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Lipase immobilization and production of fatty acid methyl esters from canola oil using immobilized lipase

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ABSTRACT

Lipase enzyme from *Aspergillus oryzae* (EC 3.1.1.3) was immobilized onto a micro porous polymeric matrix which contains aldehyde functional groups and methyl esters of long chain fatty acids (biodiesel) were synthesized by transesterification of crude canola oil using immobilized lipase. Micro porous polymeric matrix was synthesized from styrene–divinylbenzene (STY–DVB) copolymers by using high internal phase emulsion technique and two different lipases, Lipozyme TL-100L[®] and Novozym 388[®], were used for immobilization by both physical adsorption and covalent attachment. Biodiesel production was carried out with semi-continuous operation. Methanol was added into the reactor by three successive additions of 1:4 M equivalent of methanol to avoid enzyme inhibition. The transesterification reaction conditions were as follows: oil/alcohol molar ratio 1:4; temperature 40 °C and total reaction time 6 h. Lipozyme TL-100L[®] lipase provided the highest yield of fatty acid methyl esters as 92%. Operational stability was determined with immobilized lipase and it indicated that a small enzyme deactivation occurred after used repeatedly for 10 consecutive batches with each of 24 h. Since the process is yet effective and enzyme does not leak out from the polymer, the method can be proposed for industrial applications.

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1. Introduction

Biodiesel is defined as monoalkyl fatty acid ester which is produced by transesterification of oils or fats. To use vegetable oils and animal fats in diesel engines, without any modification, necessarily their fuel properties must be similar to petroleum based diesel fuel. Transesterification of oils using lipases is preferred due to their high selectivity and lower energy requirements. Enzymes have only recently become available for large-scale use in industry because of high cost of enzymes [1]. The advantages of the use of immobilized enzymes are many, and some of them have a special relevance in the area of food technology [2]. Different methods have been developed for enzyme immobilization [3]. These include deposition onto hydrophilic inorganic material, such as celite or silica gel,

encapsulation, covalent linkage to carriers, for example using epoxy functionalized polymer beads, adsorption onto polymer-based carriers and cross-linking using such as glutaraldehyde. More recently, methodologies such as adsorption and cross-linking of lipase enzyme have been exploited [4].

When considering the choice of immobilization methodologies for large-scale immobilized lipase applications, several issues have to be addressed: (a) the immobilization should preferably increase the stability of the lipase in terms of higher temperature stability and also productivity with minimum of enzyme leakage from the carrier; (b) the enzyme has to be stable during each step in the immobilization process; (c) cost-effective immobilization process; (d) the immobilization procedure should preferably be robust and reproducible; (e) production logistics-optimize production

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through put; (f) from a regulatory standpoint, the materials, if the immobilized lipase is to be used in food applications; (g) from an application standpoint, the immobilization lipase should be physically robust and preferably applicable in both batch and fixed bed processes [5].

Reversible immobilization is important protocol because it provides a very easy route for enzyme immobilization and permits the reuse of the adsorbent with the subsequent reduction of industrial waste [6,7].

Recently, enzymatic transesterification using immobilized lipase has become more attractive for biodiesel fuel production, since the glycerol produced as a byproduct can easily be recovered and the purification of fatty methyl esters is simple to accomplish [8,9].

In the present work, two different crude lipases (triacylglycerol hydrolase, EC 3.1.1.3) were immobilized onto micro porous polymeric matrix by both physical adsorption and covalent linking. Lipozyme TL-100L and Novozym 388 are well-known as the lipases with 1,3-specific and also they have high enzyme activity in several reactions [10–14] therefore experiments were conducted with these lipases. Immobilization yields were determined by Bradford method and the activity of immobilized lipase was determined with a spectrophotometric method. Immobilized enzymes were used for biodiesel production by transesterification of canola oil and methanol and the reactions were carried out with semi-continuous operation system. The essential aim of this study was to investigate the production of biodiesel by enzymatic transesterification at industrial scale. Compositions of transesterification reaction products were investigated after immobilization of two enzymes onto the STY–DVB by physical adsorption and covalent attachment. The repeated use of immobilized lipases was also investigated.

