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Biodiesel production from waste oils by using lipase immobilized on hydrotalcite and zeolites

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Abstract

In this work, hydrotalcite and four different types of zeolites were used as immobilization metarial. The size and type of the zeolite particles did not effect the amount of protein adsorbed. It was found that hydrotalcite is more efficient than zeolites studied. The amount of protein adsorbed (P_g) on hydrotalcite 13 mg/g was higher than that of zeolite as 9 mg/g. The amount of protein adsorbed on hydrotalcite was the highest at pH 8.5 and 4 °C. The immobilization of enzyme on hydrotalcite reached steady state after 5 h. Immobilized lipase retained 36% of the initial activity at 45 °C and 14% of initial activity at 55 °C, after the seventh cycle. Immobilized lipase on hydrotalcite was found to able to catalyse the transesterification of waste cooking oil with methanol to produce methyl esters. Lipase immobilized on zeolites did not show significant yields at the same reaction conditions.

Keywords: Biodiesel; Lipase; Enzyme immobilization; Hydrotalcite preparation; Transesterification

1. Introduction

There is an increasing worldwide concern for environmental protection and for conservation of non-renewable natural resources. Day by day fossil fuel resources deplete due to rapidly increasing population and industrialization all around the world. For this reason, the possibility of developing alternative energy sources to replace traditional fossil fuels has been receiving a large interest in the last few decades. Fatty acid methyl esters (FAME), which are collectively named bio-diesel and produced by methanolysis of triglycerides such as animal fats and plant oils, are shown as promising renewable sources of fuel [1]. Biodiesel can be used directly or mixed with conventional fuel for diesel engines, and as a heating fuel.

SO_2, halogens, soot and CO are major contributors to environmental pollution. The main advantage of using biodiesel as fuel is that the amounts of SO_2, halogens, soot and CO in the exhaust gas are much lower than those in petroleum diesel [2]. Furthermore, the recycling of waste oil will also contribute to alleviation of environmental problems.

Biodiesel is synthesized from direct transesterification of vegetable oils and animal fat, where the corresponding triglycerides react with a short-chain alcohol, usually methanol in the presence of a catalyst. Although conventional chemical technology using alkaline catalysts has been applied to biodiesel fuel production, there are several drawbacks to this approach, including difficulties in the recovery of glycerol and potassium and/or sodium salt, the need for catalyst exclusion, and the energy-intensive nature of the process. Utilization of lipase as a catalyst for biodiesel fuel production has great potential compared with chemical methods. Since no complex operations are needed either to recover the glycerol or to eliminate the catalyst and salt. In literature, the lipase enzyme has been used in biodiesel production in the free form or immobilized on some different materials such as ceramics, kaolinites, silica, etc. [3].

In this work, immobilized lipase enzyme was used to produce diesel fuel from waste cooking oils. Lipase enzyme was immobilized on hydrotalcite prepared by coprecipitation method and four different types of commercial zeolites. Immobilization conditions, such as temperature, pH, time and particle sizes on enzyme activity were investigated. Beside the immobilization conditions, the effects of different types of support used in immobilization on biodiesel production were also investigated.

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2. Material and methods

2.1. Chemicals

Lipase enzymes (Lipozyme-TL IM) were supplied from Novozymes, Denmark. Different type of zeolites (13-x, 5A, FM-8 and AW-300) were kindly supplied by Altek Metal Ticaret A.Ş.,Turkey, Zeochem EU and Aldrich. The restaurant grease (waste cooking oil) was a gift from Saray Catering of Kocaeli, Turkey. All other chemicals used were analytical grade and purchased either from Merck AG (Darmstadt, Germany) or Sigma Chem. Ltd. (St. Louis, USA).

