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Research Article

Immobilization of Lipase on Agarose Beads for Enzymatic Hydrolsis/Transesterification of Castor Oil

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ABSTRACT

Lipase was immobilized onto agarose beads and used for enzyme-catalyzed hydrolysis/transesterification of castor oil at room temperature without surfactants and salts. The immobilization yield was evaluated by reacting *p*-nitrophenyl butyrate and immobilized lipase. The reaction was performed in a 25 mL phosphate buffer medium (pH 7.0) and incubated for 2 hours. The absorbance of the formed *p*-nitrophenol was measured at 346 nm. The highest immobilization rate was obtained using 5.0 mg lipase with 2.5 mL glyoxal agarose beads. The reaction was performed in 25.0 mL sodium bicarbonate buffer pH 10 for 12 h. The hydrolysis/transesterification of castor oil was performed in phosphate buffer (pH 7) – ethanol medium. The main products were ricinoleic acid and ethyl ricinoleate. The highest hydrolysis/transesterification yield was 87% using 1.0 g castor oil and 2.5 mL of lipase immobilized agarose beads in the phosphate buffer (pH 7): ethanol (1: 9, V: V) at 72 hours. The reaction products were analyzed using a Fourier Transform Infrared spectrometer (FTIR) and Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC).

1. Introduction

Most industrial processes about oil hydrolysis or transformation to produce valuable products such as free fatty acids (FFAs) or their esters are carried out in harsh conditions. Enzymatic reactions can be performed under more favorable conditions than complicated processes. Lipases, triacylglycerol acylhydrolases, are mainly derived from microorganisms and have been used for many applications, such as enzymatic oil hydrolysis, modifying flavor via fatty acids, producing alcohols, etc [1].

They can catalyze transformations of various organic reactions via esterifications and transesterifications [2]. Lipases are usually used for hydrolysis and transesterification of castor oil under mild conditions. Castor oil is a triglyceride of fatty acids, and ricinoleic acid is the main hydrolysis reaction product. Ricinoleic acid has anti-inflammatory and bactericidal properties [3]. Therefore, it has received significant interest in the pharmaceutical industry. Also, its ester derivatives are used in many industrial processes, such as emulsifiers and soaps [4].

Some studies investigated the effect of solution mediums on hydrolysis and transesterification of castor oil by lipases. Yamamoto and Fujiwara used sodium phosphate buffer (pH 7.0) as the reaction medium and reported that isooctane, Triton X-I00, or PEG-6000 addition slightly improved hydrolysis yields using free lipase [5]. Kulkarni and Pandit investigated the effect of n-hexane, n-heptane, isooctane, and diethyl ether solvents and reported that they all enhanced hydrolysis yield by using free lipase [6]. Jeon et

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al. achieved 100% hydrolysis yield via isopropyl ether–water mixture (1:1) by free lipase [7]. Malhotra et al. investigated lipase-catalyzed castor oil transesterification [8]. The reported conversion rates were 95%, 90%, and 80% for hexanol, octanol, and dodecanol. Narwal et al. used lipase-immobilized silica gel for castor oil transesterification [9].
The highest yield obtained was 78%. In the present work, 90% ethanol was used as a reaction medium, and hydrolysis and transesterification products were obtained via catalysis of lipase-immobilized agarose beads.
As can be seen from the literature studies, researchers have performed hydrolysis and transesterification of castor oil using different reaction media by free and immobilized lipase. This study aims to show the availability of agarose as a support material for lipase immobilization and the ability of lipase-immobilized agarose beads to obtain both ester and hydrolysis products in an alcohol/phosphate buffer (pH 7) medium.
Using agarose as a support material provides high immobilization efficiency, easy immobilization, and increased enzyme stability. Both hydrolysis and transesterification products were obtained in mild conditions using lipase-immobilized agarose beads and ethanol-phosphate buffer (pH 7) medium.

2. General Methods

2.1. Materials

All of the used chemicals were of analytical grade. High-density glyoxal 6BCL agarose (ABT) was used as supporting material. Lipase from *Burkholderia (Pseudomonas) cepacia* was purchased from Sigma. Ricinoleic acid, as standard, was purchased from TCI chemicals. Ethanol, NaOH, acetonitrile, acetone, p-nitrophenyl, and p-nitrophenyl butyrate (PNB) were purchased from Merck Chemicals. Before HPLC analysis, the solutions were filtered with a 0.45 µm mesh cellulose esters filter(CHMLAB).