2. Materials and methods

2.1. Materials

Lipases from *Aspergillus oryzae* commercially named Novozym 388 (activity: 20,000 LU g⁻¹, LU: Lipase Unit) and Lipozyme TL-100L (activity: 100,000 LU g⁻¹, LU: Lipase Unit), (1,3-specific lipases) were used as biocatalyst. Both lipases were a gift of Novozymes Enzyme Company, Istanbul, Turkey. Methanol which was used as short-chain alcohol, pentane-1,5-dial (25% acidic aqueous solution), NaOH, styrene, divinylbenzene, potassium peroxodisulphate, calcium acetate and heptane were purchased from Merck (Merck, Darmstadt, Germany). Pyridine, monoglyceride, diglyceride and triglyceride were obtained from Sigma. Span 80, tetradecanoic acid (C:14:0), hexadecanoic acid (C:16:0), hexadec-9-enoic acid (C:16:1), octadecanoic acid (C:18:0), cis-9-Octadecenoic acid (C:18:1), cis-9,12-Octadecadienoic acid (C:18:2), cis-9,12,15-octadecatrienoic acid (C:18:3), eicosanoic acid (C:20:0), eicosenoic acid (C:20:1), docosanoic acid (C:22:0) and docosenoic acid (C:22:1) were purchased from Fluka. Refined canola oil cultivated from *Brassica napus* was obtained from AYT Company (Bursa, Turkey). The acid value as KOH was 3.5 g kg⁻¹ of oil and the water content was 5 g kg⁻¹ determined by AOAC Official

Methods [15,16]. All other chemicals were of reagent grade and used without further purification.

2.2. Immobilization of lipase onto STY–DVB and STY–DVB–PGA copolymer

Preparation of soluble polyglutaraldehyde (PGA) solution and synthesis of styrene–divinylbenzene (STY–DVB) copolymer have been described in our previous work [17]. The lipases Novozym 388 and Lipozyme TL-100L were immobilized by adsorption and covalent attachment onto STY–DVB and STY–DVB–PGA copolymers, respectively. For physical adsorption and covalent immobilization onto matrix, 10 cm³ free lipase was added into 40 cm³ of calcium acetate buffer (pH 6.0, 25 mmol dm⁻³) and prepared enzyme solutions were circulated throughout the reactor, which has 2.8 cm outer diameter, 2.3 cm inner diameter and 10 cm length, for 24 h at room temperature (25 °C) (Fig. 1). The Bradford method was used to determine the protein content in solutions. After immobilization, immobilized matrix was washed with 200 cm³ of pH 6.0 Ca(Ac)₂ buffer solution (25 mmol dm⁻³). The enzyme concentration was determined by comparing with the standard curve constructed using enzyme solutions with known concentrations. The amount of immobilized enzyme onto matrix was determined from initial protein amount present in the enzyme solution subtracting the final total protein amounts present in the remaining solution.

2.3. Removing of water from support material

Removing of water from biocatalysts was achieved using the modified method according to Ivanov and Schneider [18]. For this purpose, lipases immobilized onto STY–DVB–PGA were washed with calcium acetate buffer (pH 6.0, 25 mmol dm⁻³), then washed twice by mixtures of calcium acetate buffer and tert-butanol (40:60% v/v), followed by two washings with mixtures of calcium acetate buffer and tert-butanol (5:95% v/v).

2.4. Determination of enzyme activity for immobilized lipase

Enzyme activity assay was performed by a spectrophotometric method according to literature [19]. The transesterification reaction was carried out using p-nitrophenyl palmitate (p-NPP)

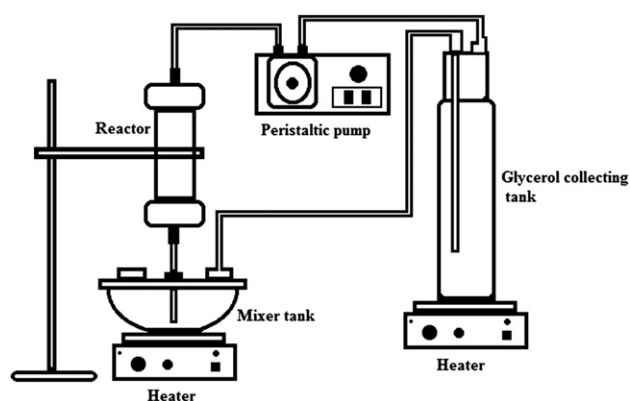


Fig. 1 – Experimental set-up.

