

University of Nebraska - Lincoln

DigitalCommons@University of Nebraska - Lincoln

---

Papers in Biomaterials

Chemical and Biomolecular Engineering  
Research and Publications

---

May 2004

## Immobilized Pseudomonas cepacia lipase for biodiesel fuel production from soybean oil,

Hossein Nouredini

*Department of Chemical Engineering, University of Nebraska-Lincoln*, hnoured@unlnotes.unl.edu

X Gao

*Department of Chemical Engineering, University of Nebraska-Lincoln*

R S. Philkana

*Department of Chemical Engineering, University of Nebraska-Lincoln*

Follow this and additional works at: [https://digitalcommons.unl.edu/chemeng\\_biomaterials](https://digitalcommons.unl.edu/chemeng_biomaterials)

 Part of the [Biomaterials Commons](#)

---

Nouredini, Hossein; Gao, X; and Philkana, R S., "Immobilized Pseudomonas cepacia lipase for biodiesel fuel production from soybean oil," (2004). *Papers in Biomaterials*. 19.

[https://digitalcommons.unl.edu/chemeng\\_biomaterials/19](https://digitalcommons.unl.edu/chemeng_biomaterials/19)

This Article is brought to you for free and open access by the Chemical and Biomolecular Engineering Research and Publications at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Papers in Biomaterials by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

## Abstract

Enzymatic transesterification of soybean oil with methanol and ethanol was studied. Of the nine lipases that were tested in the initial screening, lipase PS from *Pseudomonas cepacia* resulted in the highest yield of alkyl esters. Lipase from *Pseudomonas cepacia* was further investigated in immobilized form with in chemically inert, hydrophobic sol-gel support. The gel-entrapped lipase was prepared by polycondensation of hydrolyzed tetramethoxysilane and iso-butyltrimethoxysilane. Using the immobilized lipase PS, the effects of water and alcohol concentration, enzyme loading, enzyme thermal stability, and temperature in the transesterification reaction were investigated. The optimal conditions for processing log of soybean oil were: 35°C, 1:7.5 oil methanol molar ratio, 0.5g water and 475mg lipase with the reactions with methanol, and 35°C, 1:15.2 oil ethanol molar ratio 0.3s water, 4751ng lipase for the reactions with ethanol. Subject to the optimal conditions, methyl and ethyl esters formation of 67 and 65mol% in 1 h of reaction were obtained for the immobilized enzyme reactions. Upon the reaction with the immobilized lipase, the triglycerides reached negligible levels after the first 30min of the reaction and the immobilized lipase was consistently more active than the free enzyme. The immobilized lipase also proved to be stable and lost little activity when was subjected to repeated uses.

## Keywords:

Transesterification; Biodiesel immobilization; Entrapment; Lipase; Sol-gel; Soybean oil; *Pseudomonas cepacia*

## Introduction

Vegetable oils have attracted attention as a potential renewable source for the production of an alternative for petroleum based diesel fuel due to the diminishing petroleum reserves and environmental consequences of exhaust gases from diesel engines. Methyl and ethyl esters of fatty acids, better known as biodiesel, are nontoxic, biodegradable, and an excellent replacement for petroleum diesel. Biodiesel cetane number, energy content, viscosity and phase changes are also similar to those of petroleum-based diesel fuel (Mittelback and Tritthart, 1998). Moreover, biodiesel is essentially sulfur free and the engines fueled by biodiesel emit significantly fewer particulates, hydrocarbons, and less carbon monoxide than those operating on conventional diesel fuel. Emissions of  $\text{CO}_2$ , however, are slightly higher than those of diesel engines operating on conventional diesel fuels (Schumacher et al., 1996; Ali et al., 1995). Transesterification of vegetable oils and animal fats for the production of fatty acid alkyl esters in a well established industrial process. The conventional biodiesel technology involves the use of an inorganic base or acid catalyst at or near the boiling temperatures of the triglyceride alcohol mixture. The removal of catalyst is through neutralization and eventual separation of salt from the product esters, which is difficult to achieve. Moreover, the physicochemical synthesis schemes often result in poor reaction selectivity and may lead to undesirable side reactions. Enzymatic conversion of triglycerides has been suggested as a realistic alternative to the conventional physicochemical methods. Enzymatic transesterification of triglycerides offers an environmentally more attractive option to the conventional process.

H. Nouredini, X. Gao, R.S. Philkana, Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil, © Bioresource Technology 96 (2005) 769-777, © 2004 Elsevier Ltd.