2.2. Immobilization of lipase

The lipase immobilization was carried out according to the previously described method by Bolivar et al [10]. The glyoxal agarose beads (2.5 mL) were added to the 25.0 mL of 0.1 M sodium bicarbonate solution (pH 10) containing 2.5 – 7.5 mg lipase. The suspension was shaken with an orbital shaker at a 150 rpm agitation rate for 2 – 12 h intervals. At the end, 20.0 mg $NaBH_4$ was added to the suspension and stirred for 30 min. Then, it was washed with distilled water and stored in phosphate buffer (pH 7.0). The immobilization degree was evaluated with the hydrolysis of *p*NB.

2.3. Hydrolysis of *p*NPB

Hydrolysis of *p*-nitrophenyl butyrate (as substrate, 40 µM) was investigated using 2.5 mL of the immobilized enzyme. The reaction was performed in a 25 mL phosphate buffer medium (pH 7.0) and incubated for 2 hours. The absorbance of the product, *p*-nitrophenyl, was measured at 346 nm by a UV-Vis spectrophotometer (Shimadzu, UV-2600), and the concentrations were calculated using the calibration curve in the range of 10 – 40 µM *p*-nitrophenyl concentrations.

2.4. Hydrolysis/transesterification reaction

The hydrolysis/transesterification experiments were carried out in a 1:9 volume ratio of phosphate buffer (pH 7)-ethanol medium with different reaction times (2 – 72 h), shaking rates (30 – 200 rpm), enzyme immobilized agarose beads amount (1 – 5 mL in phosphate buffer, pH 7) and castor oil amount (0.1 – 2.0 g) in a closed erlenmeyer flask. First, an appropriate amount of castor oil was dissolved in ethanol, and then the immobilized enzyme in the buffer solution was added to the reaction medium. The total reaction volume was made up to 25 mL. Approximately 100 µL of the sample was collected with the help of an automatic pipette at the specified times. The samples were filtered with a 0.45 µm cellulose filter before HPLC analysis. All experiments were performed in three replicates. The total (hydrolysis and transesterification) conversation rates were calculated using the peak areas of TAG in HPLC analysis before and after reaction with castor oil and immobilized enzyme. The concentrations of formed ricinoleic acid and

ethyl ricinoleate were calculated using the calibration curve (4 – 80 µg/mL).

2.5. Analysis of products

The hydrolysis/transesterification products were analyzed by an RP-HPLC (Shimadzu model LC-20AD, SPD-M20A diode array detector). The acetonitrile and acetone (V/V, 2:1) mixture was used as the mobile phase. The C18 column was used (5 µm, 4.6 mm × 250 mm Intersustain) for the analysis. The flow rate was 1.0 mL/min, and the injection volume was 3.0 µL. FTIR analyses of castor oil and the products were carried out with a Perkin Elmer Spectrum Two FTIR with ATR attachment. After the reactions, the solutions were filtered with a 0.45 µm cellulose filter paper and dried in a vacuum oven at 30 °C before FTIR analysis.

3. Results and Discussion

3.1. Lipase immobilization

Enzyme immobilization experiment was carried out in 1, 2, 4, 8, and 12 hours to provide maximum immobilization. Immobilization occurred between amine groups of lipase and aldehyde groups of glyoxal agarose beads and resulted in the formation of imine groups. With the addition of $NaBH_4$, these groups were reduced, and stable multi-covalent bonds formed between lipase and glyoxal agarose beads [10, 11]. The immobilization degree was evaluated by *p*NPB hydrolysis [12]. The absorbance of the product, *p*-nitrophenyl, was measured at 346 nm. The occurred reaction is shown in Figure 1. The hydrolysis rate increased from 56% to 99% by raised times from 2 to 12 h (Figure 2). Immobilization was also tried with different enzyme amounts to reach maximum hydrolysis yields. The lipases (2.5, 5.0, and 7.5 mg) were used for immobilization with 2.5 mL of agarose. Nearly 100% hydrolysis yield was achieved using 5.0 mg of the enzyme (Figure 2.). As can be seen from the results, maximum immobilization was obtained with a 12 h reaction using 5 mg enzyme and 2.5 mL agarose beads. Further experiments were carried out with immobilized lipase obtained in a reaction of 5 mg lipase with 2.5 mL glyoxal agarose beads in 25 mL sodium bicarbonate buffer pH 10 for 12 h.

