

***Enterobacter aerogenes* Lipase immobilized on ion exchange resin D152 as a supportive carrier**

الليباز البكتيريا المعوية الهوائية المثبتة على راتنج التبادل الأيوني D152 كحامل داعم لعملية التجميد.

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تاريخ قبول البحث

2022 / 9 / 21

تاريخ تسليم البحث

2022 / 8 / 22

الملخص: تم التعبير عن البكتيريا المعوية الهوائية الليباز المستخدمة في هذه الدراسة بشكل غير متجانس بواسطة (*Pichia pastoris*. D152)، تم نخل ثلاثة أنواع من راتنجات الأنيون (D311، D301R، D380) ونوعين من الراتنجات المخلبية (D401، D418) كمصفوفة دعم. تم استخدام تفاعل التحلل المائي لتقييم الأنشطة المحددة للمثبت (EAL). وفقًا للنتائج، تم اختيار D152 لنشاط الانحلال الدموي باعتباره الإنزيم الثابت (EAL). تم اختيار D152 لنشاط التحلل المائي حيث أظهر الليباز الثابت أعلى نشاط تحلل محدد. ظروف التثبيت (تحميل الإنزيم، ووقت التثبيت، ودرجة الحرارة، وقيمة الأس الهيدروجيني). كانت أفضل النتائج هي تحميل الإنزيم 4 مجم / جم، والوقت 80 دقيقة، ودرجة الحرارة 30 درجة مئوية، ودرجة الحموضة 8، وتحت الظروف المثلى كانت كفاءة التثبيت 95٪ والنشاط النوعي 532841.34 وحدة / جم.

الكلمات الرئيسية: الخصائص الأنزيمية (*Enterobacter aerogenes* Lipase (EAL) : راتنج التبادل الأيوني D152 : الشلل : التحلل المائي : التكنولوجيا الحيوية : الليباز : كفاءة الشلل : استعادة نشاط الليباز.

ABSTRACT: *Enterobacter aerogenes* Lipase used in this study was heterologously expressed by *Pichia pastoris*. D152, D152H, D151H, D113, 724), three types of anion resins (D380, D301R, D311) and two types of chelating r activities of the immobilized (EAL). According to the results, D152 was selected for hydrolysis activity as the immobilized enzyme (EAL). The D152 was selected for hydrolysis activity since the immobilized lipase exhibited the highest specific hydrolysis activity. Immobilization conditions (enzyme loading, immobilization time, temperature, and pH value). The best results were enzyme loading 4mg/g, time 80min, temperature 30°C, and buffer pH 8; and under the optimized conditions the immobilization efficiency was 95% and the specific activity was 532841.34 U/g.esins (D401, D418) were sieved as support matrix. Hydrolysis assay was employed to evaluate the specific.

Keywords: Enzymatic Properties *Enterobacter aerogenes* Lipase (EAL); ion exchange resin D152; Immobilization; Hydrolysis; Biotechnology; lipases; Immobilization efficiency; lipase activity recovery.

1. Introduction

Resins are usually a natural sap secreted from plants. Resins can be soluble many organic solvent but they are insoluble in water. Nowadays a lot of different synthetic resins are made to use different industrial sectors. Resins are used to remove ions from water and other solutions. Resins can be also used as adsorptive supports. Different functional groups are attached with resins to make ion exchange resins [1,2]. Resins have been used as a suitable carrier for lipase immobilization in many instances. For example, macroporous adsorptive resin CRB02 was used to immobilize lipase from *Candida rugosa* for kinetic resolution of ibuprofen. It was found that CRB02 increased the enzyme activity by 50% and the enantioselectivity of the immobilized lipase was 2.2 times as much as that of the native lipase for the kinetic resolution of ibuprofen with 1-propanol in isooctane at 30°C [3]. Liu et al. immobilized the lipase from *Burkholderia cepacia* onto polystyrene macroporous resin NKA by combined strategies of bioimprinting and interfacial activation to enhance its catalytic performance. These studies showed that the supports might be promising alternatives as supports to immobilize lipase [4].

In the same context, lipases are biocatalysts of great importance due to their ability to catalyze diverse reactions. They are extensively used for the catalysis in organic solvents, which leads to multiple industrial applications [5,6]. In particular, The immobilization of *Enterobacter aerogenes* E13 strain culture medium distinguished by its lipase activity, in the ethylcellulose microcapsules is being discussed. The preparations with lipase activity can be used for the treatment of oil waste [7]. By using immobilized lipase preparations it's possible not only to degrade oil waste, but also to recycle it into bio-fuel [8,9], bio-oil and other compounds [10,11].

