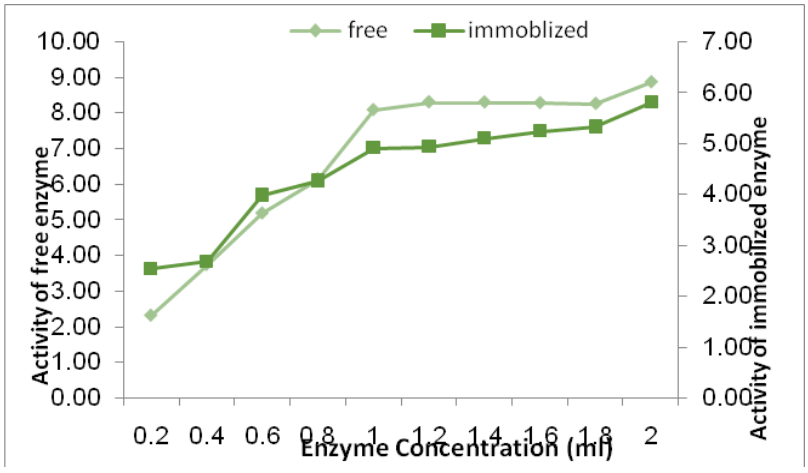

Figure 4: enzyme concentration of free and immobilized lipase.

ISSN: 0975-8585

Effect of olive oil concentration on lipase activity

In order to find the optimum substrate concentration, different amounts of olive oil was added to hydrolysis medium from 5 mM to 45 mM. In Figure 5 show that effect of olive oil concentration can be seen with comparison of free and immobilized lipase activity. The result showed that for rising substrate concentration from 5 to 45mM, there was a corresponding increase in the rate of reaction with the increase in the substrate concentration from 5 to45mM.This results were agreement with [13]who reported the increasing of substrate value indicated that the enzyme has high affinity and specificityto the olive oil .

Kinetic parameters of the hydrolytic reaction of fatty acid using the free and immobilized lipase were investigated. (Figure 6)represents the relation between initial rate and substrate concentration for the free and immobilized lipase. From the Lineweaver-Burk plot of 1/[V]versus1/[S].Michaelis-Menten constants (Km) and the maximum reaction velocity (Vmax) of thefree and immobilized enzyme were calculated. The Vmax value free lipase (0.09 µM min-1 mg-1enzyme) decreased comparing with the immobilized lipase (0.93 µM min-1 mg-^{1}enzyme). The calculated Km values of free and immobilized lipase were 0.1 and 0.093 mM, respectively.

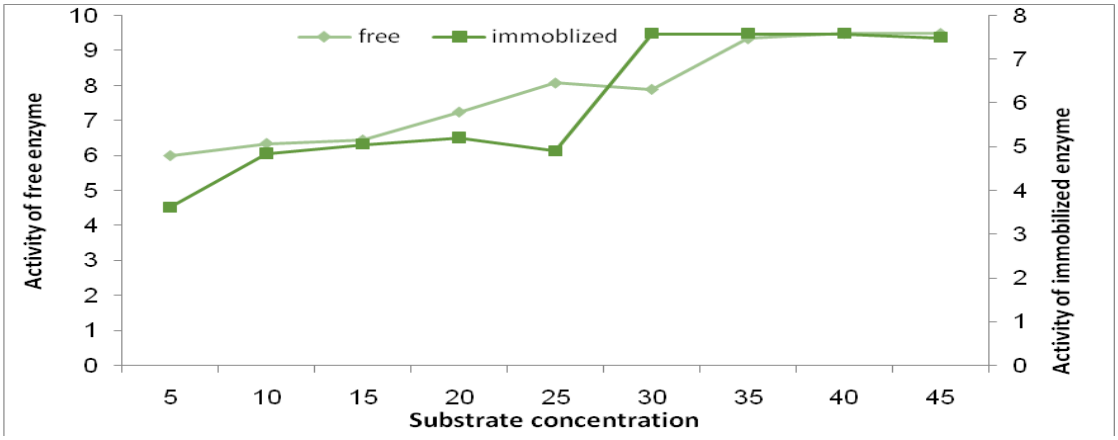

Figure 5: Substrate concentration of free and immobilized lipase.