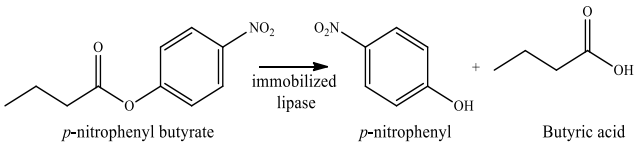

Figure 1. The catalytic reaction of *p*-nitrophenyl butyrate

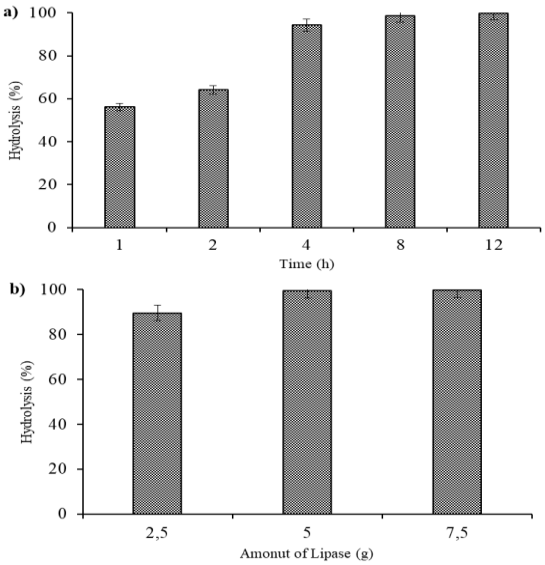

Figure 2. The immobilized enzyme activities in different immobilization times (a) (5.0 mg of lipase, 2.5 mL of glyoxal agarose) and amounts of lipase (b) (2.5 mL of glyoxal agarose, 12 h

3.2. Characterization of lipase

RP-HPLC and FTIR analyses were performed for the identification of products. RP-HPLC chromatograms are shown in Figure 3. As can be seen from the figure, castor oil is mainly triacylglycerol (TAG)(~%70), and a large proportion of TAG remained unhydrolyzed at the reaction in the buffer. The hydrolysis increased with the rising ethanol ratio, and transesterification products were also formed in the reaction. The primary hydrolysis and transesterification products were ricinoleic acid and ethyl ricinoleate. The obtained highest amounts were 0.41 g for ricinoleic acid and 0.47 g for ethyl ricinoleate using 2.5 mL of lipase-immobilized agarose beads. The reaction was performed using 1.0 g of castor oil at 150 rpm agitation rate for 72 hours in a 25 mL 1: 9 (V: V) ratio of phosphate buffer (pH 7): ethanol medium. The retention times of ricinoleic acid and ethyl ricinoleate were 3.47 minutes and 4.72

minutes. The other observed peaks were probably castor oil hydrolysates such as linoleic, oleic, palmitic, stearic, and linolenic acids and their esters.

The FTIR spectra of castor oil and product are shown in Figure 4. The 3380 and 3009 cm^{-1} peaks in the castor oil spectrum were attributed to the –OH and =CH vibrations. The 1743 and 1162 cm^{-1} peaks were assigned to C=O and C-O symmetric stretching vibrational bands, respectively. The product spectrum supports that the product was a mixture of FFAs and esters. A split band was obtained at around 1700 cm^{-1} from FFAs and esters. The C-O vibration bands of FFAs and esters appeared at 1179 and 1034 cm^{-1}, respectively. These results were agreed with Khaskheli et al [13].

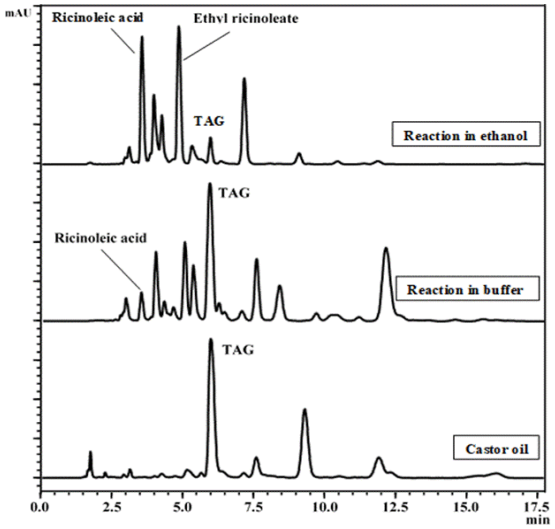

Figure 3. HPLC chromatograms of castor oil and products after hydrolysis/transesterification reactions. (72 h, 150 rpm, 2.5 mL lipase immobilized agarose beads, 1.0 g castor oil, 25 mL reaction volume)

