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RESEARCH ARTICLE

COVALENT İMMOBİLİZATİON OF *PSEUDOMONAS FLUORESCENS* LİPASE ONTO EUPERGIT CM

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ABSTRACT

Immobilization of enzymes is an important field of study as the reusable heterogeneous biocatalysts designed tremendously reduce production costs by efficient recycling and control of the catalytic processes. In the present study, the immobilization of *Pseudomonas fluorescens* lipase was performed via covalent attachment of lipase onto oxirane activated supports (Eupergit CM). Their wide specificity has rendered lipases into the most commonly-used enzymes in organic chemistry, resulting in an urgent need to effectively immobilize these enzymes. 100,0±0,2% immobilization yield and 170±1,1% activity yield were achieved by optimizing the immobilization conditions (ratio of matrix/enzyme, pH of buffer medium, molarity of buffer medium, duration of immobilization). The best results were achieved when the lipase was immobilized at pH 9 at room temperature (25 ^{o}C) for 120 hours. The operational and storage stabilities of the immobilized enzymes were determined as well. No decrease in the activity of immobilized enzyme during 20 consecutive batch reactions was observed. Furthermore, the immobilized enzymes showed high storage stability as they retained their activity for 20 days of usage. The obtained immobilized *Pseudomonas fluorescens* lipase can be used for the biodiesel production, oil hydrolysis and various important esterification reactions.

INTRODUCTION

Lipases (EC 3.1.1.3) are enzymes that are widely used for hydrolysis, alcoholysis, esterifications and transesterifications of oils (Tan *et al*., 2010). The utilization of lipases in industry is steadily growing with their major contribution being the production of oleochemicals, detergents, organics, cosmetics, perfumes and biosensors (Aravindan *et al*., 2007). The first immobilization studies of enzymes were published in 1953, and since then this field has gained increasing importance. A wide variety of immobilized enzymes are currently used in the food, pharmaceutical and chemical industries. The use of immobilized enzymes makes the large-scale continuous production of fine chemicals possible and reduces the costs of these processes (Malcata and Hill, 1991). Furthermore, immobilization of enzymes decreases the contamination of the product via residual enzymes. Immobilization also permits repeated use of enzymes and often improves their operational and storage stability. It also facilitates control of both the process and the product quality. Immobilization of *Pseudomonas fluorescens* lipase has already been studied extensively (Mustranta *et al*., 1993; Iso *et al*., 2001; Gorokhova *et al*., 2002; Kaewthong *et al*., 2005; Santos *et al*., 2008; Salis *et al*., 2009; Zarcula *et al*., 2009; Zarcula *et al*., 2010; Salis *et al*., 2010; Tomin *et al*., 2011), with adsorption beeing the most widely employed method for this purpose. However, there are important disadvantages of the adsorption method, such as low binding capacity and fast loss of immobilized activity.

Covalent immobilization is one of the ways to overcome this problem. The main advantages of covalently immobilized enzymes are their high operational and storage stability, since enzyme molecules attached to the support via strong interactions cannot leach from the support during industrial processes. Furthermore, the enzyme is no longer leached out from the immobilization support, resulting in purer products. The covalent immobilization of enzymes with oxirane-activated supports is a well-known and easy process (Katchalski-Katzir and Kraemer, 2000). Eupergit CM beads contain oxirane groups, and this resin has previously been reported to assist successful enzyme immobilization (Olofsson *et al*., 2006; Mislovicova *et al*., 2007; Olofsson *et al*., 2008; Sukyai *et al*., 2008; Forde *et al*., 2010; Chiyanzu *et al*., 2010). Enzyme molecules can covalently bind to the oxirane groups with their amino, sulfhydril and carboxyl groups in a wide pH range. Here we report the immobilization of *Pseudomonas fluorescens* lipase onto Eupergit CM to achieve high

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immobilization efficiency and a high operational and storage stability.