2.2. Hydrotalcite preparation

Hydrotalcite used for immobilization of lipase was as a synthetic $Mg(Al)O_x$ mixed oxide prepared by co-precipitation method [4]. Co-precipitation was performed in a semi-batch system composed of an insulated constant temperature water bath, a mechanical stirrer, a three-neck round-bottom reaction flask, a peristaltic pump and a pH-meter. Mg(II) nitrate and Al(III) nitrate solutions were dropwisely added to an aqueous solution sodium carbonate, which was poured into a three-neck round-bottom reaction flask and pre-heated to 60 °C. During co-precipitation, the flask was stirred with a rate of 500 rpm and the slurry was kept at pH 10 by dropwise addition of NaOH solution. After completion of Mg(II) nitrate and Al(III) nitrate solutions, the suspension was stirred at 60 °C for an hour, followed by ageing for 18 h without stirring at the same temperature. The synthesized colorless solid was filtered out, and rinsed with distilled water and dried at 80 °C for 16 h. Then, dried solid was ground and sieved to 80–250 μm size and was further calcined at 500 °C for 16 h to produce $Mg(Al)O_x$ mixed oxide.

2.3. Lipase immobilization on hydrotalcite and zeolites

In this work, hydrotalcite and four different types of zeolites were used as immobilization materials. A 0.2 g zeolite and hydrotalcite particles at four different sizes were suspended in 2 mL of lipase enzyme (Lipozyme-TL IM) solution (2.5 mg/mL) for 18 h at three different temperatures (4, 25, 37 °C). A 0.1 M phosphate buffer of pH 7.0 was used for immobilization of zeolites [5]. A 0.1 M Tris–HCl buffer of pH 8.5 was used for immobilization of hydrotalcite. The suspension was centrifuged at 10,000 rpm. The volume of supernatant was 95–97% those of the original lipase solution. Protein content and lipase activity of supernatants were determined according to methods given in Sections 2.5 and 2.6. Amounts of protein adsorbed on zeolites and hydrotalcite were compared.

2.4. Optimization of lipase immobilization on hydrotalcite

In order to immobilize lipase enzyme on hydrotalcite, immobilization conditions were optimized. To determine the effect of pH on immobilization on hydrotalcite, 0.1 M phosphate buffer at pH 6.0 and 7.0, and Tris–HCl buffer at pH range between 8.0, 8.5 and 9.0 were used. To determine the effect of temperature, hydrotalcite particles (0.2 g) were suspended in 2 mL of lipase enzyme (Lipozyme-TL IM) (2.5 mg/mL) at different temperatures (4, 25, 37 °C) for 5 h. The suspension was centrifuged at 10,000 rpm. The volume of supernatant was 95–97% those of the original lipase solution. Protein content and Lipase activity of supernatant were determined according to methods given in Sections 2.5 and 2.6. The pH at which the maximum protein adsorbed on hydrotalcite was found as 8.5 and was used for further immobilization.

To investigate the effect of time on immobilization of lipase on hydrotalcite, the mixtures were incubated with stirring. Adsorption on hydrotalcite was followed by removing small aliquots at various time intervals. The suspension was cetrifuged at 10,000 rpm and protein content was determined according to method described in Section 2.5.

The immobilized lipase was washed with cold distilled water to remove the un-entrapped enzyme, the suspension was centrifuged again and collected hydrotalcite particles were stored at 4 °C until use.

2.5. Protein measurement

Protein was measured by Commassie Blue G-250 binding method [6,7] using bovine serum albumin as the standard.

The protein adsorbed on the carrier material P_g (mg/g) was calculated from the following equation [5]:

$$P_g = \frac{C_0 V_0 - C_f V_f}{w} \quad (1)$$

where C_0 is initial protein concentration (mg/mL), C_f the protein concentration of filtrate (mg/mL), V_0 the initial volume of lipase solution (mL), V_f the volume of filtrate (mL) and w is the weight of carrier material used (g).

Degree of adsorption from Kolmogorov–Erofeev–Kazeeva–Avrami–Mampel (KEKAM) reaction model [5] was calculated as:

$$\alpha = \frac{P_g}{P_{g,m}} \quad (2)$$

where $P_{g,m}$ (mg/g) is the maximum amount of protein adsorbed on the carrier materials which were hydrotalcite and zeolites.

