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# Immobilization of lipase enzyme extracted from thermophilic *Bacillus licheniformis* 14T local isolate

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## Abstract

**Background:** Currently, lipase enzymes are considered important bio-catalysts in many industries due to their unique properties in catalyzing various types of reactions in aqueous solutions. By targeting hydrocarbons in the oil, lipase enzymes contribute to the breakdown of hydrocarbons, reducing the environmental impact of oil spills and facilitating the remediation of contaminated areas.

**Methods**: A thermostable lipase from local isolate *Bacillus licheniformis* 14T has been immobilized on four different supports that include the inactivated chitosan beads, activated chitosan beads with glutaraldehyde, inactivated chitosan-alginate beads, and activated chitosan-alginate beads with glutaraldehyde.

**Results** The purified free lipase enzyme exhibited the highest enzymatic activity at 34.6 units/ml, surpassing all immobilized enzymes. Specific activity increased to 96.25 and 79.03 unit/mg protein for activated chitosan beads and activated chitosan-alginate beads, while decreasing to 52.95 and 46.05 unit/mg protein for inactivated chitosan beads and inactivated chitosan-alginate beads compared to the free enzyme. Optimal conditions for the immobilized enzyme differed, with the highest enzyme activity achieved after 60 minutes at 60°C and pH 8, reaching 48.6, 70.23, 43.12, and 61.2 units/ml on various supports, contrasting with the free enzyme's peak activity after 30 minutes at 50°C and pH 7.

**Conclusions:** Immobilizing the lipase enzyme increases the specific activity of the immobilized enzyme on the supports of activated chitosan beads with, also the immobilization process led to a change in the optimal conditions for the activity of the immobilized enzyme compared with the optimal conditions of free enzyme.

## Introduction

Currently, lipase enzymes are considered important bio-catalysts in many industries due to their unique properties in catalyzing various types of reactions in aqueous solutions. In the past, lipase enzymes were extracted from various sources such as plants, animals, and microorganisms, but now the researchers focus their production and extraction from microorganisms, especially mesophilic and thermophilic bacteria [1-3]. The thermostable enzymes produced and extracted from thermophilic microorganisms, especially bacteria, are one of the most important bio-catalysts group in industrial applications due to their ability to resist extreme conditions of temperature and pH and these enzymes provide several advantages when used as biocatalysts in different applications such as increasing the enzyme activity, time of reaction, and rate of substrate hydrolysis [4].

The thermostable lipases consider one of the most important groups in the thermostable enzymes that are used in biotechnological applications because they have broad good properties such as working in a wide range of temperatures, pH values, reaction times, and hydrolysis of different substrates [5,6]. The vast majority of thermostable lipases that have been produced and purified from thermophilic microorganisms are extracted from different species of thermophilic *Bacillus*, this genus has some species characterized by the ability to produce the extracellular lipase enzyme and several species are important in the researches fields, and industrial applications, among these species, are *B. thermoleovorans* and *B. licheniformis*. As a result of the increased use of lipases in various industrial applications, and to increase the stability of the lipase enzyme and the possibility of its recovery after each process, many types of research have been conducted to immobilize the enzyme using different methods such as the adsorption on different supports [7-9].

Several methods were used to immobilize the lipase enzyme on solid supports namely physical entrapment, covalent attachment, adsorption, cross-linking, and adsorption on a solid support and then cross-linking. Each method has its advantages and disadvantages depending on the type of support material with the possibility of activating it or not and the nature of the enzyme in addition to the conditions of the enzymatic reaction [10,11]. Whereas, chitosan, one of the compounds derived from chitin, is considered one of the most suitable and used materials for enzymes immobilization, due to the possibility to a constituent of many physical forms such as beads, flakes, porous, gel, fiber, and membrane, and also the rate of hydrolysis is very low [12]. Therefore, the current research is aim to study and select the best support to immobilize the lipase enzyme and to investigate the effect of the immobilization process in determining the optimal conditions for the enzyme activity of the immobilized and free enzyme under the same standard conditions.