MATERIALS AND METHODS

Pseudomonas fluorescens lipase and p-nitro phenyl acetate (pNPA) were purchased from Sigma-Aldrich Chemical Co. Eupergit CM was donated by Degussa Rohm GmbH&Co. KG, Pharma Polymers (Darmstadt, Germany). 4-nitro phenol (pNP) was purschased from Fluka. Methanol was obtained from Lab-Scan and 1,4-dioxane was acquired from Acros Organics. A BCA protein assay kit was bought from Thermo Fisher Scientific Inc (Rockford, USA).

Determination of protein amounts

Before and after immobilization, the amounts of protein in the immobilization buffer and washing solutions were determined according to the BCA Assay Method (Smith *et al*., 1985) by UV/Vis (PYE UNICAM SP8-200 UV/Vis spectrophotometer) at the λ_{max} (562 nm) of a BCA/copper complex.

Determination of activity

pNPA (40 mM) and methanol (80 mM) in 1,4-dioxane (5mL) were reacted with agitation at 150 rpm with 3.87 mg free or immobilized lipase at 25 °C for 30 min in an incubator. The reaction mixture (10µL) was added to 1,4-dioxane (2 mL) and was immediately used for the determination of pNP by UV/Vis (PYE UNICAM SP8-200 UV/Vis spectrophotometer) at λ_{max} (304 nm) of *p*NP. Hydrolytic activities for powdered *Pseudomonas fluorescens* and *Pseudomonas fluorescens* immobilized on Amberzyme are defined herein as the milimoles of *p*NP produced in 1,4-dioxane per unit of weight of enzyme per time (mmol of *p*NP/min.mg).

Calculation of immobilization and activity yields

Immobilization yield was calculated using the formula:

$$\text{Immobilization Yield} = \frac{\text{mg of bounded lipase}}{\text{mg of free lipase used for immobilization}} \times 100$$

Activity yield was calculated using the formula:

$$\text{Activity Yield} = \frac{\text{Activity of immobilized lipase}}{\text{Activity of free lipase used for immobilization}} \times 100$$

Immobilization procedure

Immobilization was performed by reacting Eupergit CM (400 mg) with *Pseudomonas fluorescens* lipase (200 µL) in phosphate buffer (5 mL, 1.0 M, pH 9.0) at 25 °C with gentle shaking (150 rpm) for 24 h. Beads of immobilized enzymes were obtained after filtering and washing with phosphate buffer (5 mL, 0.1 M, pH 9.0) and plenty of aceton on a sintered glass filter by suction under vacuum. The immobilized enzyme was stored in dried form in the cooling room at 4 °C until usage.

Optimization of immobilization procedure

The optimum conditions of immobilization were determined by individually changing the conditions (amount of Eupergit C from 100 to 500 mg; pH from 5.0 to 9.0; buffer concentration from 0.25 to 1.0 M; and duration of immobilization from 24 to 120 h).

Operational and storage stability

Operational stability tests were carried out by repeating 20 batch experiments using the method described above for activity determination. Storage stability was tested for 20 days by determining the activity every other day with the activity assay method above and then keeping the immobilized enzymes in a stoppered bottle in dried form in the cooling room at 4 °C for 24 hours.

RESULTS

Determination of protein amounts

Protein amounts in the buffered lipase solutions (0.1 M, pH 7) before immobilization were found to be 3.7 mg in the 40 mg powdered lipase. According these results, 100 mg powdered lipase contains 9.68 mg protein.

Determination of activity

After pNPA (40 mM) and methanol (80 mM) in 1,4-dioxane (5mL) was reacted with agitation at 150 rpm with 3.87 mg free and immobilized lipase at 25 °C for 30 min in an incubator, pNP amounts with free and immobilized lipase were found to be 23.5 and 40 µmol respectively. Therefore, the activities of free and immobilized lipase can be calculated as 2.02×10^{-4} and 3.45×10^{-4} mmol pNP/min.mg respectively.