However, the high cost of the enzymes often makes the enzymatic processes economically unattractive. The key step in enzymatic processes lies in successful immobilization of the enzyme, which will allow for its recovery and reuse (Balcao et al., 1996). Immobilization is the most widely used method for achieving stability in lipases and to make them more attractive for industrial use (Cowan, 1996; Clark, 1994). Common immobilization techniques include physical adsorption onto a solid support (e.g. Bosley and Pielow, 1997), covalent bonding to a solid support (e.g. Walt and Agayn, 1994) and physical entrapment within a polymer matrix support (e.g. Pizarro et al., 1997). Entrapment of lipase entails capture of the lipase within a matrix of a polymer (Hartmeier, 1985). The immobilized lipase by entrapment is much more stable than physically adsorbed lipase and unlike the covalent bonding method, this method uses a relatively simple procedure and at the same time the immobilized lipase maintains its activity and stability (Kennedy and Melo, 1990). A variety of methods have been used for trapping lipases in a polymer matrix (Bickerstaff, 1971). Entrapment of enzymes in an inorganic polymer matrix is one method that has received a considerable attention in recent years. This method which was pioneered by Avnir et al. (1994) is based on sol-gel process. The application of the sol-gel material in the immobilization of lipases is well documented (e.g. Reetr, 1997). A substantial collection of research on the enzymatic transesterification of triglycerides has focused on free enzyme reactions with and without organic solvents. Nelson et al. (1996) studied the lipase-catalyzed transesterification of triglycerides in hexane, using different lipases and a variety of triglycerides and alcohols. The lipase from *Mucor miehi* (Lipozyme IM-20) was found to be the most effective in converting tallow to their respective alkyl esters with primary alcohols, whereas the lipase from *Candida antactica* was found to be most suitable for reacting with secondary alcohols, giving branched alkyl esters. Wu et al. (1999) employed response surface methodology (RSM) to optimize reaction parameters such as temperature, time, level of lipase, and molar ratio of reactants in the *Pseudomonas cepacia* lipase and *Candida antactica* lipase catalyzed transesterification reaction of restaurant grease to ethyl esters using 95.6% ethanol. Results showed a synergistic effect when the two lipases were used in sequence which surpassed the RSM predictions. Abigor et al. (2000) studied the lipase-catalyzed transesterification of two Nigerian lauric oils: palm kernel oil and coconut oil, by transesterification of oils with different alcohols using PS-30 lipase as catalyst. In the conversion of palm kernel to alkyl esters, ethanol resulted in the highest conversion of 72.04%. Other alcohols tested and their corresponding transesterification conversions included: 1-butanol 62%, 1-butanol -12%, n-propanol 42%, and iso-propanol 24%, while only 15% methyl esters was observed using methanol. Kaieda et al. (2001) studied the effect of methanol and water concentrations in the methanolysis of soybean oil using different lipases in a solvent free system. Lipase from *Pseudomonas cepacia* showed the greatest methanol resistance among the tested lipases. This lipase also exhibited the highest catalytic activity toward the transesterification reaction, even in the absence of water, methyl ester content reached 32% after 50h of reaction. Cao et al. (1992) reported the etherification and transesterification reactions catalyzed by Porcine pancreatic lipase adsorbed on glass, acetone precipitated on porous glass, kieselgur, aluminum oxide and agar beads in organic solvents. Results showed the lipase adsorbed on kieselgur and agar beads with the highest activity. Shimada et al. (1999) and Watanabe et al. (2000) used immobilized *Candida antactica* (Novozyme 435) for the conversion of vegetable oil to biodiesel. Results showed incomplete methanolysis of vegetable oil which was attributed to the inactivation of the enzyme. Stepwise addition of methanol prevented this inactivation and conversions in excess of 95% were obtained. Samoa et al. (2000) investigated the effect of the preincubation of immobilized *Candida Antarctica* lipase (Novozyme 435) in methyl oleate and soybean oil prior to the biodiesel production. Results indicated a much higher rate of methanolysis for the preincubated lipase. Hsu et al. (2001) developed a novel

H. Nouredini<sup>1</sup>, X. Gao, R.S. Philkana, Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil, © Bioresource Technology 96 (2005) 769-777, © 2004 Elsevier Ltd. procedure For the immobilization of lipase from *Pseudomonas cepacia* within a phyllosilicate sol-gel matrix. In this process phyllosilicate clay saturated with sodium ions was suspended in water and then exchanged with alkyl ammonium ions by the addition of cetyltrimethyl ammonium chloride. This mixture was then used in the entrapment of *Pseudomonas cepacia* with tetramethoxysilane (TMOS) as the polymerization precursor. The immobilized enzyme so prepared was then used in the transesterification of tallow and grease where conversions in excess of 95% were reached. In the present study, lipase PS from *Pseudomonas cepacia* was entrapped within a sol-gel polymer matrix. prepared by polycondensation of hydrolyzed TMOS and iso-butyltrimethoxysilane (iso-BTMS). The immobilized enzyme was used in the transesterification of soybean oil with methanol and ethanol.