2.6. Enzyme activity assay

Activity of immobilized lipase and free lipase were determined by the method described by Winkler and Stuckmann [8] by measuring micromoles of 4-nitrophenol released from 4-nitrophenyl palmitate. The enzymatic reaction mixture contained 75 μL of 4-nitrophenyl palmitate solution (20 mM), 5 μL enzyme and buffers (0.1 M phosphate buffer pH 7.0 for zeolite and 0.1 M Tris–HCl buffer pH 8.5 for hydrotalcite) to make a final volume of 3 mL. This mixture was incubated at 45 °C for 10 min in water bath. And then chilling at −18 °C for 8 min was employed to stop the reaction. The lipase activities were expressed in IU (international unit) where 1 IU is defined as the amount of enzyme required to produce 1 μmol of 4-nitrophenol per minute under assay conditions. Immobilization efficiency

Table 1
Properties of immobilization supports used in the study

Product type	Structure type	Major cation	Pore size	Chemical composition
5A	A	Ca^{2+}	5 Å	$Ca_{4.5}Na_3[(AlO_2)_{12}(SiO_2)_{12}]\cdot 30H_2O$
13X	X	Na^+	10 Å	$Na_{86}[(AlO_2)_{86}(SiO_2)_{106}]\cdot 276H_2O$
AW-300	Mordenite	Na^+	4 Å	$Na_{0.45}[(AlO_2)_{0.57}(SiO_2)_{0.45}]\cdot 1.64H_2O$
Zeocat FM-8	Mordenite	Na^+	3 Å	$Na_{0.17}[(AlO_2)_{1.10}(SiO_2)_{0.17}]\cdot 1.34H_2O$
Hydrotalcite	–	–	–	$Mg_6Al_2(CO_3)(OH)_{16}\cdot 4H_2O$

(%) was determined from the following equation:

$$\text{Immobilization Efficiency}\,(\%) = \left[\frac{E_0V_0 - E_fV_f}{E_0V_0}\right] \times 100 \quad (3)$$

where E_0 is initial lipase activity (IU/mL), E_f the lipase activity (IU/mL) after immobilization, V_0 the initial volume of lipase solution (mL)and V_0 is the volume of filtrate (mL).

2.7. Repeated batch hydrolysis

The immobilized enzyme was used in the repeated batch hydrolysis experiments in a water bath at 45 °C and 55 °C. The experimental conditions were the same as described above. The immobilized lipase enzyme were reused with fresh substrates after 24 h for each run. The initial activity of freshly prepared immobilized lipase in the first run was defined as 100% activity.

2.8. Enzymatic production of biodiesel

9.65 g of waste cooking oil was mixed well with lipase (4% by weight of grease) and then 1.4 g methanol was added. Alcohol to oil molar ratio was chosen as 4:1. The mixture was kept at room temperature (24 °C) and at 200 rpm. After 22–48–72–105 h, the organic phase was removed by centrifugation at 10,000 rpm.

The methyl ester contents of reaction mixture were measured on a gas chromatograph (Agilent Technologies Model 5573) equipped with a DB-35 ms capillary column (0.25 mm × 0.25 μm × 30 m; Agilent). Helium was used as carrier gas with a flowrate of 1.2 mL/min. The column temperature was kept at 190 °C for 0.5 min, then raised to 300 °C with a heating rate of 4 °C min^{-1}.

Mono-, di-, tri-gliserit, free and total glycerol measurements of products were done by using European standard test method of EN14105. The experiments were performed at TUBITAK Marmara Research Center.

3. Result and discussion

3.1. Effect of immobilization material and particle size on immobilization

Immobilization is very important in the industrial applications of enzymes due to its advantages on process control and operations. Therefore, immobilization is one of the methods used for stabilization of biocatalysts [9]. There are different materials used for immobilization of enzyme, such as natural polymeric derivatives or synthetic polymers, inorganic materials like diatomaceous earth, controlled-pore glasses, silica, zeolites, ceramics, kaolinites, etc. [10]. However, using widely different substrates and supports employed led to an enormous amount of quantitative data, which cannot be directly compared. For that reason, we have sistematicly studied with two types of materials, hydrotalcite and zeolite. Although zeolites have been used as enzyme support by several authors [5,11–13], hydrotalcite has not been studied in details. The properties and chemical compositions of zeolites and hydrotalcite studied in this study are given in Table 1.