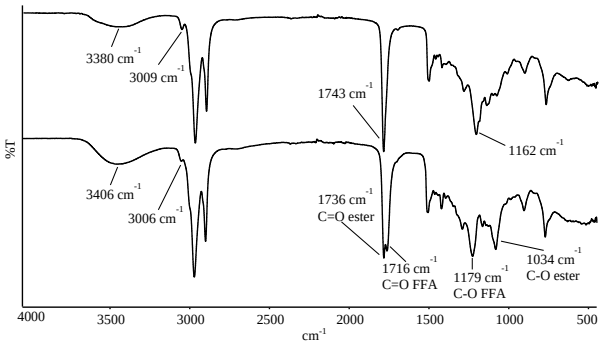

Figure 4. FTIR spectra of castor oil and product

3.3. Optimization of substrates ratio

The hydrolysis/transesterification reactions were carried out in different ethanol/buffer ratios. The enzyme-substrate interactions were feeble when only water was used as solvent due to the low solubility of oil. The ricinoleic acid (1.0%) formed when the reaction was performed in the phosphate buffer solution (pH 7).

Although the solubility problem could be overcome using surfactants, salts, or higher temperatures, there are also some disadvantages. The use of alcohols could be a solution to oil solubility but decrease yields of the hydrolysis reaction due to the occurring transesterification reaction. Both hydrolysis and transesterification efficiency increased with more dissolved castor oil by raising the ethanol ratio in the solution (Table 1). Also, ethanol acted as a substrate for transesterification and increased the reaction efficiency. The obtained maximum alcohol ratio was 90% because the synthesized lipase immobilized agarose was stored in the buffer and added to the reaction medium in buffer solution. In 1:9 (phosphate buffer (pH 7): ethanol, V: V) reaction medium, the result of the hydrolysis and transesterification reactions (Figure 5) FFAs and ester products were formed. The main products were ricinoleic acid and ethyl ricinoleate. In the enzyme: oil ratio experiments, the reaction yields decreased with the increased castor oil due to insufficient immobilized lipase in the solution (Table 2). The reaction yields were calculated from total TAG conversion rates obtained by HPLC analysis. The conversion percentage decreased from 87% using 1.0 g of castor oil to 43% using 2.0 g of castor oil with 2.5 mL lipase immobilized agarose beads at 150 rpm agitation speed and 72 h. Also, the decrease in efficiency may be due to the increased viscosity of the solution at higher oil content. As expected, the reaction yield increased when the amount of lipase-immobilized agarose beads was raised, and the castor oil was kept constant (1.0 g). The obtained efficiencies were 31% and 99% using 1 mL and 5 mL of immobilized lipase at 150 rpm agitation speed and 72 h.

Table 1. The effect of ethanol concentration on the hydrolysis/transesterification of castor oil with immobilized lipase

Ratio of phosphate buffer (pH 7):ethanol (V:V)	Ricinoleic acid (g)	Ethyl ricinoleate (g)
1:0	0.1	-
1:3	0.15	0.17
1:6	0.27	0.31
1:9	0.40	0.47

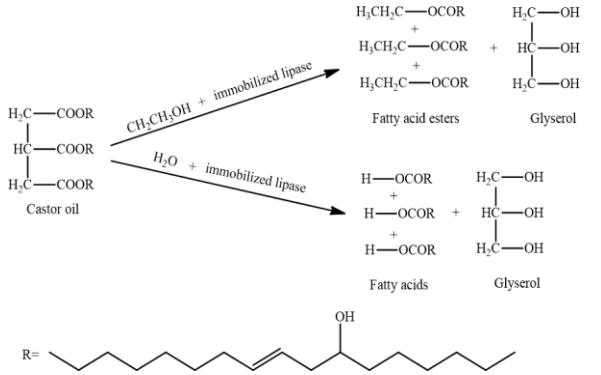

Figure 5. Hydrolysis and transesterification reactions of castor oil

Table 2. The effect of castor oil amount on the hydrolysis/transesterification of castor oil with immobilized lipase

Ratio*	Hydrolysis / transesterification(%)
2.5:0.1	99
2.5:0.5	90
2.5:1.0	87
2.5:1.5	70
2.5:2.0	43
1.0:1.0	31
2.0:1.0	70
3.0:1.0	90
4.0:1.0	96
5.0:1.0	99

*Lipase immobilized agarose beads (mL): castor oil (g) in phosphate buffer (pH 7): ethanol (1:9, V:V)

3.4. Optimization of other parameters

The optimization experiments were performed at room temperature (20 °C), and yields were calculated from total TAG conversion rates obtained by HPLC analysis. The effect of temperature was not investigated due to the low boiling point of ethyl alcohol. As shown in Figure 6, the hydrolysis/transesterification yield increased with raised time from 35% (2 hours) to 87% (72 hours) due to more enzyme-substrate interaction. Similarly, the yields were increased by the agitation rate. A liquid film was formed around the beads in the solution, and film diffusion rates of the substrate were low at the lower agitation speed. The diffusion rate of the substrate was raised by eliminating film resistance at higher agitation speeds [14]. The obtained yield increased from 30% to 87% with raised agitation speeds from 30 to 150 rpm.