## 2. Methods

### 2.1 Materials

All lipases were donated by Amano Enzyme (Nagoyri, Japan). Soybean oil was donated by Archer Daniel Midland Co. (Lincoln, NE). Methanol and ethanol were purchased from Fisher Scientific (Pittsburgh, PA). Bis-(trimethylsilyl) trifluoroacetamide (BSTFA, derivative grade), 1,2,3-tridecanoylglycerol (tricaprine: 99%), pyridine, and diatomaceous earth were purchased from Sigma Chemical Co. (St Louis, MO). Hexane (GC grade) was purchased from EM Science (Gibbstown, NJ). Tetramethoxysilane (TMOS, 95%), iso-butyltrimethoxysilane (iso-BTMS, 97%), and sodium fluoride (NaF) were purchased from the Aldrich Chemical Company (Milwaukee, WI).

### 2.2 Lipase immobilization.

A specified amount of lipase PS (optimally 1 g) was measured into a flask and 10 ml of water was added. The mixture was stirred at 100 rpm using a magnetic stirrer for about 5 min. To this mixture, 1 ml of a 1 M NaF solution and the silica precursors were added. Upon the addition of the precursors, the reaction occurred almost immediately and the gel was formed in 2 min. The flask was removed from the stirrer and left sealed at room temperature for 24 h. The flask was then uncapped and was incubated in a water bath at 33°C for about 24 h. The ceramic polymer was then broken up and grounded in a mortar. The powder was washed with 100 ml of distilled water in a 250 ml flask for 1 h at a mixing speed of 100 rpm. The mixture was then filtered. About 90 ml of supernatant was collected. This supernatant was further estimated for its residual activity (see the section on the Degree of immobilization tests). The net paste was dried again at 33°C for 24 h. The immobilized lipase was then crushed in a mortar to yield the final product. The fine powder was stored at 4°C until use. About 6 g of the sol-gel material was resulted in this procedure. Based on the degree of immobilization tests, about 95% of the enzyme was immobilized in this procedure. The actual enzyme loading was determined: 475 mg of lipase PS per 3 g of gel.

### 2.3. Reaction setup and optimization:

The reactions were carried out in a constant temperature water bath, under which a Thermodyns (Dubuque, IA) Mirnk 4-p1: Ice magnetic stirrer model #S73135 was placed to agitate the reaction mixture. Water was circulated into the bath from a Neslab (Portsmouth, NH) TTE-21 I Bath Circulator which, via an external probe, was able to control the temperature of the bath to

H. Nouredini, X. Gao, R.S. Philkana, Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil, © Bioresource Technology 96 (2005) 769-777, © 2004 Elsevier Ltd. within  $\pm 0.01$  °C. A standard set of conditions was used as the baseline in the optimization studies. These conditions were adopted from a previous study (Nouredini et al., 2002). The initial conditions were 10g soybean oil, 3g methanol (methanol to oil molar ratio of 8.2), 0.5g water, 1g immobilized lipase PS, 40°C, 700rpm. And 1 h reaction time. In the reactions with ethanol (0.5g of water and 5 g of ethanol (ethanol to oil molar ratio of 9.5) were used under otherwise identical conditions. In the optimization studies, only one reaction parameter was varied at a time. For example, when the effect of temperature was investigated, the rest of the reaction conditions were maintained at: 10g soybean oil, 3g methanol, 0.5g water, 3g immobilized lipase PS, 700 rpm. and 1 h reaction time. Lipase PS was used in all of the optimization studies.

#### 2.4. Degree of immobilization tests

The immobilized enzyme was washed with water and after filtration, about 90 ml of supernatant was collected. This supernatant may potentially contain free enzyme, partially hydrolyzed precursors, methanol, and soluble oligomers. To quantify the amount of enzyme in the supernatant, a calibration curve relating the formation of free fatty acids as a function of free Enzyme loading was constructed, which was based on the hydrolysis of soybean oil. Details about this work may be found elsewhere (Nouredini et al., 2002). By comparing the supernatant from the immobilization wash procedure with this calibration, the amount of enzyme in the supernatant and the degree of immobilization was determined. In order to mimic the exact reaction conditions, in the calibration studies, a blank gel with no enzyme was prepared. The supernatant, which was collected from washing this gel, was used as the reaction medium in the calibration experiments. Otherwise, the reaction procedure for the calibration experiments was identical to the free enzyme hydrolysis reactions. The enzyme loading was varied in the range of 0.3-30mg lipase per 1g of soybean oil for this calibration. The calibration curve showed a monotonically increasing activity as the enzyme loading was increased. The activity of the enzyme leveled off beyond the upper limit of 30mg of enzyme per 1g of soybean oil.