Effect of particle size of hydrotalcite and different type of zeolite on immobilization was investigated, in this study. Fig. 1 shows the effect of particle size on immobilization on hydrotalcite and four different types of zeolites.

The size and type of the zeolite did not effect the amount of protein adsorbed (Fig. 1). Sakaguchi et al. [14] reported that adsorption on zeolite was dependent on the number of hydrophobic interaction points on the mesopore and their morphology. It was also noted by Diaz et al. [15] that the inclusion of trypsin (38 Å) in the microporous structure is impossible since the pore size of zeolites are too small (<20 Å). According to these explanations, the amount of protein adsorbed on zeolite was not effected by pore size since the pore size of zeolite used in this work smaller than 20 Å and the number of hydrophobic interaction points on the mesophore could be same at all particle size of zeolite.

Hydrotalcite was more efficient than zeolites. OH^- ions of hydrotalcite may account for its higher efficiency. The amount of protein adsorbed (P_g) on hydrotalcite 13 mg/g was higher than that of zeolite as 9 mg/g. And the maximum immobilization efficiencies are 95.8% for hydrotalcite and 56.1% for zeolite types. Thus, hydrotalcite was used for further work.

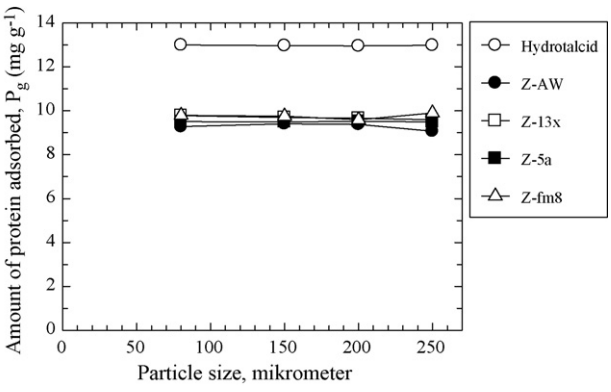

Fig. 1. Effect of particle size on immobilization.

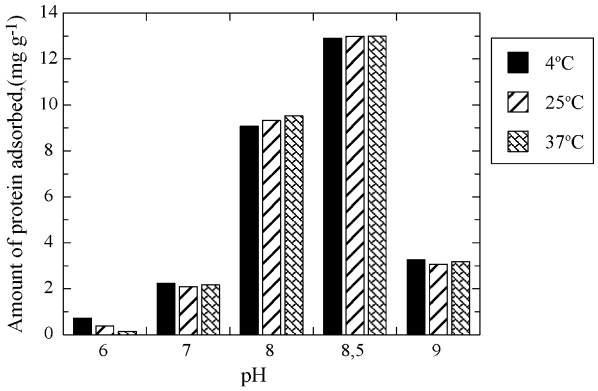

Fig. 2. Effect of pH and temperature on hydrotalcite.

3.2. Effect of pH on immobilization of lipase enzyme on hydrotalcite

Fig. 2 shows the effect of pH and temperature on the immobilization of lipase on hydrotalcite. The highest amount of protein adsorbed on hydrotalcite as 13 mg/g was obtained at pH 8.5. It can also be seen from the figure that the amount of protein adsorbed on hydrotalcite was not affected with increasing temperature from 4 °C to 37 °C. This result may suggest that the adsorption is chemical adsorption on hydrotalcite, rather than physical adsorption, due to formation of chemical bonds with OH groups of hydrotalcite.

3.3. Effect of time on immobilization on hydrotalcite

Protein adsorption as a function of time is shown in Fig. 3. The kinetic curve can be distinguished in two parts: the initial part and the plateau region. Knezevic et al. [5] explained that at the initial stage, adsorption is rapid and the amount adsorbed is proportional to time. The immobilization of enzyme on hydrotalcite reached steady state after 5 h.