This paper reports a comparison of the catalytic efficiency between free and immobilized lipases in an aqueous medium by exploring the ion exchange resin D152 immobilization strategy. Optimal conditions for enzymatic immobilization and stability were determined by comparing the catalytic efficiency and specific activity between the free and immobilized lipases.

2. Materials and methods

2.1 Materials

Enterobacter aerogenes Lipase (EAL) was purchased from Sigma. ion exchange resins D152, D152H, D151H, D113, 724, 110H, D380, D301R, D311, D401 and D418 were purchased from Tianjin Nankai Sci. & Tech. Co. Ltd., Tianjin, China. Coomassie Brilliant Blue G250 and bovine serum albumin (BSA) was purchased from Sigma. Other analytical-grade reagents, including $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, K_2HPO_4 and KH_2PO_4 , lauric acid, acetone, 1-dodecanol, ethanol, isooctane, phenolphthalein, 2-Methylimidazole (M-IM), CTAB and sodium hydroxide (NaOH), were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 Methods

2.2.1 Identification of Protein Content

Coomassie brilliant blue was prepared to define the protein content [12]. The standard curve with different (BSA) concentrations was prepared (0 as a blank control), and the concentrations started from (100, 200, 400, 600, 800, and 1000 μL). The final volume of each sample was 1000 μL . Afterward, 5 mL of Coomassie blue solution (G250) was added to each concentration and optical density was determined at 595 nm for all of the samples.

2.2.2 Resins

Characteristic of ion-exchange resin used in the present study are given in table 1:

Table 1: Characteristic of ion-exchange resin

Resin	Chemical	Resin categories	Functional group
D151H	Acrylic-DVB	Macropreticular weakly acidic acrylic type cation exchange resin	-COOH
D152H	Acrylic-DVB	Macropreticular weakly acidic acrylic type cation exchange resin	-COOH
D113	Acrylic	Macropreticular weakly acidic acrylic type cation exchange resin	-COOH
D311	Acrylic-DVB	Macropreticular weakly basic acrylic type anion exchange resin	-N(CH ₃) ₂
D301R	Styrene-DVB	Anion exchange resin, macroreticular weak basic styrene	-N(CH ₃) ₂
D401	Styrene-DVB	Macroreticular weak basic styrene chelating resin	- N(CH ₂ COONa) ₂
D380	Styrene-DVB	Macroreticular weakly basic acrylic acid type anion exchange resin	-N(CH ₃) ₂
D418	Styrene-DVB	Macroreticular weak basic styrene chelating resin	- N(CH ₂ COONa) ₂
D152	Acrylic-DVB	Macropreticular weakly acidic acrylic type cation exchange resin	-COOH

2.2.3 Preparation of Substrate For hydrolysis reaction .

Olive oil emulsion is made by Olive oil and 2% PVA with a molar ratio 1:3 respectively. Then emulsion machine used to mix them properly for 10 minutes.

2.2.4 Pretreatment of resin

Before immobilization, the resins needed to be prepared for experiment. The pretreatment of macroporous and ion exchange resins are a little bit different. At first, 0.5g of resin (macroporous or ion exchange) was taken in 50mL tube, then the resins were washed with distilled water for three times. Macroporous resins were submerged into ethanol (95%, v/v) for 4 h, and then rinsed with distilled water for three times to remove the residual ethanol. 2.5 mL 0.05M Tris-HCl buffer (pH 8) was mixed with the residual resin, the mixer was kept for 12 h at room temperature. Anion resins were treated with NaOH (4%, w/v) for 2 h and HCl (5%, w/v) for 3 h, rinsed with distilled water for three times, while cation resins were first treated with HCl (5%, w/v) for 2 h and then NaOH (4%, w/v) for 3 h, rinsed with distilled water for three times. Both types of ion exchange resins were then mixed with 2.5

mL 0.05M Tris-HCl buffer (pH 8) and kept for 12 h at room temperature. Then, the buffer was removed and added same buffer for further experiment.