## Methods

### Production and purification of lipase

According to the results of previous studies as in [13,14], which were conducted to produce lipase enzyme from thermophilic local isolate, *Bacillus licheniformis* 14T (results not shown) and then to purify the enzyme using several purification steps (results not shown). In this study, the lipase enzyme was produced and purified according to the above and used in the subsequent steps.

### Lipase activity

The titrimetric method toward 0.05 N NaOH was used to detect the activity of free and immobilized lipase enzymes by estimating the amount of free fatty acids from the hydrolysis of olive oil by the action of the enzyme, where 2 ml or 2 g from the free and immobilized enzyme respectively was added to 20 ml of the reaction mixture, then mixed well and incubated at 30 °C in a shaker water bath, 125 rpm for 30 min., thereafter the enzymatic activity was estimated based on one enzymatic unit representing the release of 1 µmol of fatty acids after one minute under the studied conditions [15].

### Protein concentration

Protein concentration in the enzyme reaction solutions and on the solutions of immobilization studies was estimated using the Lowry method according to a standard curve of bovine serum albumin as standard protein [16].

### Preparation of chitosan beads

Four grams of chitosan powder were dissolved in 96 ml of 5% acetic acid aqueous solution and then homogenized well by slightly stirring for 30 min., thereafter the mixture content was dropped slowly in 0.1 M solution of sodium hydroxyl with constant gently stirred for 24 h. at 25 °C. The formed inactivated chitosan beads are separated from the solution by filtration using Whatman 41 filter paper and then washed with distilled water several times until reaching the neutral pH. Then dried the chitosan beads by lyophilization [17].

### Preparation of chitosan-alginate beads

The 95 ml of 5% acetic acid aqueous solution was used to dissolve 2.5 g of chitosan powder with continuous stirring, and after complete dissolution, the 2.5 g of sodium alginate was added to the mixture with constant stirring for 30 min., afterward the mixture content was dropped slowly in 1 M solution of sodium hydroxyl with constant gently stirred for 24 h. at 25 °C. The formed

inactivated chitosan-alginate beads are separated from the solution by filtration using Whatman 41 filter paper and then washed with distilled water several times until reaching the neutral pH. Then dried the chitosan beads by lyophilization [10].

### Activation of chitosan and chitosan-alginate beads

Five grams from each dried bead of chitosan and chitosan-alginate hybrid were added separately to two beakers, each having 50 ml of 0.1 M sodium phosphate buffer pH 7.0 and containing 1.25 ml of glutaraldehyde. The mixture of buffer solution and beads was continued stirring for 1 h. at 25 °C to form activated chitosan and chitosan-alginate beads, which are separated from the solution by filtration using Whatman 41 filter paper, and then washed with distilled water several times and dried by lyophilization [18].

### Immobilization on activated and inactivated beads

Four different types of supports were used to immobilize the lipase enzyme included: inactivated chitosan beads, activated chitosan beads, inactivated chitosan-alginate beads, and activated chitosan-alginate beads, where 2.5 g from each support was taken and placed in a separate beaker, then added to it 50 ml of the enzymatic solution with a total enzymatic activity of 375 units to give 150 units/each gram of beads with continuous stirring for 30 minutes at 25°C, then the beads were separated and washed several times by 0.1 M sodium phosphate buffer pH 7.0, then dried by lyophilization, and kept in the refrigerator at 4°C until use [10].

### Optimal conditions of immobilized lipase

The effect of different reaction times (10, 20, 30, 40, 50, 60, 70, and 80 min.), the different temperatures (20, 30, 40, 50, 60, 70, and 80 °C), and the different pH values (5, 6, 7, 8, and 9) on the activity of the free and immobilized lipase enzyme were studied, which showed the highest enzymatic activity compared to the rest of the immobilized enzymes by determining its ability to hydrolyze the olive oil and determine the quantity of free fatty acids produce under standard conditions [14].

## Results

The thermophilic local isolate *Bacillus licheniformis* 14T, which was selected from several isolates, was used in a previous study (results not shown) as it appeared the highest ability to produce crude lipase enzyme, then several purification steps were achieved to obtain pure lipase enzyme as in previous research (results not shown). The results indicate that the enzymatic activity of the pure lipase used in the current study is 34.6 units/ml [13,14].