(10 mmol dm⁻³). The stock solution of p-NPP was prepared in 2-propanol. One volume of a 10 mmol dm⁻³ solution of p-NPP in 2-propanol was mixed just before used with 9 volumes of 25 mmol dm⁻³ Ca(AC)₂ buffer pH 6.0 containing 0.8% (kg m⁻³) Triton X-100 and 0.2% (kg m⁻³) Arabic gum. The absorbance of p-nitrophenol released was monitored at 410 nm by spectrophotometry (Shimadzu UV/Visible spectrophotometer, Japan). A calibration curve was constructed to calculate the enzyme activity using the absorbance of standard solutions of pNP in the reaction mixture. Each of the assays was performed in duplicate and mean values were presented. One unit of lipase activity was defined as 1 mol min⁻¹ m⁻³ or 1 mmol min⁻¹ kg⁻¹ of p-NPP released by enzyme in solution or immobilized solid respectively. Also immobilization yield, lipase activity and specific activity were calculated as follows,

$$\text{Immobilization yield(\%)} = \frac{\text{Amount of protein loaded}}{\text{Amount of protein introduced}} \times 100 \quad (1)$$

$$\text{Lipase activity (U g}^{-1}\text{support)} = \frac{\text{Activity of immobilized lipase}}{\text{Amount of immobilized lipase}} \quad (2)$$

$$\begin{aligned} \text{Specific activity (U mg}^{-1}\text{protein)} \\ = \frac{\text{Activity of immobilized lipase}}{\text{Amount of protein loaded}} \end{aligned} \quad (3)$$

2.5. Enzymatic transesterification

Immobilized enzymes were used for biodiesel production by transesterification of canola oil and methanol. Biodiesel production was carried out using a semi-continuous operation system at 40 °C (Fig. 1). 100 g of canola oil and three-step addition of 27 cm³ methanol with 9 cm³ in each step (a 1:4 M ratio of oil/methanol) were circulated into the reactor by a peristaltic pump at 5 cm³ min⁻¹ flow rate for 3 h at each successive step, then the reaction was continued for 24 h.

2.6. Determination of fatty acid methyl esters and glycerides

The fatty acid methyl esters (FAMES) and glycerides contents in the reaction mixtures were determined by DIN EN 14103 and DIN EN 14105 methods, respectively [20,21]. An Agilent 6890N model gas chromatograph equipped with a flame-ionization detector (FID) and a capillary column (DB-WAX; 30 m × 0.53 mm ID × 0.5 µm film thickness) was used for FAMES determinations. Helium was the carrier gas whose pressure was 20.1 kPa, and the temperatures of injector, oven and detector were 250 °C, 190 °C and 250 °C, respectively. Split flow rate was 50 cm³ min⁻¹, and injection mode was split/splitless. The injection volume was 1 µL. The column temperature was kept at 100 °C for 2 min, raised to 210 °C at 20 °C min⁻¹, and then maintained at this temperature for 15 min. The injector and detector temperatures were set at 245 °C and 250 °C, respectively. The details of the analysis are described in DIN EN 14103 method.

The glycerides were determined by the same equipment and a capillary column (15 m × 0.32 mm ID × 0.1 µm film

thickness) which covered with 95% dimethyl–5% diphenyl polysilicone as immobile phase was used. Helium was the carrier gas whose pressure was 80 kPa, and the temperatures of injector, oven and detector were 250 °C, 350 °C and 380 °C, respectively. The injection volume was 1 µL. The column temperature was kept at 50 °C for 1 min, raised to 180 °C at 15 °C min⁻¹, 230 °C at 7 °C min⁻¹, 370 °C at 10 °C min⁻¹, and then maintained at this temperature for 5 min. The injector and detector temperatures were set at 250 °C and 380 °C, respectively. The details of the analysis are described in DIN EN 14105 method. All GC measurements were performed in triplicate.

