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Lipase Immobilization Based On Biopolymer

Annysa Nur Mala Sari[1], Maharani Pertiwi Koentjoro[2], Endry Nugroho Prasetyo[1]

[1]Biomaterial and Enzyme Technology Research Group, Microbiology and Biotechnology Laboratory, Biology Department, Faculty of Natural Science, Institut Teknologi Sepuluh Nopember (ITS), Gedung H Kampus ITS Keputih Sukolilo Surabaya 60111 Indonesia
[2]Medical Laboratory Technology Study, Faculty of Health, Nadhlatul Ulama University of Surabaya, Jl. Raya Jemursari Surabaya 51-57, Surabaya-Indonesia.

Corresponding author: Dr. Endry Nugroho Prasetyo (endry@bio.its.ac.id)

Abstract. Lipase (EC 3.1.1.3) or also known as glycerol ester hydrolases are a class of enzyme to break down the hydrolysis of triglycerides into diglycerides, monoglycerides, free fatty acids, and glycerol. The usage of the biopolymer-based carrier in enzyme immobilization has advantages in terms of cheap, being obtained easily and practical. The purpose of this study is using natural polymers such as tapioca starch, gelatin, sodium alginate, and chitosan as lipase immobilization carrier which further analyzed in enzymatic activity assay, enzyme stability, enzyme storage and polymer functional group analysis using FTIR. The natural polymer could be used for lipase immobilization as a result of this study. Immobilized lipase with aminated tapioca starch biopolymer has the highest activity among all biopolymer which is 1.308,7 U/ml with the protein content of 0,207 mg/ml. FTIR result showed a bond formation on (N-H), (CN), (C-H) group in immobilized lipase. Enzyme recovery and immobilized lipase storagetesting with aminated tapioca starch resulted in the highest relative activity on 96,4% and 83,7%.

1. Introduction

Lipase (EC3.1.1.3) or also called glycerol ester hydrolases are a class of hydrolysis enzymes that break down triglycerides into diglycerides, monoglycerides, free fatty acids and glycerol [1], [2]-[6]. Lipase can be produced by plants, animals, and microbes. However, lipases produced by bacteria have better activity and stability than others [7], [8], [9]. Lipase can be applied in synthetic chemistry, namely the synthesis of polymers and enantiomers, while in food technology it used as a catalyst for modification of lipids [4], [5]. Lipase can also be applied as a catalyst to produce biodiesel with the transesterification reaction of triacylglycerides to methyl esther fatty acids [10], [3]. The applications of this enzyme still have several obstacles, including expensive with low enzyme resistance, so the reaction often less efficient when applied on an industrial scale [11], [12]. Therefore, one of solution to improve lipase work efficiency is immobilization techniques on specific careers so the enzyme can be reused [13], [14], [15], [5], [16], [17].

The enzyme immobilization technique consists of several types, by forming covalent bonds, adsorption, cross-linking, entrapment, and encapsulation [4], [18], [19], [20]. A variety of careers and new technologies made from silica and synthetic polymers have been commercialized, but have quite high prices, so it is necessary to looking for other alternative materials to reduce operating costs. The use of natural-based careers is an alternative because it has advantages that are cheap, easy to obtain and practical [11], [17], [21], [4], [11], [20], [22], [23], [10], [24]. Therefore in this study, biodegradable low-cost carrier including chitosan, gelatin, sodium alginate, and tapioca starch are used as carriers of lipase immobilization produced by Bacillus sp. SKII-5.

2. Methods

2.1. Time and Place

This research was conducted in October 2016 until January 2017 in the Microbiology and Biotechnology Laboratory, Department of Biology, FMIPA, ITS Surabaya.

2.2. Lipase Production

In the production of crude lipase, 10% (v/v) Bacillus sp. SK II-5 from the starter was grown in a fermentation medium as much as 90 ml in the 250 ml Erlenmeyer. Incubated Bacillus sp. culture at room temperature 120 rpm for 40 hours [25]. The biomass in the Medium Broth is separated using Whatman No. 1, 10 ml crude lipase was added with 90 ml ethanol 96%. The mixed solution was incubated in the refrigerator -70^{o}C for 2 hours. The combined solution was precipitated using centrifugation 8000 rpm for 10 minutes [26]. The precipitation results were dissolved with Phosphate buffer and stored in the refrigerator at 4^{o}C as a source of crude lipase [7].

2.3. Lipase Activity Assay

Lipase activity test can be done by reacting 0.1 ml triacylglycerol (TG) with 0.1 ml lipase along with 0.8 ml phosphate buffer and tween 80 0.05 ml, then incubated at 50^{o}C for 1 minute. After incubation, it was reacted with a 40 µL periodate reagent, incubated for 10 minutes. Drop formalin kit reagent and was incubated for 15 minutes. The absorbance was measured using a UV-Vis spectrophotometer with a wavelength of 550 nm [27].

2.4. Lipase Protein Content Test

Bradford reagents were made using 100 mg Coomassie Brilliant Blue G-250 dissolved in 50 ml of 95% ethanol, add 100 ml 85% (w/v) phosphoric acid. Dilute until the color dissolves all and filter using Whatman No.1 filter paper to volume 1 L [28]. The protein concentration was measured using the standard curve of Bovine Serum Albumin (BSA) protein. The measurement of sample concentration was carried out by reacting 100 µl of the enzyme sample solution with 5 ml of bradford solution, vortex and incubated at room temperature in dark conditions for 2 minutes. Then the absorbance was measured by a spectrophotometer at 595 nm [28]. The absorbance value is then entered into a standard curve to determine the concentration of protein contained in the enzyme sample.

2.5. Lipase Immobilization Using Tapioca Bio-Polymer Starch

Immobilization of lipases with starch biopolymers was carried out by oxidation and amination [29]. Starch oxidation was carried out by mixing 25 grams tapioca starch powder with 150 ml sodium metaperiodate (0.3 N). Then HCl is added to the solution until reaching pH 3. The oxidation reaction is carried out in a water bath with a temperature of 32^{o}C at 400 rpm for 1 hour. At the end of the oxidation process, the starch deposits were washed three times using distilled water. Boiled starch was immersed in 150 ml sodium metabisulphite solution 0.5% (w/v) for 1 hour to destroy residual oxidant agents (Sodium metaperiodate). Then the starch deposits are rewashed three times using distilled water, centrifugate at 3000 rpm for 10 minutes to remove remaining distilled water from starch. Natan that is formed is dried using an oven at 45^{o}C for one night. Dried starch is then filtered using a 100 mesh filter to build powdered oxidized starch [30].