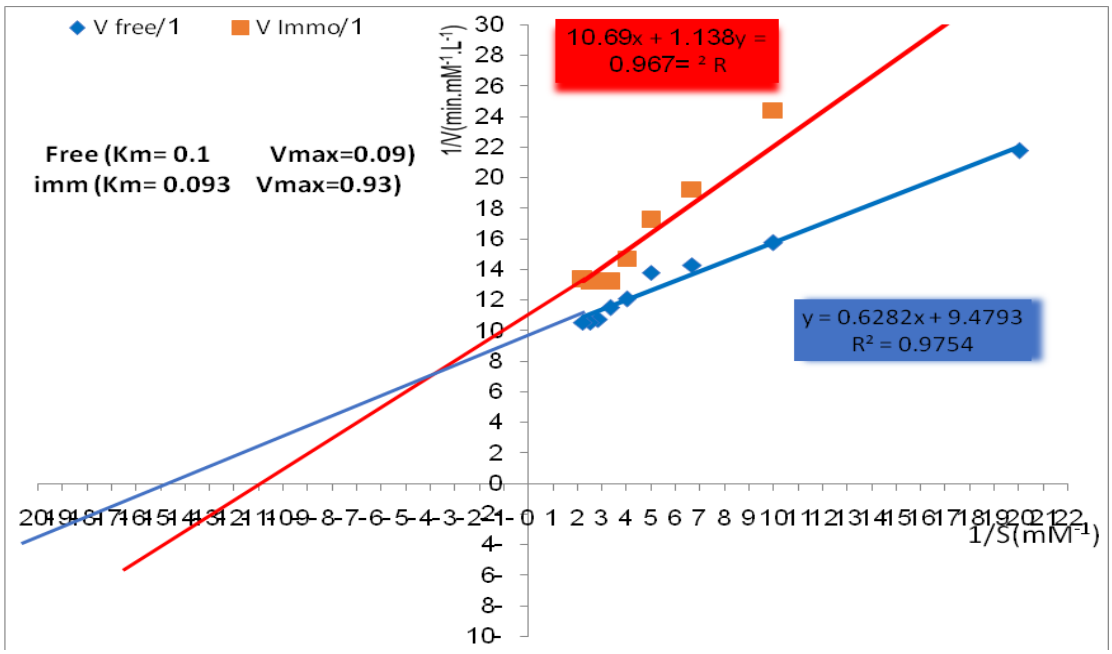

Figure 6: Kinetic constants of free and immobilized lipase using Lineweaver-Burk plot method.

ISSN: 0975-8585

CONCLUSION

Based on the results, it could be concluded that the immobilized lipase showed many benefits over the free enzyme, which could make its usage and application in industries more valuable. This was clear through the increase in thermal stability, pH level of the immobilized enzyme over the free enzyme.

REFERENCES

[1] Sharma, R., Chisti Y. and Banerjee, U.C. (2001). Production, purification, characterization, and applications of lipases. Biotechnology Advances;19:627-662.

[2] Bornscheuer U.T. (1995). Lipase-catalyzed synthesis of monoglycerols .Enzyme Microbial Technology;17:578-86

[3] Brozzoli, V., Crognale S., Sampedro I., Federici F., annibale A.D and Petruccioli M. (2009). Assessment of olive-mill will betewater as a growth medium for lipase production by Candida cylindraceain bench-top reactor. Journal of Bioresource Technology; 100:3395-3402.

[4] Kumar S., Kikon K., Upadhyay A., Kanwar S.S., Gupta R. (2005) Production, purification, and characterization of lipase from thermophilic and alkaliphilicBacillus coagulans BTS-3. Pro ExpPurif; 41: 38–44.

[5] Dalmau E., Montesinos J.L., Lotti M. and Casas C. (2000). Effect of differentcarbon sources on lipase production by Candida rugosa. Enzyme Microbial Technology; 26:657 663.

[6] Rubin B. and Dennis E.A. (1997) Lipases: Part B. Enzyme Charaterization and Utilization, Methods in Enzymol; 286:1–563

[7] Jensen R. and Detection G. (1983). determination of lipase (acylglycerolhydrolase) activity from various sources. Lipids; 18: 650–657.