### The activity of free and immobilized lipase enzyme

The results of immobilizing the lipase enzyme using four different supports show that all the immobilized enzymes gave higher values in the enzymatic activity and specific activity than the free enzyme. Also, it was found that the immobilized lipase on activated chitosan beads showed the highest ability to increase the enzyme activity depending on the enzymatic activity and specific activity, which reached 29.84 unit/ml and 96.25 unit/mg protein respectively, while it is noted that both enzyme activity and specific activity of the lipase enzyme immobilized on activated chitosan-alginate bead came in second place, reaching 21.34 unit/ml and 79.03 unit/mg protein, respectively.

| Lipase types | Protein concentration (mg/ml) | Enzyme activity (unit/ml) | Specific activity (unit/mg protein) | Percentage of enzyme binding (%) |
|---|---|---|---|---|
| Purified free enzyme | 0.5 | 34.6 | 69.2 | - |
| Immobilized on inactivated chitosan beads | 0.21 | 11.12 | 52.95 | 42 |
| Immobilized on activated chitosan beads | 0.31 | 29.84 | 96.25 | 62 |
| Immobilized on inactivated chitosan-alginate beads | 0.18 | 8.29 | 46.05 | 36 |
| Immobilized on activated chitosan-alginate beads | 0.27 | 21.34 | 79.03 | 54 |

**Table 1:** The enzymatic activity and specific activity of the free lipase enzyme and the immobilized lipase enzyme on different supports.

The reason of decreasing in the enzymatic activity and specific activity for the immobilized lipase enzyme on the inactivated supports is that the amount of proteins that bind to it is less than that bound to the activated supports, the results in Table (1) indicate that the protein concentration binds in activated supports reached 0.31 and 0.27 mg/ml respectively, while in inactivated supports reached 0.21 and 0.18 mg/ml respectively. Also, the results of calculating the percentage of lipase enzyme binding with different supports used in the current study, which was calculated based on the amount of protein in each experiment, indicated that the highest percentage of enzyme binding was 62% when using activated chitosan, followed by 54% when using deactivated chitosan, while non-activated supports gave the lowest percentage, where the enzyme binding was 42% and 36% for chitosan and alginate, respectively.

### Effect of reaction conditions on lipase activity

Studying the effect of reaction time, temperature and pH indicates a change in the optimal factors affecting the enzymatic activity of the immobilized enzyme compared

with the free enzyme, where the optimum reaction time shifted from 30 minutes for the free enzyme to 60 minutes for all immobilized enzymes, Figure (1).

The results also showed clear differences in the enzymatic activity of the immobilized enzymes, and the lipase enzyme bound on activated chitosan beads gave the best enzymatic activity for all reaction periods compared with the free enzyme and other immobilized enzymes, where the highest value of 62.7 unit/ml was obtained after 60 minutes.

The results show in Figure (2) that immobilization of lipase enzyme on the four supports led to a change in the optimum temperature for enzyme activity as the highest enzymatic activity of immobilized enzymes was at 60 °C, while the free enzyme was at 50 °C, this means that the immobilization process led to increasing the stability of the enzyme, and also the lipase enzyme bound on activated chitosan beads gave the greater enzymatic activity of 67.43 units/ml compared to the other free or immobilized enzymes.

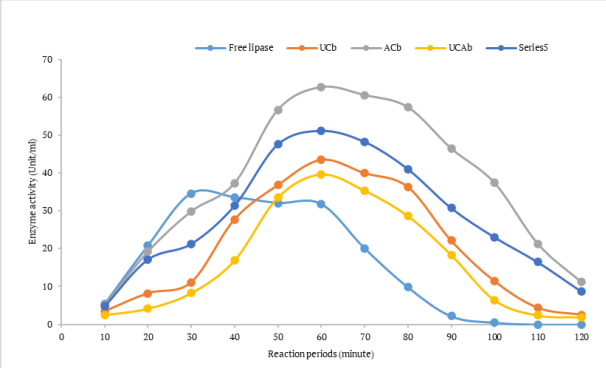


**Figure 1:** Effect of the reaction time on the enzymatic activity of the free lipase enzyme and the immobilized lipase enzyme produced from *Bacillus licheniformis* 14T.