The oxidized starch then amended by reacting 10 grams of starch oxidized with 40 ml of 25% ammonium hydroxide and 10 grams of nickel II Chloride as a catalyst for starch amination. The formed mixture is heated with hydrogen pressure in autoclave 121^{o}C for 5 hours. The results of the contaminated starch obtained were evaporated using a hotplate to obtain a contaminated starch paste [29]. After receiving the contaminated starch, lipase immobilization is then carried out. The first step is done as much as 2 grams of contaminated starch as a career, dissolved the carrier with 5 ml of glutaraldehyde 5% and 1 ml HCl 1%, the mixed solution was incubated for 1 hour then washed using distilled water three times to pH 7. The dyed starch was washed, then added 5 ml lipase and phosphate buffer pH 7 then incubated for 3 hours then dried at room temperature. Immobilized lipases are stored at 4^{o}C.

2.6. Lipase Immobilization Using Gelatin Bio-Polymer

Lipase immobilization using gelatine biopolymers was carried out using the cross-linking method. Two grams of gelatin dissolved and heated with aquades as much as 10 ml until the gel is formed. Then gelatin was added with 5ml glutaraldehyde 5% and 1ml HCl 1%; the mixed solution was incubated for 1 hour then washed using distilled water three times. Five ml of lipase was added and incubated for 3 hours, then washed three times using a phosphate buffer pH 7 to remove nonimmobilized lipases. Immobilized lipases are stored at 4^{o}C [31].

2.7. Immobilization Using Sodium Alginate Bio-Polymer

Lipase immobilization using Sodium Alginate biopolymers can use the entrapment method [32]. The first step is to make a 2.5% sodium alginate solution by mixing 2.5 grams of sodium alginate in 100 ml of distilled water. Then 4 ml of lipase mixed with 16 ml of sodium alginate solution. The mixture of lipase and sodium alginate was dropped into 20 ml of $CaCl^2$ solution using a syringe, so the gel beads with a diameter of 3 mm are formed. After 20 minutes, Ca-Alg beads formed were separated from $CaCl^2$ solution and washed with phosphate buffer (pH 7) [6], [33].

2.8. Lipase Immobilization Using Chitosan Bio-Polymer

Lipase immobilization using chitosan biopolymers was carried out by cross-linking method. Two grams of chitosan as a career was added with 5ml glutaraldehyde 5% and 1 ml HCl 1%, the mixed solution was incubated for 1 hour then washed using distilled water three times. Added 5 ml of lipase and incubated for 3 hours, washed three times using a phosphate buffer pH 7 to remove nonimmobilized lipases. Immobilized lipases are stored at 4^{o}C [31].

2.9. Lipase Activity Assay

Lipase activity test can be done by reacting 0.1 ml TG with 0.1 ml lipase along with 0.8 ml phosphate buffer and tween 80 0.05 ml, then incubating at 50 ^{o}c for 1 minute. After incubation reacted the enzyme with a 40 µL reagent periodate incubated for 10 minutes, add one drop of formalin kit reagent and incubated for 15 minutes. The absorbance was measured using a UV-Vis spectrophotometer at 550 nm [27].

2.10. Lipase Protein Content Test

Bradford reagents were made using 100 mg Coomassie Brilliant Blue G-250 dissolved in 50 ml of 95% ethanol, add 100 ml 85% (w/v) phosphoric acid. Dilute until the color disappears all and strain using Whatman No.1 filter paper to volume 1 L [34]. The protein concentration was measured using the standard curve of Bovine Serum Albumin (BSA) protein. The measurement of sample concentration was carried out by reacting 100 µl of the enzyme sample solution with 5 ml of Bradford solution, vortexed and incubated at room temperature in dark conditions for 2 minutes. Then measured the absorbance with a spectrophotometer at 595 nm [34]. The absorbance value is then entered into a standard curve to determine the concentration of protein contained in the enzyme sample.

2.11. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Samples from free lipases and immobilized lipases can be compared for their protein content using FTIR analysis [34]. Wavelength with a range of 400 to 4000 nm were used for evaluation of enzyme immobilization procedures.

2.12. Lipase Reusability And Storage Ability Test

The immobilized lipase reusability test was carried out by activity tests as much as 8 repetitions and represented on a graph to determine the level of decline in activity that occurred. In the immobilized lipase shelf life test, the immobilized lipase activity test was carried out four times in 30 days storage intervals and represented on a graph to determine the level of decline in activity that occurred.

3. Results and Discussions

3.1. Lipase Immobilization Strategy in Different Careers

The lipase immobilization strategy in carriers of tapioca starch, gelatin, and chitosan used the crosslinking method by utilizing glutaraldehyde as a cross-linker agent visualized in Figure 1.

