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Electrospun PVA fibrous mats immobilizing lipase entrapped in alkylsilicate cages: Application to continuous production of fatty acid butyl ester

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ABSTRACT

Electrospun polyvinyl alcohol (PVA) fibers are used to immobilize lipase entrapped in organosilicate cages. The cages are prepared from dimethyldimethoxysilane and tetramethoxysilane (called Fiber-SilicaLip in this paper) and are used as the active component of a flow-through reactor for the continuous production of fatty acid butyl esters by butanolysis of rapeseed oil. The catalytic activity of the catalyst, defined by reaction rate during the first 4 h of reaction, depended on the content of water in the mixture of rapeseed oil and n-butanol (molar ratio of triglyceride and n-butanol = 1:3). The highest catalytic activity was detected at 0.5% (w/w) water addition. At this level, Fiber-SilicaLip showed a 57-fold increase in catalytic activity over purchased lipase powder and 3.7-fold higher catalytic activity than PVA fibers enclosing non-treated lipase. In addition, the reaction rate per amount of catalyst (matrix + protein lipase) detected at 0.5% (w/w) water addition was 1.6 times greater than the maximum value detected for Novozym 435 with no added water. The Fiber-SilicaLip continued to work as a catalyst for a 14 day run as a membranous component of a continuous fatty acid butyl esters production system.

1. Introduction

Electrospinning is a simple and versatile method for producing fibers with diameters ranging from several microns down to 100 nm or less. Recently, electrospun fibers have attracted much attention as novel carriers for immobilized enzymes. Methods of immobilization used either bind the enzymes to the surface of fibers and or entrap them within the fibers [1]. Such fibers have a large surface area-to-volume ratio, and small diffusion resistance for both substrate and products due to an intrinsically short diffusion path. Another desirable feature is that non-woven fabrics composed of the fibers can be easily manufactured into membranous reaction components for continuous operation bioreactors.

Lipase (triacylglycerol hydrolase, EC 3.1.1.3) is an enzyme which has been shown the effectiveness when immobilized in electrospun fiber [2–5]. In nature, the enzyme catalyzes the hydrolysis of triglycerides at the interface of oil and water. The enzyme also catalyzes reactions in organic media including transesterification, esterification, aminolysis, and acyl exchange [6]. An emerging application of lipase is in biodiesel production, where the enzyme catalyzes the transesterification of triglycerides. Biodiesel made from vegetable oil or animal fat has recently attracted significant attention as a replacement for fossil diesel fuel. Environmental issues concerned with the formation of greenhouse gases from the combustion of fossil fuels encourage the investigation of biodiesel as a carbon neutral fuel.

Recently, we developed an electrospun fibrous membrane composed of poly(vinyl alcohol) (PVA) fibers of approximately 1 μm in diameter, with immobilized lipase entrapped in organosilicate cages. The cages are in the order of several hundreds of nanometers in size, i.e. much smaller than the fibers. We revealed that the lipase immobilized in silicate cages composed of dimethyldimethoxysilane (DMDMOS) showed an initial transesterification rate of (S)-glycidol to glycidyl n-butyrate with vinyl n-butyrate in isooctane, more than ten times faster than a non-immobilized equivalent [7]. The objective of the present study was to investigate the feasibility of the immobilized lipase for biodiesel production from vegetable oil in a solvent-free system (containing only the mixture of substrates). We used n-butanol (producible from sustainable materials) as the alcohol for the transesterification of triglycerides. Methanol is the most widely investigated alcohol for biodiesel production; however, methanol is a stronger denaturing agent than longer aliphatic alcohols such as butanol and hence has a greater capacity to inactivate enzymes [8]. In addition, methanol is

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commonly manufactured from natural gas, which is far from ideal from the viewpoint of sustainability. The higher alcohol butanol is attracting attention as a biofuel precursor [9]. UK oil company BP and the US chemical company DuPont have jointly developed a process for producing butanol from feed stocks such as sugarcane, maize and wheat.