3. Results and discussion

3.1. Lipase immobilization efficiency

The efficiency of polymer support to bind and express the lipase activity can be calculated in terms of percentage immobilization yield. The enzyme activity of immobilized lipase is expressed as enzyme units g⁻¹ of polymer support. The immobilization of enzymes on different support materials has several advantages for industrial applications [22]. Immobilized enzyme is more effective than free enzyme. Support surface and immobilization method are also important on catalytic efficiency of enzyme [23]. In this study, two different lipases, Lipozyme TL-100L and Novozym 388, were used for immobilization by both physical adsorption on the styrene–divinylbenzene (STY–DVB) copolymer and covalent attachment on the styrene–divinylbenzene–glutaraldehyde (STY–DVB–GA) and styrene–divinylbenzene–polyglutaraldehyde (STY–DVB–PGA) copolymers. Lipase immobilization yield on each support, lipase activity, protein loading per gram support and specific activity of immobilized lipases were investigated. The results indicated that the highest immobilization yield was observed as 34.66% for Lipozyme TL-100L and 36.99% for Novozym 388 on the micro porous STY–DVB–PGA copolymer by covalently (Table 1). However, Lipozyme TL-100L and Novozym 388 lipase were immobilized by physical adsorption as 23.70% and 11.65% immobilization yield on the STY–DVB copolymer respectively. It can also be seen from Table 1 that when glutaraldehyde or polyglutaraldehyde enter into the support material (covalent immobilization), the amount of enzyme immobilized on the support material increased but activity of lipases decreased for both enzymes. This can be attributed to protein–protein interaction. Thus, active center of lipases is inactivated. Lipase activity and also specific activity of Lipozyme TL-100L and Novozym 388 which were immobilized on the styrene–divinylbenzene (STY–DVB) copolymer were calculated higher than the others. On the other hand, the highest protein loading capacity observed (5.81 mg g⁻¹support and 4.19 mg g⁻¹support respectively) with styrene–divinylbenzene–polyglutaraldehyde (STY–DVB–PGA) copolymer (Table 1). The results indicate that covalent immobilization can load more enzymes on support than absorption but lost some activity due to the inactivity of active sites of enzymes. Similar behaviors have been reported in our previous work for immobilization of Lipozyme TL-100L on STY–DVB–PGA [11].

Table 1 – Adsorption capacity, immobilization yields and activity of the Lipozyme TL-100L and Novozym 388 immobilized on different support materials.

Matrix – (Enzyme)	Immobilized enzyme (mg g ⁻¹ support)	Immobilization yield (%)	Lipase activity (U g ⁻¹ support)	Specific activity (U mg ⁻¹ protein)
1. STY–DVB – (Lipase TL-100L)	4.79	23.70	22.20	4.63
2. STY–DVB – (Lipase N388)	0.99	11.65	20.29	20.49
3. STY–DVB–GA – (Lipase TL-100L)	5.04	26.48	19.37	3.84
4. STY–DVB–GA – (Lipase N388)	1.77	26.21	18.28	10.33
5. STY–DVB–PGA – (Lipase TL-100L)	5.81	34.66	17.54	3.02
6. STY–DVB–PGA – (Lipase N388)	4.19	36.99	17.69	4.22

The activity of the immobilized enzymes depends on several parameters such as lipase type, surface of support particle and immobilization protocol. Between the lipase enzymes, the Novozym 388 showed the best performance, producing immobilized lipase samples with the activity of 20.29 U g⁻¹support and specific activity of 20.49 U mg⁻¹protein, which corresponds to an immobilization yield of 11.65%. The use of glutaraldehyde and polyglutaraldehyde as activating agent decreased the lipase activity by about 20% (4 U g⁻¹support) indicating that different chemical modifications were produced by each activating agent. Concerning the activation with glutaraldehyde and polyglutaraldehyde, the most probable mechanism occurred between the activating agent and STY–DVB which is initially physical adsorption and later a formation of imine bond with an aldehyde group of the glutaraldehyde and an NH₂ group of the enzyme as proposed in Fig. 1. This hypothesis was confirmed by analyzing the FTIR spectrum and scanning electron microscopy. High specific activity was observed with Novozym 388 by adsorption and covalent immobilization corresponding to the high enzyme loading and immobilization yield. Based on these results, we realize that the specific activity must increase when the immobilized enzyme increased in the case of enzymes immobilized by covalently on the micro porous STY–DVB–GA and STY–DVB–PGA copolymers. There may be a much stronger interaction between the Novozym 388 lipase and the STY–DVB–PGA, which leads to greater activity loss at low loading due to lipase spreading.