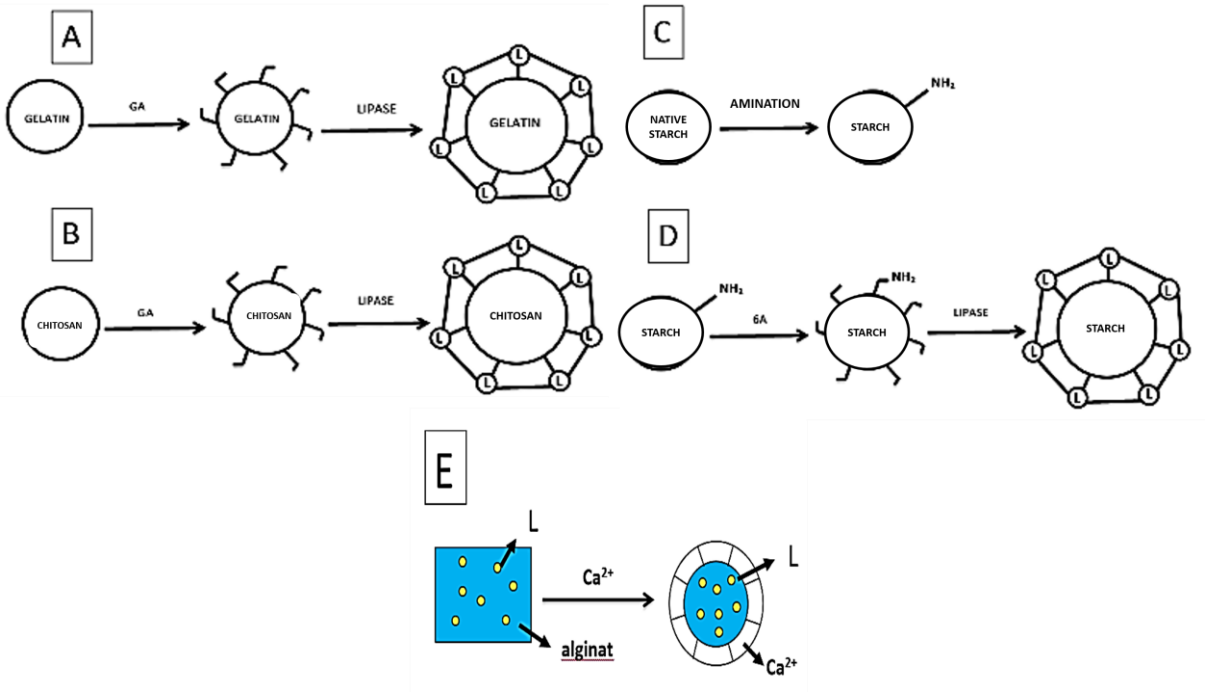

Figure 1. Schematic of Lipase Immobilization Using Different Carriers (A) Immobilization of Lipase Using Biopolymer Gelatin, (B) Chitosan, (C) Amination of Starch, (D) Immobilization of Lipase Using Aminated Tapioca Starch, (E) Lipase Immobilization Using Sodium Alginate Carrier. "L" Lipase, "GA" Glutaraldehyde

In Figure 1, almost all career activations use diantaranyaglutaraldehyde as a cross-linker agent. Glutaraldehyde is the most effective and accessible compound in enzyme immobilization [35]. This method is straightforward, efficient, and can improve the stability of the enzyme by producing covalent bonds from the reaction of aldehyde glutaraldehyde groups and amine groups so that it has a stronger bonding structure [35]-[39].

Figures 1 (A) and (B) shows a scheme of immobilization using careers of gelatin and chitosan that utilize cross-linker glutaraldehyde agents to strengthen the covalent bonds formed [20]. According to [13], [40], [16], [31], [41], [42] polymers which do not contain amine groups must be amended first so that a bond between carbon and amine occurs in glutaraldehyde and polymer, so that the polymer tapioca starch is carried out aminization using an ammonium hydroxide precursor (Figure 1 C) before reacting with glutaraldehyde (Figure 1 D).

Lipase immobilization in sodium alginate was carried out by the entrapment method (Figure 1 E), sodium alginate, and lipase solution was dropped on salt Ca^{2+} to strengthen the settlement. Cation Ca^{2+} acts as a cross-linking agent for alginate, and precipitation of droplets forms beads where enzymes are trapped in its tissues [33].

The results of immobilization are influenced by the environment and content of the career. Figure 2 shows the shape of the results of lipase immobilization using a variation career aminated tapioca starch, gelatin, alginate, and chitosan.

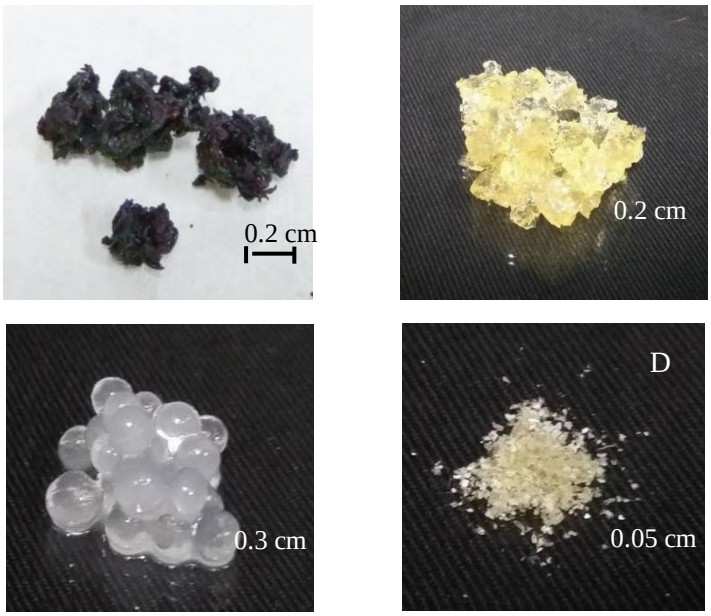

Figure 2. Visualization of the Forms of Lipase Immobilization Using Biopolymer Variations: (A) Aminated Tapioca Starch, (B) Gelatin, (C) Sodium Alginate, and (D) Chitosan.

Immobilized lipases using starch biopolymers have a black structure, resemble a solid paste, and has a diameter of 0.2 cm. Lipase immobilization using gelatin biopolymers formed beads which were gelatinized with a water-soluble diameter of 0.2 cm. Chitosan biopolymers that have solid granule form are also water-insoluble with a diameter of 0.05 cm, so they are reusable and easily separated from the substrate. In lipase immobilization using sodium alginate biopolymer formed the results of beads with a diameter of 0.3 cm, which is non-water soluble.

3.2. Lipase immobilization in different biopolymers

Lipase immobilization in this study used variations of biopolymers, consists of tapioca starch, gelatin, sodium alginate, and chitosan. The crude lipase activity before and after immobilization is listed in Table 1. The highest activity in crude lipase before immobilization was 3,353.6 U/ml with a protein content of 0.419 mg/ml; the activity indicated that lipase could hydrolyze 3,353.6 µmol triacylglycerols (TG) in 1 minute [43], [44].