The results in Figure (3) confirm that the process of immobilizing lipase on different types of supports led to a change in the stability of the enzyme towards both temperature and pH, which led to a shift in the optimum pH of the enzyme to 8 instead of 7 for the free enzyme. The results also indicate that the lipase enzyme bound on activated chitosan beads gave the maximum enzymatic activity of 70.23 units/ml compared to the other free and immobilized enzymes for all studied pH values. Also, the results show that the lipase enzyme bound on activated supports retained about 43.65% of its enzyme activity at pH 9, while the free enzyme lost 88.42% of its activity at pH 9.

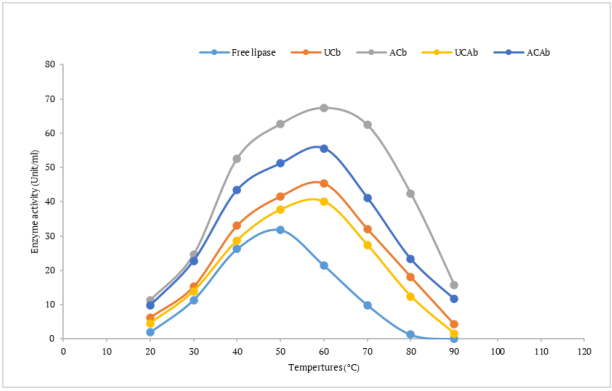


**Figure 2:** Effect of the temperatures on the enzymatic activity of the free lipase enzyme and the immobilized lipase enzyme produced from *Bacillus licheniformis* 14T.

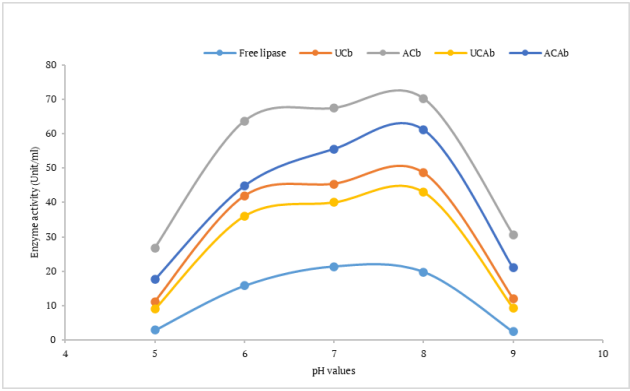


**Figure 3:** Effect of the pH values on the enzymatic activity of the free lipase enzyme and the immobilized lipase enzyme produced from *Bacillus licheniformis* 14T.

## Discussion

Generally, there are two methods to immobilize enzymes onto the solid supports called physical and chemical methods to increase the stability of enzymes toward the temperature, pH, and reaction time, whereas the adsorption method on solid materials is considered the simplest and most economical way, so it was considered one of the best methods for immobilizing lipase enzyme on a large scale, whether at the research or production level [10]. In general, the process of binding enzymes with polycationic supports leads to a change in the chemical properties of these enzymes and thus a change in the environmental conditions affecting them. Although the chitosan belonged to this group of supports, the immobilization of enzymes to this type of support does not lead to any change in the optimum pH toward the acidity [19,20].

Gohel *et. al.*, found that the immobilized lipase enzymes produced from *Bacillus subtilis* xrf11 and *Bacillus licheniformis* xrf12 can give a good enzymatic activity of up to 80% of the activity of the free lipase enzyme and these two immobilized enzymes can give