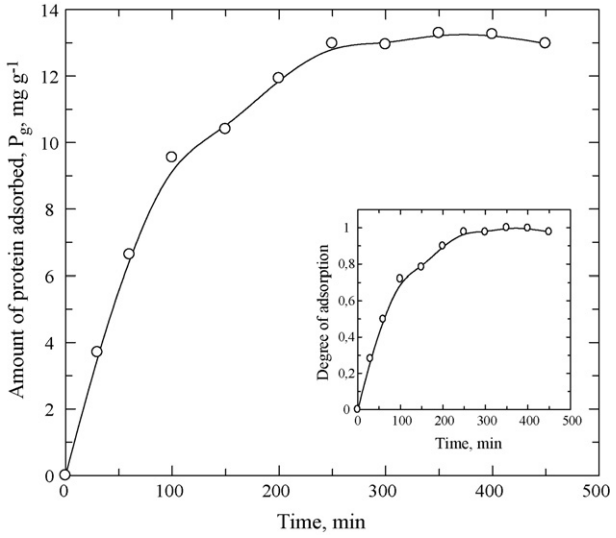

Fig. 3. Effect of time on immobilization of lipase enzyme on hydrotalcite.

3.4. Effect of temperature and stability of immobilized lipase on hydrotalcite in repeated use

The optimum temperature was found to be 45 °C (data were not shown). The relative activities at other temperatures were estimated considering the activities at 45 °C as 100%. Activity of immobilized lipase on hydrotalcite was not affected from temperature changes. The relative activity at 30 °C and 60 °C were 89.6% and 86.8%, respectively.

The immobilized enzyme on hydrotalcite was used in the repeated batch hydrolysis experiments in a water bath at 45 °C and 55 °C. The results are shown in Table 2. Immobilized lipase could be used with little loss of activity for up to two times at 45 °C. Immobilized enzyme retained 36% its original activity, after seven cycles. At 55 °C, the enzyme lost its activity after one usage. At the end of seventh cycle, only 14% of its original activity remained.

3.5. Enzymatic production of biodiesel

Activity of lipase enzyme (Lipozyme IM) immobilized on hydrotalcite and zeolite particles was measured in the transesterification reaction of waste vegetable cooking oil and methanol. The waste oil had 1.05% free fatty acid and an acid value of 2.1 mg KOH/g, which is slightly above the 1% limit for satisfactory transesterification reaction using conventional alkaline catalyst. Free fatty acid content of waste oil was determined by a standard titrimetry method [16]. Reaction temperature and time, types of enzyme and support are the parameters of transesterification of waste oil with methanol investigated in this study. The highest yield of 95% was obtained by using Lipozyme-TL IM free enzyme solution at room temperature after 105 h. It was found that Lipozyme-TL IM immobilized on hidrotalcite gave a slightly low yield (92.8%) than free enzyme solution. However, comparing the enzyme amounts take place in the reaction, immobilized enzyme showed much higher activity than free enzyme. The reason for this may be support material hydrotalcite itself is also an active site for transesterification reaction. MgAl hydrotalcites are recently used as solid base heterogeneous catalysts in transesterification reaction of edible oils. In the study of Xie et al. [17], 67% conversion of soybean oil was obtained by using MgAl hydrotalcite solid base catalyst. Cantrell et al. [18] showed the relation between hydrotalcite structure and catalytic activity

Table 2
Stability of immobilized lipase in repeated use

Number of cycle[a]	Relative activity	
	45 °C	55 °C
1	100	100
2	90	64
3	72	50
4	66	40
5	55	30
6	49	22
7	36	14

[a] In each cycle, enzymatic reaction was carried out at pH 8.5 for 10 min.