Table 1. Immobilized Lipase Activity and Protein Levels

Lipase	Lipase Activity (U/ml)	Protein Content (mg/ml)
Crude lipase	3.353,6	0,419
Lipase + tapioca starch	1.308,7	0,207
Lipase + Gelatin	1.169,6	0,205
Lipase + sodium alginate	302,9	0,153
Lipase + chitosan	579,7	0,116

Based on Table 1, lipase activity after immobilization showed a significant decrease; this is most likely due to a decrease in the total amount of protein after immobilization. [45 and [46] state that the enzyme will decrease in activity after immobilization process due to a reduction in the levels of enzyme proteins and also the partial denaturation of bound proteins.

The highest activity of immobilized lipase was achieved in careers of tapioca starch and gelatin, 1,308.7 U/ml and 1,169.6 U/ml with protein levels of 0.207 mg/ml and 0.205 mg/ml. Tapioca starch and gelatin using cross-linking method to produce covalent bonds of amine and aldehyde groups so that they have a stronger bonding structure. based on the statement of [35] that the aldehyde group contained in glutaraldehyde is very reactive with the amine group by forming a ring structure and producing strong bonds. The more amine groups present in careers, the higher the covalent bonds that occur between careers and enzymes.

In addition to tapioca starch and gelatin, chitosan also used the cross-linking method in the process of immobilizing lipase but showed lower lipase activity results of 579.7 U/ml with a protein content of 0.116 mg/ml, which could be influenced by the small number of clusters binding function. Chitosan is known to have a small number of amine groups that can covalently bind with lipase [13], [40], [16], [31], [41], [42]. The lowest lipase activity was found in lipase immobilization using sodium alginate biopolymer which showed of 302.9 U/ml with protein content of 0.153 mg/ml which used the entrapment method which had several disadvantages such as cell leakage, high immobilization costs, diffusion limitations, deactivation enzymes during the immobilization process and have low loading capacity [47], [48], [24], [49], [50]. This method can cause the activity produced by immobilized lipases using the entrapment is lower than immobilized lipase using the cross-linking process.

3.3. FTIR Analysis of Amination Starch

Tapioca starch does not have an amine group; this makes it difficult to combine covalently between enzymes with carrier [51], [52], so amination is carried out before the amine group is added. FTIR test results of aminization of tapioca starch with variations in the concentration of oxidant agents (sodium metaperiodate) are listed in Figure 3.

Figure 3 shows the control group (tapioca starch without amination) with an absorbance range of 3,200-3,550 cm-1 (red circle). It shows the vibrations of the hydroxyl groups which indicate the absence of an amine group. Otherwise, the vibration stretch amine (N-H) which is in the absorbance range of 3,100-3,500 cm-1 (red rectangle) is indicated by a group of tapioca starch which is aminated by the treatment of sodium metaperiodate with an ammonium hydroxide precursor. The closer to the wavelength of 3,500 cm-1, the more the amine groups were detected. This is because the increasing number of dialdehyde starch (DAS) is produced along with the increased concentration of sodium metaperiodate to convert aldehyde, hydroxyl and carboxyl groups into amine [29], [30].

The higher the amine group formed in tapioca starch, the greater the lipase proteins are bound to material support so that more significant lipase activity will be obtained. The starch amination process using sodium metaperiodate oxidation has the disadvantage of degradation of polymeric units from starch, which disrupts the gelatinization process, so it has resulted in irregular particles [30].

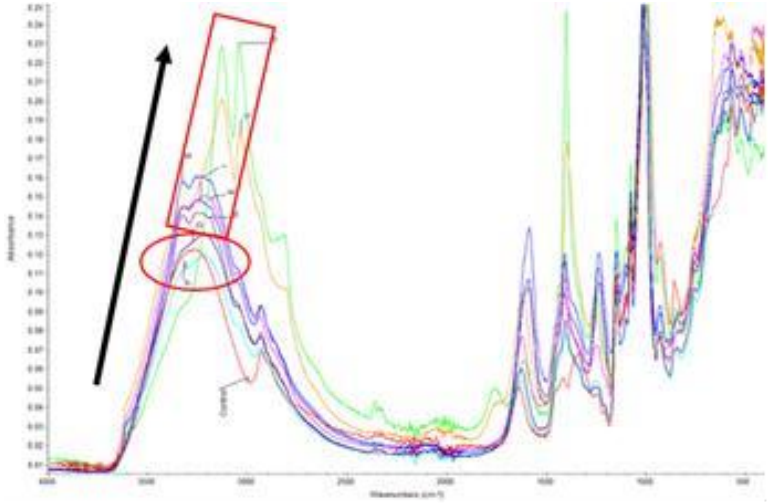

Figure 3. Spectra of FTIR Aminated Tapioca Starch (Arrows Shows Addition of Concentration, Rectangular Signs Shows Amine Cluster Peak Movement, Circle Signs Shows Peak Cluster (O-H)).

3.4. FTIR Analysis of Polymers After Lipase Immobilization

The characteristics of FTIR spectra of immobilized lipase functional groups are shown in Figure 4.

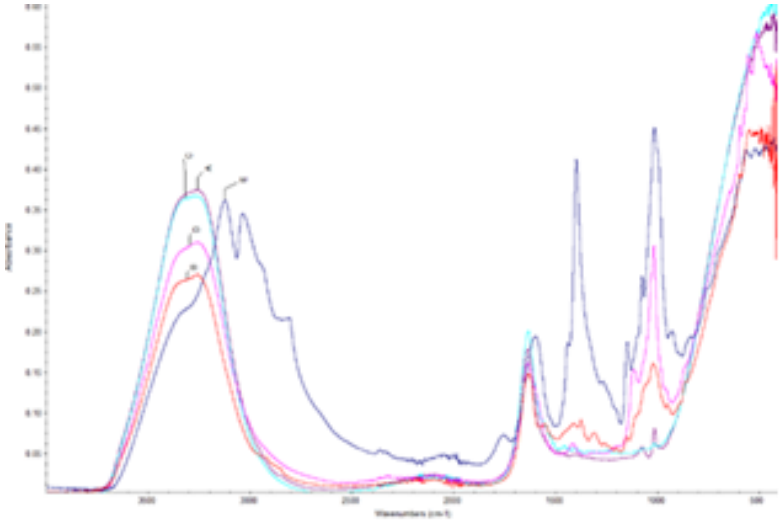

Figure 4. FTIR Spectra of Immobilized Lipase (A) Lipase Without Immobilization, (B) Chitosan, (C) Gelatin, (D) Sodium Alginate (E) Aminated Tapioca Starch.