2.2.5 Immobilization reaction

After the pretreatment, 1 mL of (EAL) solution was loaded into the tube. The tube was then stirred in a water bath with a speed of 200 rpm at 37°C for 1 hour. The suspension was then separated from the tube. The immobilized EAL (resins adsorbing the lipase) was washed with 1 mL 0.05M Tris-HCl buffer (pH 8) two times to remove loosely bound enzyme from the carrier. Finally, the obtained immobilized EAL was dried in a FD-1D 50 vacuum desiccator for 4.5 h and stored at 4°C before use.

2.2.5.1 Lipase activity assay

Lipase activity was tested by both hydrolysis and esterification reaction to figure out the suitable supports for (EAL). The hydrolysis activity was determined by Olive oil emulsion. One unit of activity (U) was defined as the amount of enzyme necessary to produce 1 μmol of free fatty acid per min. The esterification activity was measured for lauric acid and dodecanol. The immobilization efficiency (%), specific activity (U/g protein) (hydrolysis activity), and lipase activity recovery (%) were estimated by following equations (1-3).

$$\text{Immobilization efficiency (\%)} = \frac{\text{immobilized protein}}{\text{total loading protein}} \times 100\% \quad (1)$$

$$\text{Specific activity (U g}^{-1} \text{ protein)} = \frac{\text{initial activity}}{\text{protein content of immobilized lipase}} \times 100\% \quad (2)$$

$$\text{Activity recovery (\%)} = \frac{\text{specific activity of immobilized lipase}}{\text{specific activity of adding free lipase}} \times 100\% \quad (3)$$

2.2.5.1.1 Hydrolysis assay

10 mg of immobilized resin was used for hydrolysis reaction. The substrate for hydrolysis test should prepare before the reaction otherwise it loses its conformation. At first, 4ml of substrate solution was taken in a 50ml conical flask, and then 6ml of Tris-HCl buffer (pH 8.0, 0.05M) was added to the flask. The mixture was preheated for 5 minutes before adding immobilized resin. The reaction was conducted in a water bath at 60°C with 150 rpm for 10 minutes. After 10 minutes 15ml of stop solution was added to finish the reaction. Free fatty acid was then checked by titration using 0.05M NaOH. The test was conducted triplets.

3.Result:

3.1 Screening of suitable immobilization supports for (EAL)

In this study, five types of cation resins (D152, D152H, D151H, D113, 724), three types of anion resins (D380, D 301R, D311) and two types of chelating resins (D401, D418) were screened for immobilization of EAL. Their properties are given at table 1.

Hydrolytic activity was done to find suitable carrier for (EAL). The immobilization efficiency of different types of resins are presented in (Fig. 3.1). An immobilization efficiency in ion exchange resins D418 showed lower than another resins. Only three types of resin D152, D152H and D380 reached more than 85%. Among them, D380 exhibited the highest immobilization efficiency (86%).

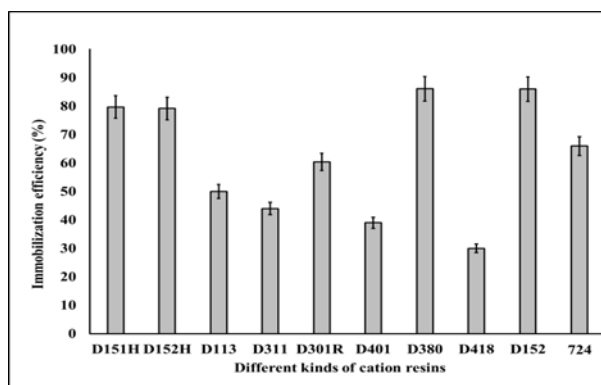


Fig.3.1. Effect of resin type on immobilization efficiency of immobilized lipase conditions: enzyme loading 1 ml, Tris-HCl buffer (pH 8.0, 0.05M), reaction time 1 hour, temperature 37°C, stirring

Among the cation resins, D152 only showed high level of hydrolytic activity 528105.5 U/g. D380 exhibited the highest amount of specific activity 437040.5 U/g shown in (Fig. 3.2). It was found that D301R, D311, D401 showed little level of activity while, 724 and D418 do not have any hydrolytic activity.