Table 3
FAME compositions of biodiesel products

Enzyme	Enzyme amount (mg)	Yield (%)	FAME	Structure[a] fatty acid	FAME (wt%)
Lipozyme TL (immobilized) hydrotalcite	5	92.8	Methyl myristate	14:0	0.4
			Methyl palmitate	16:0	9.0
			Methyl stearate	18:0	4.5
			Methyl oleate	18:1	69.7
			Linolenic acid ME	18:3	1.1
			Arachidic acid ME	20:0	1.0
Lipozyme TL (free)	386	95	Methyl myristate	14:0	0.9
			Methyl palmitate	16:0	18
			Methyl stearate	18:0	6.5
			Methyl Oleate	18:1	57.8
			Linolenic acid ME	18:3	1.3
			Arachidic acid ME	20:0	0.7
Lipozyme TL (immobilized) FM-8	5	No yield	Methyl palmitate	16:0	0.04
			Methyl stearate	18:0	0.03
			Methyl oleate	18:1	0.6

[a]Carbons in the chain: double bonds.

Table 4
Comparison of Gliserid, free and total glycerol contents of biodiesel produced with EN14214 standard

	Monoglyceride content % (m/m)	Diglyceride content % (m/m)	Triglyceride content % (m/m)	Free glycerol % (m/m)	Total glycerol % (m/m)
Lipozyme TL (immobilized) hydrotalcite	0.198	1.15	0.018	0.023	0.243
Lipozyme TL (free)	0.157	0.844	0.036	0.003	0.171
EN14214 limit (max)	0.8	0.2	0.2	0.02	0.25

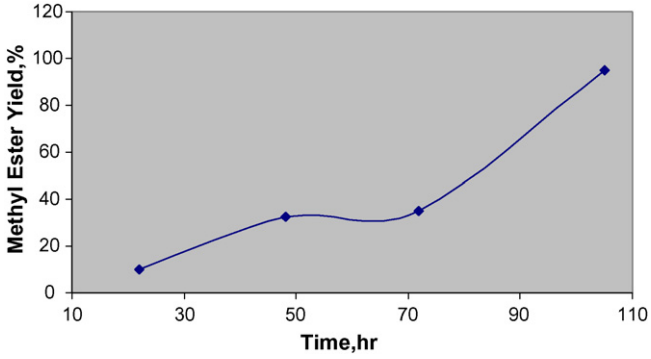

Fig. 4. Effect of reaction time on methyl ester yield.

in the transesterification of glyceryl tributyrate and a maximum conversion of 74.8% was obtained with the hydrotalcite catalyst containing a Mg:Al ratio of 2.93:1. Fatty acid methyl esters profile of biodiesels produced in this study are shown in Table 3. On the other hand enzymes immobilized on zeolites did not show significant yields at the same reaction conditions as shown in Table 3. The inactivity of these biocatalysts may be caused from conformational changes or blocking of lipase active sites during lipase immobilization process.

In order to see the effects of reaction time, transesterification experiments were performed at room temperature (24 °C) for 22–105 h by using Lipozyme-TL IM free enzyme solution. As shown in Fig. 4 methyl ester yield increased with time, but reached 95% yield after 105 h. Therefore, all other transesterification reactions were performed for 105 h in this study.

Table 4 presents the results of glycerides, free and total glycerol content measurements of biodiesels produced in this study comparing with European Standard of EN14214. All values are in limits except diglyceride contents.

4. Conclusions

In this study, immobilization conditions of lipase enzyme on hydrotalcite prepared by coprecipitation and four different types of commercial zeolites was investigated. Temperature, pH, time and particle sizes on enzyme activity were the immobilization conditions investigated. Hydrotalcite was found to be the best support material for lipase than the other four types of zeolites. The highest amount of protein adsorbed on hydrotalcite as 13 mg/g was obtained at pH 8.5 and 4 °C. The key step in enzymatic processes lies in succesfull immobilization of the enzyme which will allow for its recovery and reuse. It was found that the immobilized lipase on hydrotalcite yielded a lipolytic activity equivalent to 36% of initial activity of lipase that was measured after the seventh using. The present study also demonstrates that immobilized lipase on hydrotalcite are able to catalyse the transesterification of waste cooking oil with methanol to produce methyl esters.

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