In the present study, lipase was used as an immobilization agent for glyoxal agarose beads. Easy and direct lipase immobilization occurred in an alkaline medium via multi-covalent attachment. Therefore, a stable structure is formed against the various external effects. The synthesized lipase immobilized agarose beads can be used for castor oil hydrolysis and transesterification. The reaction products, ricinoleic acid, and its derivatives could be used as a surfactant, plasticizer, emulsifier, soap ingredient, etc [15]. The obtained results were compared to the literature study in Table 3. It is seen that the obtained results are compatible with the literature. No study has been found on the hydrolysis of castor oil using lipase-immobilized glyoxal agarose beads in phosphate buffer(pH 7)-ethanol medium.

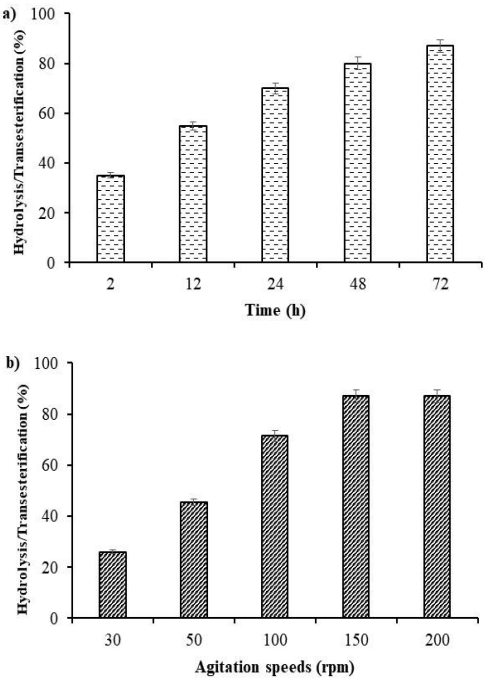

Figure 6. The effect of reaction time (1.0 g castor oil, 2.5 mL of immobilized lipase, 150 rpm), agitation speed (1.0 g castor oil, 2.5 mL of immobilized lipase, 72 h) on the reaction yields

Table 3. Comparison of the obtained results with the literature studies

Method	Reaction	Conditions	Yield
Free lipase [5]	Hydrolysis	Phosphate buffer (pH 7.0), 40 °C, 72 h, 1 g lipase	71.3%
Free lipase [6]	Hydrolysis	Isooctane, pH 7, 55°C, 24 h 175U lipase	~90%
Free lipase [7]	Hydrolysis	Isopropyl ether:water(1:1), 30 °C, 24 h, pH 6, 4% lipase	~100%
Immobilized [8]	Transesterification	1-hexanol, 50 °C, 48 h, RMIM+Novozym 435 (2.5%+5% w/w oil)	95%
Immobilized [9]	Transesterification	Methanol, 55 °C, 96 h, 84 U lipase	78.13%
Immobilized [this study]	Hydrolysis / transesterification	Ethanol:phosphate buffer(9:1) pH 7, 72 h, 2.5 mL immobilized lipase suspension	87%

4. Conclusion

An enzymatic procedure was evaluated for hydrolysis/transesterification of castor oil by lipase-immobilized glyoxal agarose beads. Immobilization efficiency improved at a 12-hour reaction using 5.0 mg enzyme and 2.5 mL agarose beads. A nearly 100% hydrolysis yield was achieved against the p-NPB using lipase-immobilized agarose beads. The highest conversion yield was obtained using 2.5 mL of lipase immobilized agarose beads and 1.0 g of castor oil at a 150 rpm agitation rate, 72 hours in 25 mL 1: 9 (V: V) ratio of phosphate buffer(pH 7): ethanol medium. The obtained main products were ricinoleic acid and ethyl ricinoleate. The other reaction products probably were linoleic, oleic, palmitic, stearic, and linolenic acids and their esters. The reaction products were analyzed using RP-HPLC and FTIR.

Article Information Form

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Authors' Contribution
S. Yılmazer Keskin: Supervision, Project administration, Conceptualization, Writing-Original Draft.
K. Karakaya: Performed the experiments and collected data.

The Declaration of Conflict of Interest/ Common Interest
No conflict of interest or common interest has been declared by the authors.

The Declaration of Ethics Committee Approval
This study does not require ethics committee permission or any special permission.

The Declaration of Research and Publication Ethics
The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

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