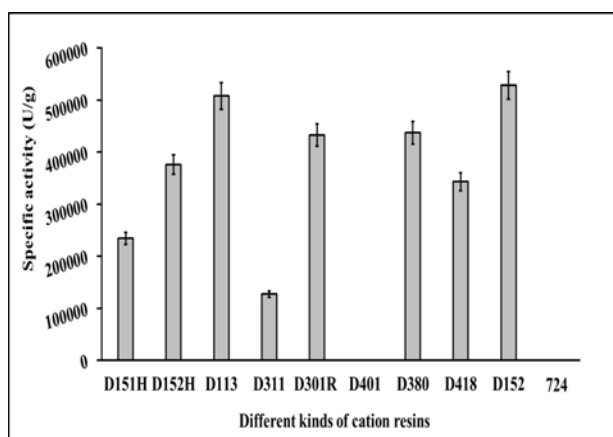


Fig.3.2 Effects of resin type on specific activity of hydrolysis reaction of immobilized lipase. Immobilization conditions: enzyme loading 1 ml, Tris-HCl buffer (pH 8.0, 0.05M), reaction time 1 hour, temperature 37°C, stirring speed 200rpm. Hydrolysis reaction conditions: immobilized resin 10mg, reaction time 10 mins, temperature 60°C, stirring speed 150 rpm, substrate 4mL, Tris-HCl buffer (pH 8.0, 0.05M) 6mL, stop solution 15mL.

3.2 Optimization of immobilization conditions on ion exchange resin D152

3.2.1 Effect of enzyme loading on the (EAL) immobilization

In this study, the effect of enzyme loading from (2mg/g to 7mg/g) on the (EAL) immobilization efficiency and specific activity was studied and the results are presented in (Fig.3.3). A decrease in (D152-EAL) immobilization efficiency, specific activity and activity recovery is observed when the enzyme loading is over 4mg/g. The highest specific activity (5,54,531.6 U/g) was achieved when the enzyme loading is 4mg/g. whereas, the highest immobilization efficiency (93%).

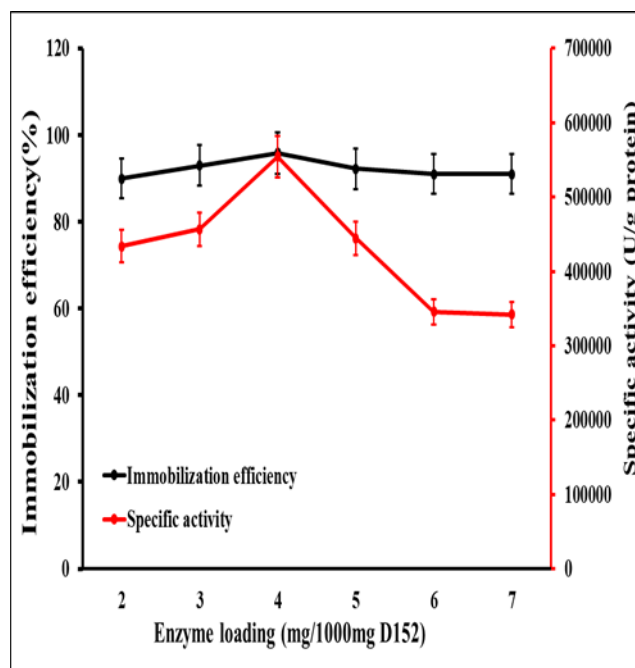


Fig.3. 3 Effects of enzyme loading on the (EAL) immobilization on D152 resin. Immobilization conditions: enzyme loading (2mg/g to 7mg/g), Tris-HCl buffer (pH 8.0, 0.05M), reaction time 1 hour, temperature 30°C, stirring speed 200rpm. Hydrolysis reaction conditions: immobilized resin 5mg, reaction time 10 mins, temperature 60°C, stirring speed 150 rpm, substrate 4mL, Tris-HCl buffer (pH 8.0, 0.05M) 6mL, stop solution 15mL.

3.2.3 Effect of reaction time on (EAL) immobilization

The specific activity, activity recovery and immobilization efficiency was all affected to a point by the immobilization time. To examine the effect of reaction time on the (EAL) immobilization, the reaction was done for 120 minutes where time interval was at 20 minutes. The results showed that the maximum D152-EAL specific activity, activity recovery and immobilization efficiency were achieved at the reaction time of 80 minutes (Fig.3. 4). After 80 minutes the D152 lipase specific activity and activity recovery started to decrease gradually.