Based on Figure 4, the absorbance range of lipases in FTIR spectra is at a wavelength of 4,000400 cm-1. The range of absorbance of 3,200-3,550 cm-1 shows the vibration of the group (O-H) contained in crude lipase. The absorbance of groups (O-H) in crude lipase before immobilization has a lower intensity, 3,257.79 cm-1. Whereas in crude lipase immobilized using gelatin, sodium alginate and chitosan have a peak with a higher absorption intensity, 3,258.51 cm-1, 3,258.11 cm-1, 3,258.75 cm-1 and decreased absorption intensity in crude lipase immobilized using tapioca starch, 3,124.98 cm-1.

The range of absorbance of 3,100-3,500 cm-1 also shows stretch group vibrations (N-H) which are indicated by the appearance of new peaks in the absorbance range, the wave of stretch groups (N-H) is evident in crude lipase immobilized using tapioca starch at 3,124.98 cm- 1, this indicates that lipase has a free amine group which can play an essential role in forming covalent bonds with carriers and glutaraldehyde as an active aldehyde group [53].

The absorption band in the range of 1,590-1,655 cm-1 shows the vibration of (N-H) group bonds. The absorbance of group bonds (N-H) on crude lipase before immobilization showed a lower intensity at a wavelength of 1,635.93 cm-1 whereas the immobilized crude lipase using gelatin and chitosan showed a higher absorption intensity at 1,636.59 cm-1 and 1,636.51 cm-1. Immobilized crude lipase using a career of sodium alginate and aminated tapioca starch showed higher absorption, 1,637.09 cm-1 and 1,697.99 cm-1 which indicate that more amine group bonds occur in immobilized crude lipase. Hydroxyl and amine groups present in careers can facilitate bonding with enzymes and glutaraldehyde, used as a cross-linking agent, can improve the stability of bonds in carriers with enzymes [16], [54], [55].

The absorption band in the range of 1,000-1,300 cm-1 shows the vibration of the group (C-O). Absorption group (C-O) in crude lipase before immobilization shows a lower intensity at 1,015.82 cm-1. While immobilized crude lipase using aminated tapioca starch, sodium alginate, and chitosan showed peak movement with higher absorption intensity, at wavelengths of 1,016.03 cm-1, 1,018.24 cm-1, and 1,023.51 cm-1.

The absorption band in the range of 1,000-1,250 cm-1 shows bond vibration of (C-N), the bond of the group in crude lipase before immobilization shows a lower intensity, at 1,015.82 cm-1. Whereas in immobilized crude lipase using aminated tapioca starch, sodium alginate and chitosan showed peak movement with higher absorption intensity, at 1,016.03 cm-1, 1,018.24 cm-1, and 1,023.51 cm-1. The bond indicates the occurrence of covalent bonds between polymers and lipases due to the reaction of amines and carboxyls [56]. Also, the absorption band in the range 1,300-1,500 cm-1 shows the bond

vibration of (C-H). Immobilized crude lipase using chitosan carrier showed a wavelength of 1,377.20 cm-1 and increased in aminated tapioca starch and sodium alginate, at 1,398.86 cm-1 and 1,420.27 cm-1.

Immobilized crude lipase using a carrier of aminated tapioca starch showed the absorption bands at wavelengths of 1,075.96, showed stretch group vibrations (C-N) and formed absorption bands at wavelength 1,149.27 cm-1 which showed vibrations of (C-F) group and at a wavelength of 3,035.61 cm-1 shows the vibration of (C-H) group.

3.5. Lipase Reusability and Storage Stability Test

The reusability test of immobilized lipases is listed in Figure 5. Immobilized lipase activity at the first repetition in hydrolyzing the substrate is considered 100%. The ability to reuse immobilized lipases using carriers of tapioca starch, gelatin, sodium alginate, and chitosan showed a significant decrease for eight repetitions. Tapioca starch carriers have the highest stability with the lowest decline of 69.4%, 65% in the sodium alginate, 48.2% in the chitosan and the most significant decrease in gelatin reaching 15%, this can be due to enzyme inactivation, protein denaturation and enzyme leakage from carriers during reuse [57], [58].

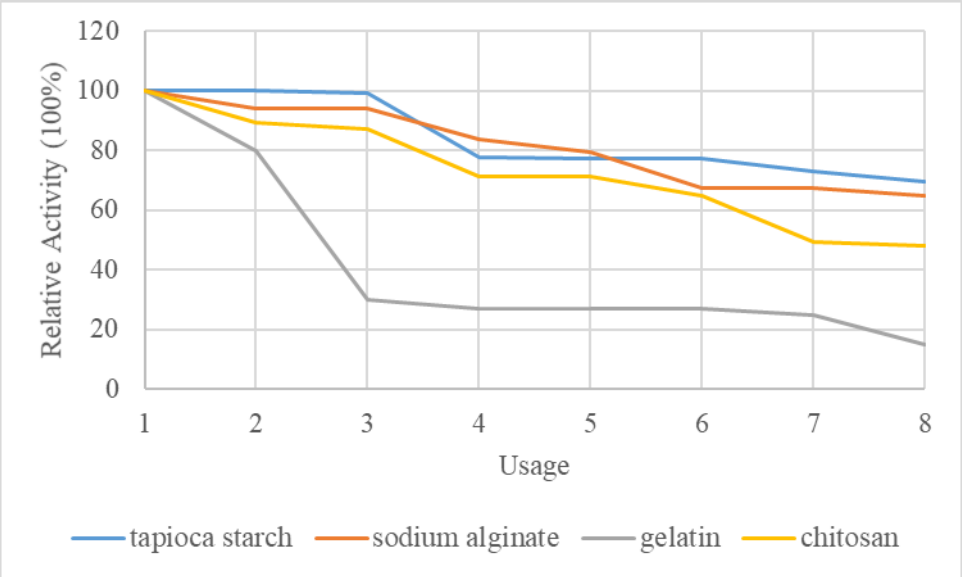

Figure 5. Percentage of Immobilized Lipase Activities in Reuse of Hydrolizing TG.