Moreover, the high lipase activity yield of STY–DVB–GA and STY–DVB–PGA polymers can be explained by their micro porous nature. The SEM results showed that the polymer particles contain large numbers of micropores. Micro porous polymer structures primarily support the diffusion of enzyme molecules inside the pores and further facilitate the movement of enzymes toward the reactive aldehyde groups of polymer. The reduction in diffusional limitations also assists the rapid biocatalytic reactions by inhibiting substrate or product accumulation inside the pores. The increase in immobilization yield of STY–DVB–PGA particles can be correlated with the corresponding increase in small and more regular pores, which can facilitate the fixation of the enzyme on the support. Similar behaviors have been reported in literature for lipase immobilization using the same source of support and *Candida rugosa* lipase [14]. According to these authors, the enzyme from *Candida antarctica* has a structure that is much more difficult to distort, making it less likely to spread on the support surface. It is possible that this is also the case for Novozym 388.

3.2. Enzymatic transesterification

Transesterification reaction of canola oil was carried out with methanol and lipase enzymes (Lipozyme TL-100L and Novozym 388) immobilized onto STY–DVB and STY–DVB–PGA copolymers. Composition of transesterification reaction products using Lipozyme TL-100L and Novozym 388 enzymes immobilized by adsorption onto micro porous STY–DVB copolymer and immobilized by covalently onto STY–DVB–PGA copolymer are given in Tables 2 and 3. The highest FAME yields were 92.41% with physical adsorption onto STY–DVB and 84.69% with covalent attachment onto STY–DVB–PGA using Lipozyme TL-100L. The amount of immobilized Lipozyme TL-100L was also higher than Novozym 388 on the same supports (Table 1). The FAME yield was enhanced by increasing immobilized lipase. Novozym 388 is known as a lipase with 1,3-positional specificity, so theoretically the highest FAME yield should be only 67%. Therefore acyl transfer has been thought to occur during the transesterification reaction, which resulted in the FAME yield of 83.15% and 80.85% could be obtained by adsorption and covalent attachment of the supports respectively. On the other hand, FAME yield of canola oil using Novozym 388 was lower than that of the Lipozyme TL-100L which results from the significant inhibitory effect of glycerol on the reaction, although there is no negative effect of free fatty acids (FFA). The FFAs were calculated as 0.79% and 1.77% based on the oil in adsorption and covalent attachment. In another work, a method for immobilization of lipase within hydrophilic polyurethane foams using polyglutaraldehyde was developed for the immobilization of Lipozyme TL-100L lipase to produce biodiesel with canola oil and methanol [11]. The new proposed method with STY–DVB support increased the FAME yield of the reaction. The immobilized enzymes were rinsed with tert-

Table 2 – Composition of transesterification reaction products using Lipozyme TL-100L and Novozym 388 enzymes immobilized by adsorption onto micro porous STY–DVB copolymer.

Composition (wt%)	Lipozyme TL	Novozym 388
Free fatty acids	2.98	0.79
Monoglycerides	3.50	1.50
Diglycerides	1.08	4.65
Triglycerides	0.03	9.91
Fatty acid methyl esters	92.41	83.15

Table 3 – Composition of transesterification reaction products using Lipozyme TL-100L and Novozym 388 enzymes immobilized by covalently onto micro porous STY–DVB–PGA copolymer.

Composition (wt%)	Lipozyme TL	Novozym 388
Free fatty acids	6.61	1.77
Monoglycerides	3.81	3.70
Diglycerides	3.24	4.88
Triglycerides	1.65	8.80
Fatty acid methyl esters	84.69	80.85

butanol to remove the remaining glycerol on the support material. The other advantage of the proposed method is to perform the transesterification reaction in a continuous system with a reactor configuration as depicted in Fig. 1. The system allows the reaction in the separate reactor and collects the glycerol as byproduct in different medium, so the percentage of the FAME is increased on the immobilized support with less amount of glycerol and other reaction products.