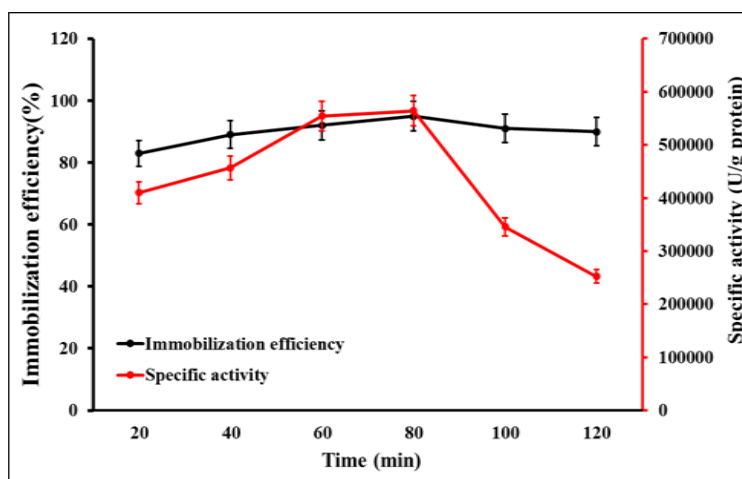


Fig.3.4 Effects of immobilization time on the (EAL) immobilization on D152 resin. Immobilization conditions: enzyme loading 3mg/g, Tris-HCl buffer (pH 8.0, 0.05M), reaction time (20 to 120) minutes, temperature 30°C, stirring speed 200rpm. Hydrolysis reaction conditions: immobilized resin 5mg, reaction time 10 mins, temperature 60°C, stirring speed 150 rpm, substrate 4mL, Tris-HCl buffer (pH 8.0, 0.05M) 6mL, stop solution 15mL.

3.3.3 Effect of immobilization temperature on the (EAL) immobilization

The effect of temperature on immobilized D152-EAL was tested in the range of 20-45°C. It was observed that at 30°C, the D152-EAL obtained the highest specific activity, recovery and the immobilization efficiency (Fig.3.5). The specific activity, activity recovery and immobilization efficiency were achieved respectively 5,62,325.3 U/g, over 100% and 95%. It was found that after reaching the higher point at 30°C the specific activity and activity recovery started to decrease steadily

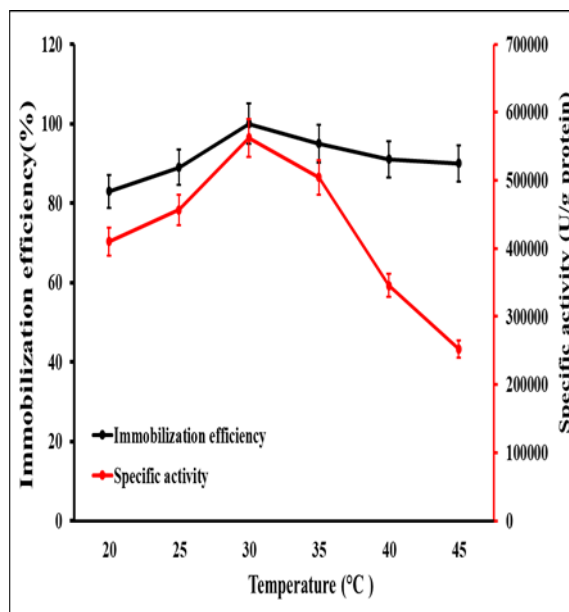


Fig.3.5 Effects of immobilization temperature on the (EAL) immobilization on D152 resin. Immobilization conditions: enzyme loading 3mg/g, Tris-HCl buffer (pH 8.0, 0.05M), reaction time 1h, temperature (20-45°C), stirring speed 200rpm. Hydrolysis reaction conditions: immobilized resin 5mg, reaction time 10 mins, temperature 60°C, stirring speed 150 rpm, substrate 4mL, Tris-HCl buffer (pH 8.0, 0.05M) 6mL, stop solution 15mL.

3.3.4 Effect of buffer pH on the (EAL) immobilization

In experiment was also conducted with three buffer solutions with different pH range to check the effect of pH on (D152-EAL). The pH range was 6.0-10.5. The pH of the buffer solution is a significant factor which can be affected the immobilization efficiency and specific activity. Fig.3.6 showed the effect of buffer pH on immobilization efficiency. It was observed that the highest immobilization efficiency (91%) was obtained at pH 6.0 by PBS, whereas immobilization efficiency (95%) was seen at pH 7.5 by Tris-HCl buffer. On the other hand, The Gly-NaOH showed very low immobilization efficiency which is less than 40%. The highest specific activity was obtained at pH 8.0 with PBS buffer which is 527590.6 U/g, whereas Tris-HCl at pH 8.0 showed lipase specific activity of 532841.34 U/g.