Based on the reusability test of immobilized lipases, it was shown that immobilized lipases were reusable, as shown in Figure 6.

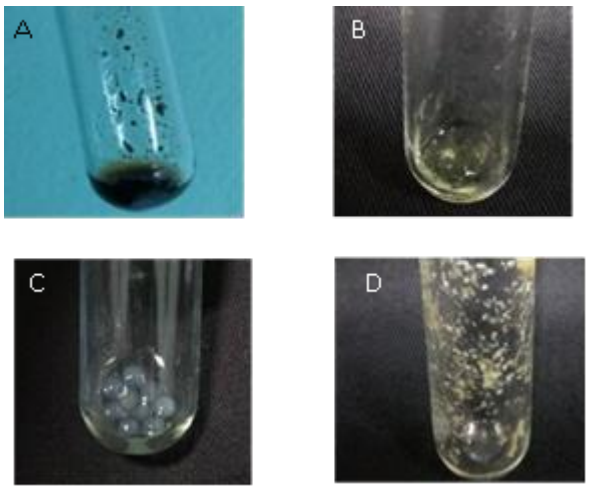

Figure 6. Visualization of Reusable Forms of Lipase Immobilization: (A) Aminated Tapioca Starch, (B) Gelatin, (C) Sodium Alginate and (D) Chitosan

Immobilized lipase using tapioca starch biopolymers (Figure 6 A), gelatin (Figure 6 B), sodium alginate (Figure 6 C) and chitosan (Figure 6 D) can be separated from the substrate after being used to hydrolyze triacylglycerides. This ability shows that the use of immobilized lipases can improve efficiency on an industrial scale.

The shelf life of immobilized lipases is an essential factor in the application of industrial scale [16]. The test results for the immobilized lipase are shown in Figure 4.7. The shelf life of immobilized lipases using tapioca starch biopolymers, gelatin, sodium alginate, and chitosan showed a significant decrease in 30 days. Tapioca starch carriers have the highest shelf life ability characterized by the lowest decline of 83.7%, followed by immobilized lipase using sodium alginate and chitosan biopolymers 81.8% and 50.7% respectively. The shelf life of immobilized lipases using sodium alginate has the highest results can be caused by the small diameter of alginate porous [33].

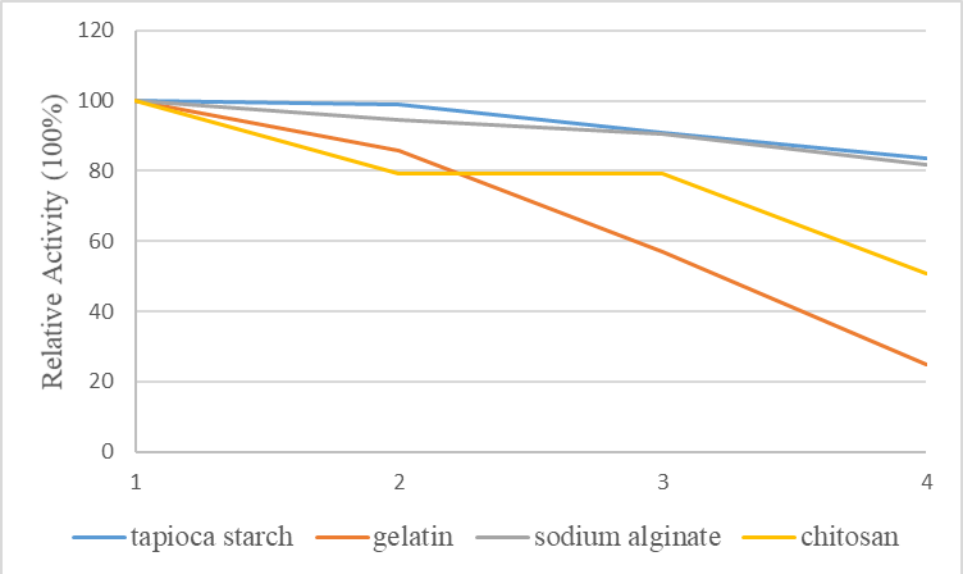

Figure 7. Percentage of Storage Ability of Immobilized Lipase.

The highest decrease in the shelf life of gelatin polymers, 24.7%, was probably due to the instability of covalent bonds formed by the less amine groups compared to tapioca starch [56].

4. Conclusions

This study concluded that lipase immobilization in a carrier of contaminated tapioca starch had the highest activity of 1,308.7 U/ml with a protein content of 0.207 mg/ml. The results of FTIR analysis on immobilized lipases using careers of tapioca starch, gelatin, alginate, and chitosan showed the presence of group (N-H), (C-N), and (C-H) bonds which indicated the results of carrier and protein immobilization. Immobilized lipase using aminated tapioca starch also has high reuse and shelf life ability of 69.4% and 83.7% respectively.

References

[1] Bora, L. Bora, M. 2012. Optimization Of Extracellular Thermophilic Highly Alkaline Lipase From Thermophilic Bacillus Sp Isolated From Hotspring Of Arunachal Pradesh, India. Brazilian Journal of Microbiology, 30-42.

[2] Sharma, R. Chisti, Y. and Banerjee, U. C. 2001. Production, purification, characterization, and applications of lipases. Biotechnology Advances, 70 :1-15.D

[3] Ma, F. and Hanna, M. A.. 2002. Biodiesel production: a review. Technology, 67(6):2138–2142.

[4] Ghaly, A. E. Dave, D. Brooks, M.S. and Budge, S. 2010. Production of Biodiesel by Enzymatic Transesterification: Review. American Journal of Biochemistry and Biotechnology, 6 (2): 54-76

[5] Lee, K. Y. and Mooney, D. J. 2012. Alginate: properties and biomedical applications. Prog Polym Sci, 37(1): 106–126.