Calculation of immobilization and activity yields

According to the formula described in the methods, after optimization of immobilization conditions, the obtained immobilized activity yield was 170±1,1% of free lipase (3.45×10^{-4} mmol pNP/min.mg/2.02×10^{-4} mmol pNP/min.mg).

Optimization of immobilization procedure

Effect of amount of Eupergit CM

As it is known that the amount of imobilization support is one of the critical optimalization parameters, the amount of Eupergit CM (100–500 mg) used for the immobilization of *Pseudomonas fluorescens* lipase solution (200 µL) was varied.

Table 1. Effect of Eupergit CM Amount on Immobilization and Activity Yields

Eupergit CM (mg)	Immobilization Yield (%)	Activity Yield (%)
100	28,5±0,3	76±1,2
200	31,2±0,5	119±1,4
300	34,9±0,6	130±1,3
400	40,4±0,4	140±1,5
500	42,4±0,2	132±1,3

***** 3,87 mg dissoved enzyme in 200 µL 0,1 M phosphate buffer (pH 7,0) were reacted with different amounts of supports in 5mL of phosphate buffer (1,0 M, pH 7,0) at 150 rpm and room temperature in an incubator for 24 hours.

****** Free (3,87mg) and dry immobilized enzymes were reacted with 5 mL substrate solutions (40 mM p-nitro phenyl acetate and 80 mM methanol in 1,4-dioxane) at 150 rpm and room temperature in an incubator for 30 minutes.

In Table 1 it becomes obvious that the amount of immobilized enzyme is increased by increasing the amount of immobilization support, reaching a maximum of immobilization yield (42.4±0,2%) at 500 mg of Eupergit CM. The highest activity yield (140±1,5%) was obtained for 400 mg of Eupergit CM with a decrease in activity with higher amounts of immobilization support. Further optimalization was performed with the ratio 400 mg of Eupergit CM:3.87 mg lipase as the highest activity yield was obtained with this ratio.

Effect of pH of immobilization buffer

Table 2 shows that the immobilization yield (62,4±0,4%) and activity yield (155±1,5%) reached a maximum at pH 9.0.

Table 2. Effect of pH on Immobilization and Activity Yields

pH	Immobilization Yield (%)	Activity Yield (%)
5	57,8±0,6	133±1,1
6	52,7±0,4	137±1,3
7	40,4±0,4	140±1,5
8	48,5±0,3	142±1,4
9	62,4±0,4	155±1,5

***** 3,87 mg dissoved enzyme in 200 µL 0,1 M phosphate buffer (pH 7,0) were reacted with 400 mg support at different pHs of 5 mL phosphate buffer (1,0 M) at 150 rpm and room temperature in an incubator for 24 hours.
****** Free (3,87mg) and dry immobilized enzymes were reacted with 5 mL substrate solutions (40 mM p-nitro phenyl acetate and 80 mM methanol in 1,4-dioxane) at 150 rpm and room temperature in an incubator for 30 minutes.

Effect of molarity of immobilization buffer

Buffer concentrations and salts such as ammonium sulphate also influence the immobilization efficiency considerably in immobilization with oxirane groups (Martin *et al*., 2003). In our experiments, the highest immobilization and activity yields are 62,4±0,4% and 155±1,5% respectively, in phosphate buffers of 0.75 M (Table 3).

Table 3. Effect of Immobilization Buffer Concentration on Immobilization and Activity Yields

Concentration of Immobilization Buffer	Immobilization Yield (%)	Activity Yield (%)
0,25	53,8±0,3	44±1,4
0,5	52,7±0,5	68±1,2
0,75	62,4±0,4	155±1,5
1,0	40,4±0,2	147±1,3

***** 3,87 mg dissoved enzyme in 200 µL 0,1 M phosphate buffer (pH 9,0) were reacted with 400 mg of support at different concentrations of 5mL phosphate buffer (pH 9,0) at 150 rpm and room temperature in an incubator for 24 hours.
****** Free (3,87mg) and dry immobilized enzymes were reacted with 5 mL substrate solutions (40 mM p-nitro phenyl acetate and 80 mM methanol in 1,4-dioxane) at 150 rpm and room temperature in an incubator for 30 minutes.