50% of their initial activity after being used for 20 cycles of reaction and then the activity will be decreased due to the hydrolysis of the enzyme supports as a result of their consumption during the cycles of enzymatic reactions [8]. The results of many studies showed the ability of bacterial isolates of the genus *Bacillus* to produce the enzyme lipase [4], and many studies indicated that many species of the *Bacillus* genus, which are thermophilic possess the ability to produce the enzyme lipase [5,21]. Many kinds of research have been conducted to purify the lipase enzyme using different purification steps, where the studies have shown the possibility of purifying the enzyme with several purification times of 40-80 times, depending on the type of bacterial isolate and the purification method used [22,23]. Carneiro and his group used different concentrations of both pure chitosan and chitosan supplemented with alginate to immobilize pure lipase enzyme extracted from selected fungal isolate, where the results showed that chitosan supplemented with alginate did not improve the efficiency of the lipase enzyme immobilization process and the pure chitosan showed the higher efficiency than other supports, also, the best concentration of activated chitosan by alginate was found when using 5% of chitosan, which gave 97% of the activity of the free enzyme, and the stability of the enzyme reached 154 times more than the free enzyme [24]. Several natural polymers were used to immobilize the lipase enzyme produced from *Bacillus* sp. isolate such as tapioca starch, gelatin, sodium alginate, and chitosan. The results indicated that the best natural polymer for immobilizing the enzyme was tapioca starch, which gave the highest enzymatic activity reaching 1.308,7 U/ml with a protein concentration of 0,207 mg/ml compared with other natural polymers used in this study [23]. 3% of chitosan was used to immobilize 0.3% of the lipase enzyme, as it was found that chitosan was able to immobilize78% of the enzyme, and the immobilization led to the retention of the immobilized enzyme by about 108% of the enzyme's activity, and it also led to an increase in the stability of the enzyme towards temperature and pH (27). Partially pure lipase enzyme, which was produced by the thermophilic isolate *Bacillus* sp., where the optimal conditions for its activity were studied. It was found that the highest activity was at 60°C and pH 8.0 for the free enzyme, while it was noted that the enzyme immobilized on silica and HP-20 beads gave the best enzymatic activity at 65 ° C and pH 8.5 of the reaction solution, meaning that the immobilization led to a shift in the optimal conditions for the enzyme's activity, in addition to extending the storage life of the immobilized enzyme to 2.5 times compared to the free enzyme [2].

The results of the current research indicate that the process of immobilizing the lipase enzyme on the used supports led to an increase in the specific activity of the immobilized enzyme on the supports of activated chitosan beads with glutaraldehyde, inactivated chitosan-alginate beads, and activated chitosan-alginate beads with glutaraldehyde, while it led to a decrease in the specific activity of the lipase enzyme which immobilized on inactivated chitosan beads, also the immobilization process led to a change in the optimal conditions for the activity of the immobilized enzyme compared with the optimal conditions of free enzyme.

## Author Contributions

Saad Hussein Khudhair: : devised the idea for the study and sample collection and enzyme purification.
Melad Khalaf. Mohammed, microbial identification, enzyme assay.
Ahmed Darweesh Jabbar : Biodegradation assay
All authors contributed in preparing the initial manuscript draft

## Conflict of Interest

The authors declare that there is no conflict of interestregarding the publication of this paper.

## References


1. Patel K, Parikh S. Identification, production, and purification of a novel lipase from Bacillus safensis. Journal of Applied Biology and Biotechnology, (2022); 10(4): 73-76.
2. Nawani N, Singh R, Kaur J. Immobilization and stability studies of a lipase from thermophilic Bacillus sp: The effect of process parameters on immobilization of enzyme. Electronic Journal of Biotechnology, (2006); 9(5): 559-565.
3. Kılınç A, Teke M, Önal S, Telefoncu A. Immobilization of pancreatic lipase on chitin and chitosan. Preparative biochemistry & biotechnology, (2006); 36(2): 153-163.
4. Bora L, Bora M. Optimization of extracellular thermophilic highly alkaline lipase from thermophilic Bacillus sp isolated from hotspring of Arunachal Pradesh, India. Brazilian Journal of Microbiology, (2012); 43: 30-42.
5. Ugras S. Characterization of a thermophilic lipase from Bacillus licheniformis Ht7 isolated from Hayran Thermal Springs in Giresun. Romanian Biotechnological Letters, (2017); 22(1): 12297-12306.
6. Chandra P, Enespa, Singh R, Arora PK. Microbial lipases and their industrial applications: a comprehensive review. Microbial cell factories, (2020); 19(169):1-42.
7. Rashid FAA, Rahim RA, Ibrahim D, Balan A, Bakar NMA. Purification and properties of thermostable lipase from a thermophilic bacterium, Bacillus licheniformis IBRL-CHS2. J Pure Appl Microbiol, (2013); 7: 1635-1645.
8. Gohel H, Ghosh S, Bragazna V. Production, Purification and Immobilization of extracellular lipases from thermophilic Bacillus Subtilis XRF 11 and Bacillus Licheniformis XRF 12 FOR Production of alkyl esters. Int J Life Sc Bt & Pharm Res, (2013); 2(3): 278-286..
9. Sharma P, Sharma N, Pathania S, Handa S. Purification and characterization of lipase by Bacillus methylotrophicus PS3 under submerged fermentation and its application in detergent industry. Journal of Genetic Engineering and Biotechnology, (2017); 15(2): 369-377.
10. Carneiro LABC, Costa-Silva TA, Souza CRF, Bachmann L, Oliveira WP, Said S. Immobilization of lipases produced by the endophytic fungus *Cercospora kikuchii* on chitosan