2. Materials and methods

2.1. Materials

Rhizopus oryzae lipase (Lipase F-AP) containing lipase at 65.0% was purchased from Wako Pure Chemicals (Osaka, Japan), and was used without further purification. According to the manufacturer, the declared activity of the enzyme is more than 150,000 FIP-units/g (One unit of enzyme activity is defined as that quantity of a standard lipase preparation that liberates the equivalent of 1 μmole of fatty acid from olive oil per minute by hydrolysis under Federation Internationale Pharmaceutique assay conditions). A commercially available control material, Candida antarctica lipase B immobilized on acrylic resin (Novozym 435) was obtained. According to the manufacturer, the declared activity of Novozym 435 is more than 10,000 PL-units/g (propyl laurate units per gram). PVA with MW 146,000–186,000 and 98-99% hydrolyzed was obtained from Sigma (MO, USA). Tetramethoxysilane (TMOS) and DMDMOS were purchased from Tokyo Kasei (Tokyo, Japan). Rapeseed oil was purchased from Riken (Saga, Japan) and dehydrated with a molecular sieve before use.

2.2. Immobilization of lipase in PVA fibers

Electrospun fibrous mats with immobilized lipase (Fiber-Lip), with lipase entrapped in silica cages (Fiber-SilicaLip) and without entrapment in silicate cages (Fiber-NakedLip) were prepared using a previously reported method [7]. Briefly, the lipase was exposed to the sol–gel process: 0.545 mmol of DMDMOS, 0.136 mmol of TMOS, 23.3 μl of distilled water, and 1.5 μl of 40 mM HCl were mixed in a glass tube at room temperature. After obtaining a homogeneous mixture resulting from hydrolysis of the precursors, the resultant solution was cooled in an ice bath. Then, 167 μl of 100 mM phosphate buffer solution (PBS, pH 7.5) was added. Immediately after mixing for several seconds, a mixture of 50 μl of PBS and 150 mg lipase was mixed with the solution. The resultant mixture became a sol with whitish color resulting from condensation of the hydrolyzed precursors. Subsequently, the sol containing lipase was mixed with 15 ml of 10% (w/w) PVA solution. The resultant solution was loaded into a plastic syringe equipped with a 20-gauge stainless steel needle and extruded at 0.8 ml/h. The needle connected to the DC supply was located 12 cm away from the earthed counter-electrode (this is the tip-to-collector distance). A high-voltage DC generator was used to apply +15 kV. The resultant fibrous mats were vacuum-dried for 1 day. The size of the silicate cages measured using a dynamic light scattering particle size analyzer after dissolving the resultant fibrous mats in distilled water was 410 ± 118 nm.

2.3. Transesterification experiments

Batch and flow-through reactors were used to perform the transesterification of rapeseed oil. For the batch reactions, vacuum-dried Fiber-Lip mats were cut into small pieces of about 3 mm × 3 mm square using scissors. Experiments were performed using either cut specimens (200 mg-dry weight containing 13 mg of protein lipase), non-treated lipase reagent powder (Powder-Lip, 20-mg dry weight containing 13 mg of protein lipase), or Novozym 435 (200-mg dry weight). The test materials were placed in 30-ml screw-capped glass vials containing 20 ml of reaction medium. The reaction medium was a mixture of rapeseed oil and n-butanol at molar ratio of triglyceride to n-butanol of 1:3. The water content in the reaction media was controlled by adding an appropriate amount of distilled water–the amount of water initially contained in the mixture of rapeseed oil and n-butanol was 800 ppm and the amount of subsequently added water was less than that required to cause phase-separation. The glass vessels were shaken at 1300 rev/min and 35 °C using a temperature controlled incubator-shaker. The initial transesterification rate was determined from the increase in concentration of n-butyl ester during the first 4 h of reaction, with measurements taken every hour.

For the flow-through reactor, 45 mm diameter circular disks of about 0.5 mm thickness were cut from the Fiber-SilicaLip mats. Eight of these disks (650 mg) were placed on a filter support screen in a 47 mm internal diameter filter holder. The medium used for the flow-through reaction experiment contained triglyceride and n-butanol at a molar ratio of 1:3 and 0.5% (w/w) water addition. The experiment was performed with a volumetric flow rate of 0.15–1.34 ml/min.