3.3. Repeated use of immobilized lipases

The main advantage of immobilization of an enzyme is that an expensive enzyme can be repeatedly used. Experiments were performed to examine the reusability and the stability of the immobilized enzymes. The changes of enzymatic activities for Lipozyme TL-100L and Novozym 388 are shown in Fig. 2. Reactions were conducted with 10 batches. One batch reaction time was 24 h. The immobilized lipases were rinsed with *tert*-butanol between each batch. The residual activity determined after 24 h was expressed as relative conversion. The conversion achieved in the first batch was set to 100, and the other transesterification reaction was calculated as relative to the first batch. Novozym 388 and Lipozyme TL-100L

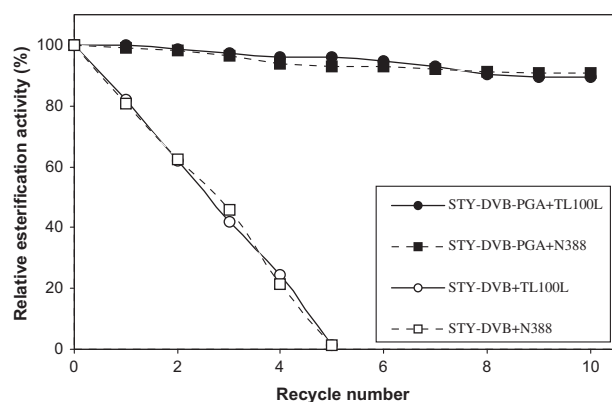


Fig. 2 – Relative esterification activity of Lipozyme TL-100L and Novozym 388 immobilized by covalently on micro porous STY–DVB–PGA copolymer and physical adsorption on micro porous STY–DVB copolymer by repeated use. The reaction conditions; 20% enzyme based on oil weight (100 g); oil/alcohol molar ratio 1:4; reaction temperature: 40 °C and reaction time 24 h. (Methanol was added into the reactor by three successive additions of 1:4 M equivalent of methanol to avoid enzyme inhibition).

lipases were compared which were immobilized by covalently onto STY–DVB–PGA copolymer proved to be stable after even 10 reuse and lost little activity when was subjected to repeated use. Similar results were obtained with Lipozyme TL-100L when different support material was used for immobilization in the literature [23]. However, the enzymes immobilized by physical adsorption onto STY–DVB were enabled only 5 reuse. Decrease in the enzyme activity of the lipases immobilized onto STY–DVB is because of leaching of enzyme from support particle or due to inactivation of enzyme.

4. Conclusion

Lipase immobilization onto micro porous STY–DVB copolymer was studied. Two lipases were used for immobilization onto support. Micro porous STY–DVB–PGA copolymer which contains aldehyde functional groups was synthesized by polymerizing the continuous phase of a high internal phase emulsion of styrene–divinylbenzene–glutaraldehyde monomers. Lipozyme TL-100L and Novozym 388 lipases were immobilized on micro porous copolymer by physical adsorption and covalent linking. The maximum immobilization efficiency was obtained as 36.99% for Novozym 388 and 34.66% for Lipozyme TL-100L when immobilized onto STY–DVB–PGA copolymer. The highest lipase activity obtained was 22.20 U g^{−1} support for Lipozyme TL-100L and 20.29 U g^{−1} support for Novozym 388 when immobilized onto STY–DVB copolymer. Biodiesel production was carried out by semi-continuous operation. Methanol was added into the reactor by three successive additions of 1/3 M equivalent of methanol. Canola oil and methanol mixture circulated into reactor by a peristaltic pump for 24 h. The optimum conditions of the transesterification reaction were found as follows; oil/alcohol molar ratio 1:4; temperature 40 °C and reaction time 6 h. According to chromatographic analysis, Lipase Lipozyme TL-100L resulted in the highest yield of methyl ester as 92%. Novozym 388 and Lipozyme TL-100L lipases which were immobilized by covalently on micro porous copolymer proved to be stable after even 10 reused and lost little activity when was subjected to repeated use. However, the immobilized lipases by physical adsorption (without polyglutaraldehyde) onto STY–DVB copolymer lost its whole activity after 5 repeated reuses.

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