[8] Aravindan R., Anbumathi P, Viruthagiri T. (2007). Lipase applications in food industry. Ind J of Biotec; 6: 141-158

[9] Vulfson, E.N. (1994) . Industrial applications of lipases. In: Woolley P, Petersen SB, editors. Lipases their structure, biochemistry and application. Cambridge, UK: Cambridge University Press;271-288.

[10] Veerapagu M., Shankara A., Ponmurugan K., Jeya R. (2013). Screening selection identification production and optimization of bacterial lipase from oil spilled soil. Asian J Pharm and Clin Res; 6: 62–67

[11] Castilho L.R., Polato C.M., Baruque E.A. et al. (2000). Economic analysis of lipase production by Penicilliumrestrictumin solid-state and submerged fermentations. BiochemEng J; 4: 239-247,

[12] Leal M.C., Cammarota M.C., Freire D.M. et al (2002). Hydrolytic enzymes as coadjuvants in the anaerobic treatment of dairy will bete waters. Braz J ChemEng; 19: 175-180.

[13] Kazlauskas R.J. and Bornscheuer U.T. (1998). Biotransformations with lipases, inRehm: HJ, Pihler G, Stadler A, Kelly PJW. (Eds.), Biotechnology, VCH, New York. 37–192.

[14] Lee W.M., Kim K.J., Kim MG, Lee S.B. (1995). Enzymatic resolution of racemic ibuprofen esters: effects of organic solvents and temperature. J Ferment Bioeng; 6: 613–615.

[15] Becker P., Abu-Reesh I., Markossian S., Antranikian G. and Maerkl H. (1997). Determination of the kinetic parameters during continuous cultivation of the lipase-producing thermophile Bacillus sp. IHI-91 on olive oil. Appl. Microbiol. Biotechnol; **48**: 184–190.

[16] Geraats S (1994). Scaling-up of lipase fermentation process: a practical approach. In: Galindo E, Ramirez OT, eds. Advances in Bioprocess Engineering. Dordrecht: Kluwer Academic Publishers; 41–46.

[17] Markossian S., Becker P., Maerkl H., Antranikian G. (2000). Isolation and characterization of lipid-degrading Bacillus thermoleovoransIHI-91 from an Icelandic hot spring. Extremophiles; **4**: 365–371.

[18] Barabino RC, Gray DN, Keyes MH (1978). Coupled reaction of immobilized enzymes and immobilized substrates: clinical application as exemplified by amylase assay. Clin. Chem. 24(8):1393-1398.

[19] Huge-Jensen B, Galluzzo DR, Jensen RG. Partial purification and characterization of free and immobilized lipases from Mucormiehei. Lipids 1987; 22: 559–565.

[20] ZHANG, S., SHANG, W., YANG, X., ZHANG, S., ZHANG, X. & CHEN, J. 2013. Immobilization of lipase using alginate hydrogel beads and enzymatic evaluation in hydrolysis of p-Nitrophenol butyrate. Bulletin of the Korean Chemical Society, 34**,** 2741-2746.

[21] Awang, R., GhazuliRafaei, M., &Baari, M. (2007). Immobilization of lipase from Candida rugosa on palm-based polyurethane foam as a support material. American Journal of Biochemistry and Biotechnology, 163-6.

[22] Bailey JE, Ollis DF. Biochemical engineering fundamentals. 2nd edn. United States of America: McGraw-Hill Book Company. 1986.

[23] Jermsuntiea, W.; Aki, T.; Toyoura, R.; Iwashita, K.; Kawamoto, S.; Ono, K. Purification and characterization of intracellular lipase from the polyunsaturated fatty acid-producing fungus Mortierellaalliacea. New Biotechnol. 2011, 28, 158–164.

[24] FENG, X., PATTERSON, D. A., BALABAN, M. & EMANUELSSON, E. A. C. 2013. Enabling the utilization of wool as an enzyme support: enhancing the activity and stability of lipase immobilized onto woolen cloth. Colloids and Surfaces B: Biointerfaces, 102**,** 526-533.