[6] Minovska, V. Winkelhausen, E. and Kuzmanov, S. 2005. Lipase Immobilized by different techniques on various support materials applied in oil hydrolysis. J. Serb. Chem. Soc, 70 (4): 609–624.

[7] Ramani, K. Chockalingam, E. and Sekaran, G. 2010. Production of a novel extracellular acidic lipase from Pseudomonas gessardii using slaughterhouse waste as a substrate. J Ind Microbiol Biotechnol. 37:531–535.

[8] Bayoumi, R.A. Atta, H.M. and El-Sehrawy, M.H. 2012. Bioremediation of Khormah Slaughter House Wastes by Production of Thermoalkalistable Lipase for Application in Leather Industries. Life Science Journal, 9(4).

[9] Houde, A. Kademi, A. and Leblanc, D. 2004. Lipases and Their Industrial Applications. Applied Biochemistry and Biotechnology, Vol. 118.

[10] Fukuda, H. Kondo, A. and Noda, H. 2001. Biodiesel Fuel Production by Transesterification of Oils. Journal Of Bioscience And Bioengineering, Vol. 92, No. 5,405-416.

[11] Souza, R. R. Ferreira, R. D. M. T. and Elias, B. 2014. Immobilization of Lipase from Candida Rugosa on Hydrophobic and Mesoporous Support by Adsorption (MCM 41). Chemical Engineering Transactions, Vol, 37: 565-570.

[12] Zhang, L. Zhang, W. Yin, F. Zhou, X. Li, J. Xu, R. Chen, Y. and Liu, S. 2010. Lipase Catalyzed Production of Biodiesel. Laboratory of Advanced Technique and Preparation for Renewable Energy Materials. 978-1-4244-4813-5.

[13] Mohamad, N. R. Marzuki, N. H. C. Buang, N. A. H. F. and Wahab, R. A. 2015. An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. Biotechnology & Biotechnological Equipment, Vol. 29, No. 2, 205220.K

[14] Christensen, M. W. Andersen, L. and Husum, T. L. Kirk, O. 2003. Industrial lipase immobilization. Eur. J. Lipid Sci. Techno, 105 : 318–321.

[15] Ren, Y. Rivera, J. G. He, L. Kulkarni, H. Lee, D. K. and Messersmith, P. B. 2011. Facile, high efficiency immobilization of lipase enzyme on magnetic iron oxide nanoparticles via a biomimetic coating. BMC Biotechnology, 11:63.

[16] Datta, S. Christena, L. R. and Rajaram,Y. R. S. 2013. Enzyme immobilization: an overview on techniques and support materials. Biotech, 3:1–9.

[17] Nigam, S. Mehrotra, S. Vani, B. and Mehrotra, R. 2014. Lipase Immobilization Techniques for Biodiesel Production: An Overview. International Journal of Renewable Energy & Biofuels, Vol 2014

[18] Cheetham, P. S. J. Blunt, K. W. and Bucke, C. 1979. Physical Studies on Cell Immobilization Using Calcium Alginate Gels. Biotechnology and Bioengineering, Vol. XXI, Pp. 2155-2 168.

[19] Klibanov, A. M. 2014. Immobilized Enzymes and Cells as Practical Ctalysts. Science, Vol 219.

[20] Malcata, F. X. Reyes, H. R. Garcia, H. S. Hill, C. G. and Amundson, C. H. A. 1990. Immobilized Lipase Reactors for Modification of Fats and Oils-A-Review. JADCS, Vol 97, No 12.

[21] Zhao, X. Qi, F. Yuan, C. Du, W. and Liu, D. 2015. Lipase-catalyzed process for biodiesel production: Enzyme immobilization, process simulation and optimization. Renewable and Sustainable Energy Reviews 44 :182–197.

[22] Fernandez-Lafuente, R. Armisen, P. Sabuquillo, P. Fernandez-Lorente, G. and Guisan., J. M. 1998. Immobilization of lipases by selective adsorption on hydrophobic supports. Chemistry and Physics of Lipids, 93 :185–19..

[23] Jegannathan, K. R. and Abang, S. 2008. Consumer acceptance meat quality aspects. Production of Biodiesel Using Immobilized Lipase—A Critical Review. Critical Reviews in Biotechnology, 28:253–264.

[24] Gao, W. Diao, X. Luo, G. and Dai, Y. 2010. Effect of pore diameter and cross-linking method on the immobilization efficiency of Candida rugosa lipase in SBA-15. Bioresour. Technol, Vol 101: 3830-3837.

[25] Rajendran, A. and Thangavelu, V. 2012. Optimization and Modeling of Process Parameters for Lipase Production by Bacillus brevis. Food Bioprocess Technol 5:310–322.

[26] Estuğrul, S. Dönmez, G. and Takac, S. 2007. Isolation of lipase producing Bacillus sp. from olive mill wastewater and improving its enzyme activity. Journal of Hazardous Materials. 149 : 720–724

[27] Kuhn, J. Müller, H. Salzig, D. and Czermak, P. 2015. A Rapid Method For An Offline Glycerol Determination During Microbial Fermentation. Electronic Journal of Biotechnology, Vol 18: 252–255.

[28] Bradford, M. M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry (1976) 72, 248-254

[29] Watts, L. W. J. and Austin, T. 1979. United States Patent: Aminated Starch Derivatives. Texaco Development Corporation, 828,799.

[30] Wongsagon, R. Shobsngob, S. and Varavinit, S. 2005. Preparation and Physicochemical Properties of Dialdehyde Tapioca Starch. Biotechnology, 166–172

[31] Xie, R. Cui, C. Chen, B. and Tan, T. 2015. Immobilizing Yarrowia lipolytica Lipase Lip2 via Improvement of Microspheres by Gelatin Modification. Appl Biochem Biotechnol.

[32] Klibanov, A. M. 2014. Immobilized Enzymes and Cells as Practical Ctalysts. Science, Vol 219.