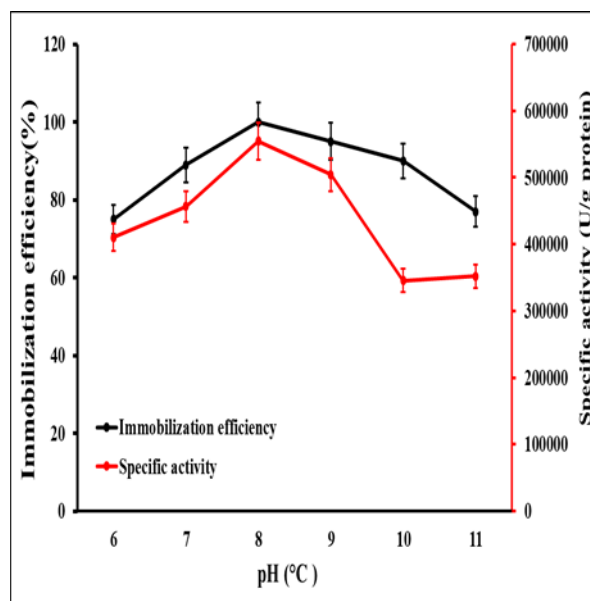


Fig.3.6 Effects of pH value on immobilization efficiency on the (EAL) immobilization on D152 resin. Immobilization conditions: enzyme loading 3mg/g, PBS (pH 6.0-8.0) Tris-HCl (pH 7.5-8.5) Glycine-NaOH (pH 8.5-10.5), reaction time 1h ,temperature 25°C, stirring speed 200rpm. Hydrolysis reaction conditions: immobilized resin 5mg, reaction time 10 mins, temperature 60°C, sirring speed 150 rpm, substrate 4mL, Tris-HCl buffer (pH 8.0,0.05M) 6mL, stop solution 15mL.

3. Discussion:

The D152 showed the highest activity in the hydrolysis system and also the highest activity recovery. It was observed that cation resins did not exhibit any esterification activity, while they have hydrolytic activity. Whereas, the anion resins did not show any hydrolysis activity but they have esterification activity. This result indicates that resin type show specificity for catalytic activity. Hence, it is important to choose specific resins for specific activity assays. As it was found that D152 has good activity on hydrolysis assay, the following experiment was conducted by using it[13,14,15].

In the same context, it was found that the high level of enzyme loading decreased the specific activity. This is due to the diffusional limitation because of the porous diameter. According to the results, 4mg/g enzyme loading was chosen for the further experiment of (EAL) immobilization.

Liu et al. higher lipase loading can increase viscosity by increasing the reaction rate. Therefore, it seems that a high level of enzyme loading evoked the reaction kinetics to downplay because of the mass transfer efficiency, resulting to a lower lipase activity [4]. The decrease of lipase specific activity after 80min could be due to the effect of the denaturalization which was emerged with increasing time. It seems to a common phenomenon as other authors reported the same condition. According to above results, the optimum immobilization time for (EAL) immobilization on D152 is considered 80mi. These results indicated that the immobilization efficiency, specific activity and activity recovery also varies because of different buffer solution. It was observed that Gly-NaOH buffer did not exhibit any activity, which can be because of the Na⁺. The Gly-NaOH either could not interact with resin or maybe it changed the structure of resin, leading the low immobilization efficiency and no specific activity. This study showed that the optimal pH for (EAL)L immobilization on D152 resin is pH 8.0 of phosphate buffer (PBS) [14,16].

In this work, all these above results showed that higher enzyme loading increased the aggregation which leads the decreasing level of immobilization efficiency, specific activity and activity recovery. After a certain time the

specific activity and activity recovery was also started to decrease. Moreover, even though high temperature increases the reaction rate, at a point higher temperature may destroy the lipase structure and leading a low specific activity. It was also observed that pH of different buffer can make a difference on specific activity, immobilization efficiency and activity recovery[17,18,14].

4. Conclusion:

The (EAL) was successfully immobilized on D152 under optimal conditions. The immobilized lipases also showed higher stability compared with other types and it was successfully.

5. Acknowledgments:

This work is supported by faculty of Arts and Sciences, Ghemins, University of Benghazi, Libya.