Effect of immobilization time

The time of immobilization reaction is of importance: while the immobilization yield changed from 62,4±0,4% to 93,6±0,5%, the activity yield changed from 155±1,5% to 165 in 24 and 96 hours respectively (Table 4). After immobilization for 120 hours, all of the soluble enzyme used for immobilization was bound to the Eupergit CM and the highest activity yield (170±1,1%) was obtained.

Table 4. Effect of Immobilization Time on Immobilization and Activity Yields

Duration of Immobilization (hours)	Immobilization Yield (%)	Activity Yield (%)
24	62,4±0,4	155±1,5
48	73,4±0,4	159±1,3
72	89,0±0,6	163±1,4
96	93,6±0,5	165±1,6
120	100,0±0,2	170±1,1

***** 3,87 mg dissoved enzyme in 200 µL 0,1 M phosphate buffer (pH 7,0) were reacted with 400 mg of supports in 5mL of phosphate buffer (0,75 M, pH 9,0) at 150 rpm and room temperature in an incubator for 24 hours.
****** Free (3,87mg) and dry immobilized enzymes were reacted with 5 mL substrate solutions (40 mM p-nitro phenyl acetate and 80 mM methanol in 1,4-dioxane) at 150 rpm and room temperature in an incubator for 30 minutes.

Operational and storage stabilities

The operational and storage stability of *Pseudomonas fluorescens* lipase immobilized on Amberzyme at pH 9, buffer concentration of 0.75 M and for 120 hours was tested. The immobilized enzyme retained its activity during 20 consecutive batch reactions, each lasting for 30 min at 25 ◦C. The immobilized enzyme was also able to retain its initial activity for 20 days when used every second day.

DISCUSSIONS

The decrease in activity with higher amounts of Eupergit CM is possibly due to multiple attachments and reactions with groups associated with the active site and those responsible for the tertiary structure of the enzyme, as discussed in the literature (Katchalski-Katzir and Kraemer, 2000). Although oxirane groups can react with various reactive groups of enzymes in a wide pH range (1–12), immobilization of many enzymes resulted in the highest yield at their optimal pH range (Katchalski-Katzir and Kraemer, 2000). Since maximum activity yield obtained at pH 9,0 it can be expected for the lipase of *Pseudomonas fluorescens* optimum pH probably is 9.0. Immobilized lipase shows high operational and storage stability. It can be concluded that immobilization of the enzyme at a higher pH and temperature (20 °C) and with a longer incubation time led to the formation of new covalent linkages between the enzyme molecules and the support. In this way a more stable biocatalyst was produced (Mateo *et al*., 2007). Similar results were also obtained in our previous study (Aslan *et al*., 2013), however, the activity yield using Eupergit CM as the immobilization support is higher than with the Amberzyme used in the previous study of Miletic *et al*. (2009) showed that the hydrolytic activity of Cal-B strongly depends on the particle size and the pore size of the support. The activity yield increased by decreasing the particle size and increasing the pore size. The particle sizes of Amberzyme and Eupergit CM are 235 μm and 130-280 μm, respectively, and the pore sizes of Amberzyme and Eupergit CM are 220 Å and 500 Å, respectively. Therefore, the higher activity yield of immobilized *Pseudomonas fluorescens* lipase immobilized on Eupergit CM rather than Amberzyme arises from particle and pore size differences.

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Conclusion

In the present study, *Pseudomonas fluorescens* lipase was covalently immobilized onto Eupergit CM with high immobilization yield (100%) and activity yield (170%). Furthermore, immobilized *Pseudomonas fluorescens* lipase with high operational and storage stability was obtained via immobilization at pH 9, at room temperature (25 ^{o}C) for 120 hours. The obtained immobilized *Pseudomonas fluorescens* lipase can be used for the biodiesel production, oil hydrolysis and various important esterification reactions.

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