[33] Kavardi, S. S. S. Alemzadeh, I. and Kazemi, A. 2012. Optimization Of Lipase Immobilization. IJE Transactions C: Aspects, Vol. 25, No. 1.

[34] Bruno, L. M. Filho, J. L. L. and Castro, H. F. 2008. Comparative Performance of Microbial Lipases Immobilized on Magnetic Polysiloxane Polyvinyl Alcohol Particles. Brazilian Archives Of Biology And Technology, 51, 5 : 889-896.

[35] Migneault, I. Dartiguenave, C. Bertrand, M. J. and Waldron, K. C. 2004. Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. BioTechniques 37:790-802.

[36] Nazari, T. Alijanianzadeh, M. Molaeirad, A. and Khayati, M. 2016. Immobilization of Subtilisin Carlsberg on Modified Silica Gel by Cross-linking and Covalent Binding Methods. Biomacromol. J., Vol. 2, No. 1, 53-58.

[37] Adlercreutz, P. 2013. Immobilisation and application of lipases inorganic media. Chem.Soc.Rev, Vol 42, 6406—6436

[38] Betancor, F. Hidalgo, N. A. M. R. Fernández, L. and Guisán, J.M. 2006. Enzyme Microbiology. Enzyme Tech. 39. 877.

[39] Gallego, F. L. Betancor, L. C. M. Hidalgo, N. A. M. Ortiz, D. Guisán, J.M. and Lafuente, F. J. 2005. Biotechnol. 119. 70.

[40] Nawani, N. Singh, R. and Kaur, J. 2006. Immobilization and stability studies of a lipase from thermophilic Bacillus sp: The effect of process parameters on immobilization of enzyme. Electronic Journal of Biotechnology, Vol.9 No.5.

[41] Bello, J. Bello, H. R. and Vinograd, J. R. 1962. The Functional Groups In The Gelation Of Gelatin. Biochim. Biophys. Acta, 57. 222-229.

[42] Tokuyasu, K. Kaneko, S. Hayashi, K. and Mori, Y. 1899. Production of Recombinant Chitin Deactylation in the Culture Medium of Escherichia coli Cells. Journal FEBS. 458: 23–26.

[43] Willerding, A. L. Oliveira, L. A. Moreira, F. W. Germano, M. G. and Chagas, A. I. F. 2011. Lipase Activity among Bacteria Isolated fromAmazonian Soils. SAGE-Hindawi Access to Research Enzyme Research. Article ID 720194: 5..

[44] Kundu, M. Basu, J. Guchhait, M. and Chakrabarti, P. 1987. Isolation and Characterization of an Extracellular Lipase from the Conidia of Neurospora crassa. Journal of General Microbiology. 133, 149-153.

[45] Kress, J. Zanaletti. R. Amour, A. Ladlow, M. Frey, J.G. and Bradley, M. 2002. Enzyme accessibility and solid supports: which molecular weight enzymes can be used on solid supports?An investigation using confocal Raman microscopy. Chem Eur J 8:3769–377.

[46] Homaei, A. A. Sariri, R Vianello, F and Stevanato, R. 2013. Enzyme Immobilization: An Update. J Chem Biol (2013) 6:185–205.

[47] Krekeler, C. Z. and Klein, J. 1991. Influence of physicochemical bacterial surface properties on adsorption to inorganic porous supports. Appl. Microbiol. Biotechnol. 35:484-490.

[48] Song, S. H. Choi, S. S. Park, K. and Yoo, Y. Y. 2005. Novel hybrid immobilization of microorganisms and its applications to biological denitrification. Enzyme Microb. Technol. 37:567-573..

[49] Stolarzewicz, I. Bialecka-Florjañczyk, E. Majewska, E. and Krzyczkowska, J. 2011. Immobilization of yeast on polymeric supports. Chem. Biochem. Eng, Vol, 25:135-144. H., D. de Oliveira, M. A. Mazutti, M. Di Luccio, J. V. Oliveira. “A Review on Microbial Lipases Production”. Food Bioprocess Technol. (2010) 3:182–196.

[50] Martins, S. C. S. Martins, C. M. Fiúza, L. M. C. G. and Santaella, S. T. 2013. Immobilization Of Microbial Cells: A Promising Tool For Treatment Of Toxic Pollutants In Industrial Wastewater. Afr. J. Biotechnol. Vol. 12(28), pp. 4412-4418.

[51] Jesionowski, T. Zdarta, J. and Krajewska, B. 2014. Enzyme immobilization by adsorption: a review. Adsorption: 18 June 2014.

[52] Neelam, K. Vijay, S. and Lalit, Singh. 2012. Various Techniques For The Modification Starch And The Applications Of Its Derivates. International Research Journal Of Pharmacy, 3 (5).

[53] Ahmad, R. and Sardar, M. 2015. Enzyme Immobilization: An Overview on Nanoparticles as Immobilization Matrix. Biochem Anal Biochem, Vol 4:2.

[54] Elcin, Y. M. 1995. Encapsulation of Urease Enzyme in Xanthan-Alginate Spheres. Biomaterials, 16(15), 1157-1161.

[55] Flores-Maltos, A. Rodríguez-Durán, L. V. Renovato, J. Contreras, J. C. Rodríguez, R. and Aguilar, C. N. 2011. Catalytical Properties of Free and Immobilized Aspergillus niger Tannase. Enzyme Research, Vol. 2011: 6.

[56] Sachan, N. K. Pushkar, S. Jha, A. and Bhattcharya, A. 2009. Sodium Alginate: The Wonder Polymer For Controlled Drug Delivery. Journal of Pharmacy Research, 2(8),1191-1199.

[57] Ali, Z. Tian, L. Zhao, P. Zhang, B. Ali, N. and Khan, M. 2016. Immobilization Of Lipase On Mesoporous Silica Nanoparticles With Herarchichal Fibrous Pore. Journal of Molecular Catalysis B:Enzymatic, Vol 134, 129-135.

[58] Nickpour, M. and Pazouki, M. 2014. Synthesis and Characteristics of Mesoporous Sol-gels for Lipase Immobilization. Ije Transactions A: Basics, Vol. 27, No. 10, 495-1502.