The concentration of C18 butyl esters was determined with a gas chromatograph (Shimadzu GC-2010, Kyoto, Japan) using methyl laurate as internal standard based on the calibration curve for n-butyl ester obtained from butyl oleate. The content of C18 fatty acids in the rapeseed oil used in this study is 93.2% (supplier's data). Conversion was calculated from following equation: Conversion (%) = [(C18 butyl esters concentration (mM)]/[2421 mM (total fatty acids concentration in fresh reaction medium) × 0.932] × 100.

3. Results and discussion

3.1. The effect of water content on catalytic activity

PVA is an attractive material to electrospin fibers for entrapping enzymes because it can be spun from aqueous solution at neutral pH, conditions with little risk of damaging the enzymes. However, absorbed water may hinder diffusion of hydrophobic substrates through the PVA matrix due to the highly hydrophilic nature of the material. It is also well-known that an appropriate amount of water is required for enzymatic action [6] and that the water content in the solvent is an important factor in controlling a lipase-catalyzed transesterification reaction [8]. In this study, we compared catalytic activities of lipases contained in Powder-Lip, Fiber-NakedLip, and Fiber-SilicaLip by measuring an initial reaction rate. This rate was defined as the production rate of n-butyl ester during the first 4 h of reaction per mg of protein lipase. The initial reaction rate detected for Powder-Lip was increased over 200-fold by increasing the amount of water addition from nil (initial reaction rate = 2.5×10^{-4} μmol/min/mg-lipase) to 2.0% (w/w) (0.064 μmol/min/mg-lipase) (Fig. 1a). This result means that the 800 ppm of water initially contained in the reaction medium was insufficient for lipase enzymatic action. Even without the addition of water, Fiber-Lip (Fiber-SilicaLip: 0.245 μmol/min/mg-lipase and Fiber-NakedLip: 0.087 μmol/min/mg-lipase), showed a 300-fold faster initial reaction rate compared with Powder-Lip (2.5×10^{-4} μmol/min/mg-lipase). One crucial factor is the highly hydrophilic nature of the PVA surrounding the lipase which is important in maintaining a catalytically active enzyme conformation. This dependence of the initial reaction rate on the reaction system water content was the reason for the significant difference between Powder-Lip and Fiber-Lip. Both Fiber-NakedLip and Fiber-SilicaLip showed their individual fastest initial reaction rates at 0.5% (w/w) water addition (0.110 and 0.402 μmol/min/mg-

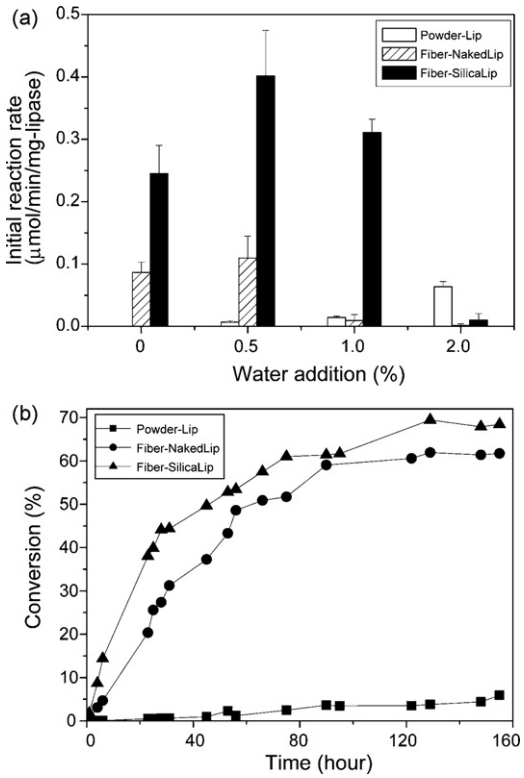

Fig. 1. (a) Initial reaction rates of Powder-Lip, Fiber-NakedLip, and Fiber-SilicaLip per mg-protein lipase at 35 °C with different water contents. The bars represent the mean ($n = 3$) ± standard deviation. (b) Typical time-conversion plots of triglyceride to butyl esters at 0.5% (w/w) water addition.