microparticles. Brazilian Archives of Biology and Technology, (2014); 57578-586.
11. Barik A, Sen SK, Rajhans G, Raut S. Purification and optimization of extracellular lipase from a novel strain Kocuria flava Y4. International Journal of Analytical Chemistry, (2022); 2022:1-10.
12. Egwim EC, Adeshina AA, Oyewole OA, Okoliegbe IN. Optimization of Lipase immobilised on Chitosan Beads for Biodiesel Production. (2012) ; 2(2):103-112..
13. Khudhair SH. Isolation and optimization of thermophilic lipase producing bacteria from soil contaminated with used engines oil. Journal of Wasit for Science and Medicine, (2016); 8(4): 66-74.
14. KHUDHAIR SH, MOHAMMED MK. PURIFICATION AND CHARACTERIZATION OF LIPASE ENZYME EXTRACTED FROM Bacillus licheniformis 14T LOCAL ISOLATE. PLANT CELL BIOTECHNOLOGY AND MOLECULAR BIOLOGY, (2021); 22(69-70): 169-177.
15. Sirisha E, Rajasekar N, Narasu ML. Isolation and optimization of lipase producing bacteria from oil contaminated soils. Advances in Biological Research, (2010); 4(5): 249-252.
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. Journal of biological Chemistry, (1951); 193(1): 265-275.
17. Mendes AA, de Castro HF, de S. Rodrigues D, Adriano WS, Tardioli PW, et al. Multipoint covalent immobilization of lipase on chitosan hybrid hydrogels: influence of the polyelectrolyte complex type and chemical modification on the catalytic properties of the biocatalysts. Journal of Industrial Microbiology and Biotechnology, (2011); 38(8): 1055-1066.
18. Rodrigues DS, Mendes AA, Adriano WS, Gonçalves LR, Giordano RL. Multipoint covalent immobilization of microbial lipase on chitosan and agarose activated by different methods. Journal of Molecular Catalysis B: Enzymatic, (2008); 51(3-4): 100-109.
19. Mohamad NR, Marzuki NHC, Buang NA, Huyop F, Wahab RA. An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. Biotechnology & Biotechnological Equipment, (2015); 29(2): 205-220.
20. Nazari T, Alijanianzadeh M, Molaeirad A, Khayati M. Immobilization of Subtilisin Carlsberg on modified silica gel by cross-linking and covalent binding methods. Biomacromolecular Journal, (2016); 2(1): 53-58.
21. Sharma Chander K, Kanwar Shamsher S. Purification of a Novel Thermophilic Lipase from B. licheniformis MTCC-10498. ISCA Journal of Biological Sciences, (2001);1, 43-48.
22. Saun NK, Mehta P, Gupta R. Purification and physicochemical properties of lipase from thermophilic Bacillus aerius. Journal of oleo science, (2014); 63(12): 1261-1268.
23. Sari ANM, Koentjoro MP, Prasetyo EN. Lipase Immobilization Based On Biopolymer. Surabaya international Health conference; (2019); 406-418.
24. Carneiro EA, Bastos AKP, Oliveira UMFd, Matos LJBLd, Adriano WS, et al. Improving the catalytic features of the lipase from Rhizomucor miehei immobilized on chitosan-based hybrid matrices by altering the chemical activation conditions. Química Nova, (2020); 431234-1239.