6. References

- Bertram, M., Manschot-Lawrence, C., Flöter, E., & Bornscheuer, U. T. (2007). A microtiter plate-based assay method to determine fat quality. *European Journal of Lipid Science and Technology*, 109(2), 180-185.
- Wang, H., Zong, M. H., Wu, H., & Lou, W. Y. (2007). Novel and highly regioselective route for synthesis of 5-fluorouridine lipophilic ester derivatives by lipozyme TL IM. *Journal of biotechnology*, 129(4), 689-695
- Yu, H., Wu, J., & Ching, C. B. (2004). Enhanced activity and enantioselectivity of *Candida rugosa* lipase immobilized on macroporous adsorptive resins for ibuprofen resolution. *Biotechnology Letters*, 26(8), 629-633.
- Liu, K., Zhao, G., He, B., Chen, L., & Huang, L. (2012). Immobilization of pectinase and lipase on macroporous resin coated with chitosan for treatment of whitewater from papermaking. *Bioresource technology*, 123, 616-619.
- Li, X., Huang, S., Xu, L., & Yan, Y. (2013). Improving activity and enantioselectivity of lipase via immobilization on macroporous resin for resolution of racemic 1-phenylethanol in non-aqueous medium. *BMC biotechnology*, 13(1), 1-9
- Salis, A., Svensson, I., Monduzzi, M., Solinas, V., & Adlercreutz, P. (2003). The atypical lipase B from *Candida antarctica* is better adapted for organic media than the typical lipase from *Thermomyces lanuginosa*. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, 1646(1-2), 145-151.
- Sun, J., Jiang, Y., Zhou, L., & Gao, J. (2010). Immobilization of *Candida antarctica* lipase B by adsorption in organic medium. *New Biotechnology*, 27(1), 53-58.
- Dizge, N., & Keskinler, B. (2008). Enzymatic production of biodiesel from canola oil using immobilized lipase. *Biomass and bioenergy*, 32(12), 1274-1278.
- Sharma, R., Chisti, Y. & Banerjee, U.C. "Production, purification, characterization, and applications of lipases". *Biotechn. adva.* vol. 19, pp.627-662, 2001.
- Ferreira-Dias, S., Sandoval, G., Plou, F. & Valero, F. "The potential use of lipases in the production of fatty acid derivatives for the food and nutraceutical industries". *Elect. J. Biotechn.* 16(3), pp.12-12, 2013.
- Kappe, C. O., Shishkin, O. V., Uray, G., & Verdino, P. (2000). X-ray structure, conformational analysis, enantioseparation, and determination of absolute configuration of the mitotic kinesin Eg5 inhibitor monastrol. *Tetrahedron*, 56(13), 1859-1862.
- Bradford, M.M. "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding". *Anal. biochem.* vol. 72, pp.248-254,1976.
- Jaeger, K.E. & Reetz, M.T. "Microbial lipases form versatile tools for biotechnology". *Trends in biotechn.* vol. 16, pp.396-403, 1998.

- H Jurado, E., Camacho, F., Luzón, G., Fernández-Serrano, M., & García-Román, M. (2008). Kinetics of the enzymatic hydrolysis of triglycerides in o/w emulsions: study of the initial rates and the reaction time course. *Biochemical Engineering Journal*, 40(3), 473-484.
- Sörensen, M. H., Ng, J. B., Bergström, L., & Alberius, P. C. (2010). Improved enzymatic activity of *Thermomyces lanuginosus* lipase immobilized in a hydrophobic particulate mesoporous carrier. *Journal of colloid and interface science*, 343(1), 359-365.
- Svendsen, A. (2000). Lipase protein engineering. *Biochimica et Biophysica Acta (BBA)/Protein Structure and Molecular Enzymology*, 1543(2), 223-238.
- Khan, N.A., Jung, B.K., Hasan, Z. & Jhung, S.H. "Adsorption and removal of phthalic acid and diethyl phthalate from water with zeolitic imidazolate and metal–organic frameworks". *J. Hazardous Mat.*, vol. 282, pp.194-200, 2015.
- Abian, O., Grazú, V., Hermoso, J., González, R., García, J. L., Fernández-Lafuente, R., & Guisán, J. M. (2004). Stabilization of penicillin G acylase from *Escherichia coli*: site-directed mutagenesis of the protein surface to increase multipoint covalent attachment. *Applied and environmental microbiology*, 70(2), 1249-1251.