lipase, respectively) and were 16 and 57 times faster than the rate detected for Powder-Lip (0.007 μmol/min/mg-lipase) under equivalent conditions. A possible explanation for the increase of initial reaction rates for Fiber-NakedLip and Fiber-SilicaLip when the water content is increased to 0.5% (w/w) is increase in molecular diffusion rate through the PVA layer. This increase is the result of the reaction media absorbing water and swelling up, leading to a looser microscopic matrix structure. As is expected from the initial reaction rates, the time necessary for achieving the equilibrium conversion state was the shortest for Fiber-SilicaLip system (Fig. 1b). The results shown in Fig. 1b also show that entrapping lipase in alkylsilicate cages is effective for enhancing equilibrium conversion. Further increase in water content decreased the initial reaction rates. At 2.0% (w/w) water addition, Powder-Lip (0.064 μmol/min/mg-lipase) showed a 6-fold increase in initial reaction over Fiber-Lip (Fiber-SilicaLip: 0.010 μmol/min/mg-lipase and Fiber-NakedLip: 0.001 μmol/min/mg-lipase). The higher catalytic activity of Powder-Lip over Fiber-Lip is explained by an excess amount of water adsorption by the PVA matrix resulting in hindrance of diffusion through the carrier to the enclosed lipase. Comparison of the images obtained by scanning electron microscope (SEM) examination of the Fiber-SilicaLip just after electrospinning and after the experiments indicates the occurrence of absorption of water by Fig. 2 the PVA fibers (Fig. 2). The PVA fibers used in the reaction with 2.0% (w/w) water addition showed morphology typical of swelling due to water absorption (Fig. 2c). Compared with Fiber-Lip, Fiber-SilicaLip showed higher catalytic activity than Fiber-NakedLip over the complete range of water contents. This result clearly demonstrates the effectiveness of the encapsulation of lipase in alkylsilicate cages contained within individual electrospun fibers for enhancing catalytic activity for fatty acid butyl esters by transesterification of triglycerides. The enhanced catalytic activity can be explained by a possible interaction between the lipophilic domains of the lipase and the hydrophobic regions of the alkylsilicate resulting in more active conformation of the enzyme in organic medium [10,11].

Novozym 435 is one of the most widely used lipase-immobilizing catalysts for biodiesel production [12]. We compared the catalytic activity of Fiber-SilicaLip and Novozym 435 for the initial reaction rate per mg of lipase-immobilizing catalyst (matrix + protein lipase). As shown in Table 1, Novozym 435 showed the highest catalytic activity at nil water addition (1.60×10^{-2} μmol/min/mg-catalyst). This value was almost the same as that detected for Fiber-SilicaLip (1.59×10^{-2} μmol/min/mg-catalyst). The catalytic activity

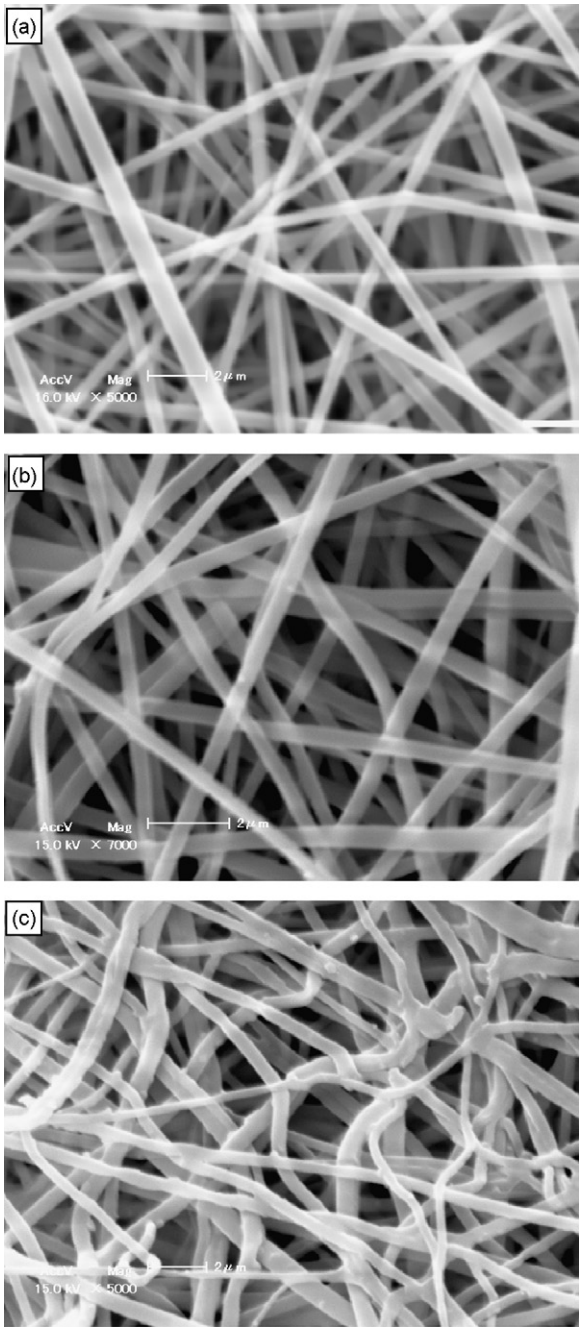

Fig. 2. SEM micrographs of Fiber-SilicaLip (a) before and after use for biodiesel production, (b) nil water addition and (c) 2.0% of water addition. Bars represent 2 μm.

Table 1
Initial reaction rates of Fiber-SilicaLip and Novozym 435 for fatty acid butyl ester production with different water contents.

	Water addition (%)			
	0	0.5	1.0	2.0
Fiber-SilicaLip ($\times 10^{-2}$ μmol/min/mg-catalyst)	1.59 ± 0.29	2.61 ± 0.47	2.02 ± 0.14	0.07 ± 0.06
Novozym 435 ($\times 10^{-2}$ μmol/min/mg-catalyst)	1.60 ± 0.38	0.45 ± 0.12	0.36 ± 0.12	0.36 ± 0.17

200 mg of lipase-immobilizing catalyst (support matrix + lipase) were used. Data: mean ± S.D. (n = 3).

detected for Fiber-SilicaLip was highest at 0.5% (w/w) of water addition (2.61×10^{-2} μmol/min/mg-catalyst). In contrast, that for Novozym 435 decreased with increasing water content in a similar fashion to that reported when used for the transesterification of Jatropha oil with methanol [13]. These results demonstrate the feasibility of Fiber-SilicaLip immobilized Rhizopus oryzae lipase for biodiesel production from vegetable oil and n-butanol. However, the result of our preliminary study indicates that the feasibility is limited to systems using long chain alcohols such as n-butanol. The most commonly used alcohols for biodiesel production are short chain alcohols, such as methanol. For the methanol system, the result of our preliminary study was that the catalytic activity of Fiber-SilicaLip was less than a fiftieth of that detected using n-butanol. In contrast, it was reported that Novozym 435 showed higher activity in the methanol system than when used with n-butanol [13].

3.2. Flow-through biodiesel production performance

Flow-through reactors are more economically attractive for mass production compared with batch reactor systems. Enzymes immobilized in electrospun non-woven fabrics are more suitable as the active component of a flow-through reactor than for the equivalent batch reactors because of the sheet-like geometry of the catalytic substrates. Fig. 3 shows the variation of the degree of conversion to biodiesel with the flow rate of the medium. As is usual with flow-through reactors, the yield of conversion increased as the flow rate decreased. The maximum conversion yield determined in this study (8%) was not high compared with the equilibrium conversion measured in the batch reaction shown in Fig. 1b, but triglycerides were successfully converted to butyl esters. One simple and well recognized way to increase the yield of conversion is by increasing the quantity of catalyst within the reactor. Further studies for optimizing the content of catalyst and reaction conditions are in progress. We also studied the stability of the catalytic activity of Fiber-SilicaLip exposed to a continuous flow-through biodiesel production system. This experiment was performed under a fixed flow rate of substrate solution giving about 2% of conversion yield for the catalyst in a 4 h run. The reaction cycle of the enzymes proceed

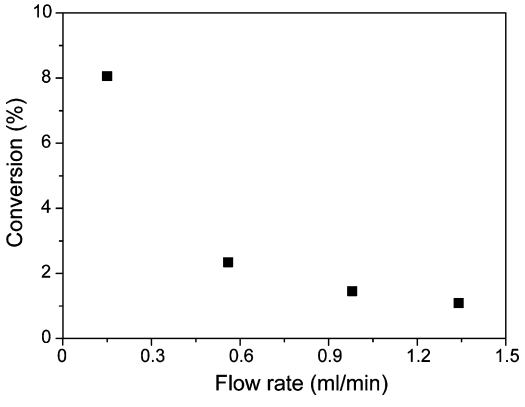

Fig. 3. Effect of flow rate on degree of conversion of flow-through reactor using a Fiber-SilicaLip membrane.

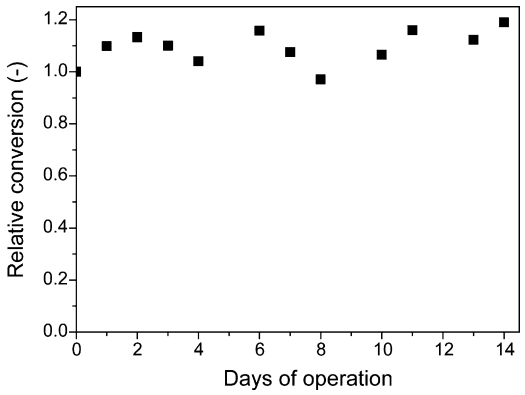

Fig. 4. Transition of conversion yield to biodiesel in a flow-through reactor using a Fiber-SilicaLip membrane. Relative conversion is a dimensionless value normalized against the value at 4 h after start of operation.

faster under these conditions, but give a lower conversion yield. As shown in Fig. 4, a reduction in conversion by the Fiber-SilicaLip membrane was not seen during a 2 week run. Moreover, the conversion rate measured after the first day of operation was 10% higher than that measured after 4 h. This finding can be explained by the achievement of an equilibrium state of water adsorption by the PVA with the solution of 0.5% (w/w) water.

Continuous reactors are very applicable for large-scale production, though the most commonly studied experimental reactor type for biodiesel production using lipase is a batch type continuously stirred tank reactor [12]. The results of this study reveal the potential application of PVA electrospun fibrous membrane containing immobilized lipase entrapped in organosilicate cages as catalyst for continuous biodiesel production. In this study we studied production of fatty acid butyl esters through butanolysis of rapeseed oil using immobilized Rhizopus oryzae lipase. Scale-up of the reaction component for a higher conversion yield of biodiesel is the subject of further study. In addition, evaluation of the effectiveness of the immobilization as Fiber-SilicaLip for the other lipases will also be studied because reactivity and stability of lipases strongly depend on their origin [8,13].

4. Conclusion

We have examined the feasibility of Fiber-SilicaLip for biodiesel production from vegetable oil and n-butanol when used as a membrane-shaped active component in a flow-through reactor. Encapsulation of lipase in alkylsilicate cages was effective for enhancing catalytic activity of the enzyme for butanolysis. The catalytic activity depended on the content of water in the reacting solution and showed maximum activity at 0.5% (w/w) of water addition. The catalytic activity of a membrane-shaped active component was observed over 14 days in a flow-through biodiesel production system. From these results, we conclude that Fiber-SilicaLip is a possible candidate as a catalyst for continuous biodiesel production from rapeseed oil as a reaction component in flow-through reactors.

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