#### 2.5. Sampling Analysis:

Samples were 0.8-1.2 ml in volume and were collected in 5-ml sample vials. Samples from the reactions were initially heated to ensure enzyme denaturation, freeze dried to remove excess water and alcohol, and finally derivatized with BSTFA. The silylating agent reacts with the carboxyl groups of fatty acids and results in trimethylsilylated fatty acids which are readily separated and quantified. The derivatized samples were then analyzed by a gas chromatograph (GC) to determine the concentration of fatty acid esters, free fatty acids, mono, di, and triglycerides. A Hewlett-Packard (Wilmington; DE) 6890 Series GC system was used for the chromatography work and a Hewlett-Packard Chemstation software was used for the data analysis. The GC was equipped with a Hewlett-Packard (part number 19091.1-012) HP-5 column. Sample volumes were 2 $\mu$ l, the carrier gas was helium, and the GC was operated in constant flow mode with a flow rate of 12.0ml/min. A split injector was used with a split ratio of 15:1 and a temperature of 325°C. The FID detector was operated at 350°C and used a helium makeup flow to maintain a constant detector flow of 25.0ml/min. The oven was initially held at 80.0°C and was then heated to 180°C at 15.0°C/min, to 250°C at 1.00°C/min, and finally to 325°C at 8.0°C/min. The oven was held at this temperature for 22.95min before returning to 80.0°C. Total run time for this method was 53.0min. Calibration of the

H. Nouredini<sup>1</sup>, X. Gao, R.S. Philkana, Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil, © Bioresource Technology 96 (2005) 769-777, © 2004 Elsevier Ltd. GC method was carried out by analyzing standard solutions of mixed glycerol, fatty acid esters, free fatty acids; mono-, di- and triglycerides. The standards were derivatized in the same fashion as the reaction samples. More details about the sample preparations and analysis procedures are explained elsewhere (Wagner, 1999).

### 2.6 Data Analysis :

Experimental results are presented in Fig. 1-8. In these figures, the activity of the lipases is presented by the formation of esters and other reaction products in terms of molar percent of the components. The data presented in Figs. 1-6 were replicated at least three times. The mean values for the replicated data are presented in the graphs. There was no error analysis for Figs. 7 and 8. In Figs. 1-6, the standard deviations for fatty acids were approximately 5% of the mean values and are not shown in the figures. The data was analyzed by Microsoft Excel build-in functions.

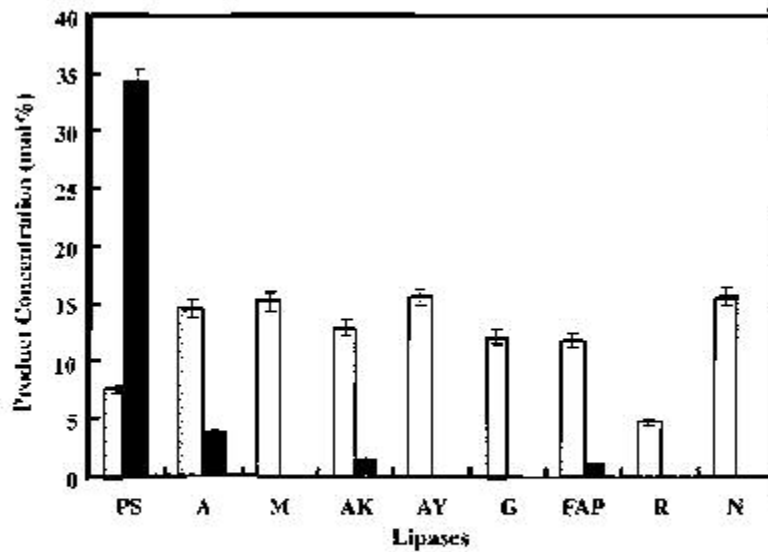


Fig. 1. Lipase screening on transesterification of soybean oil, a loading of 250 mg free lipase, 3.0 g methanol, 0.5 g water, 10 g oil, a stirring rate of 700 rpm and a 1-h reaction at 35 °C. (■) Methylesters and (□) free fatty acids.

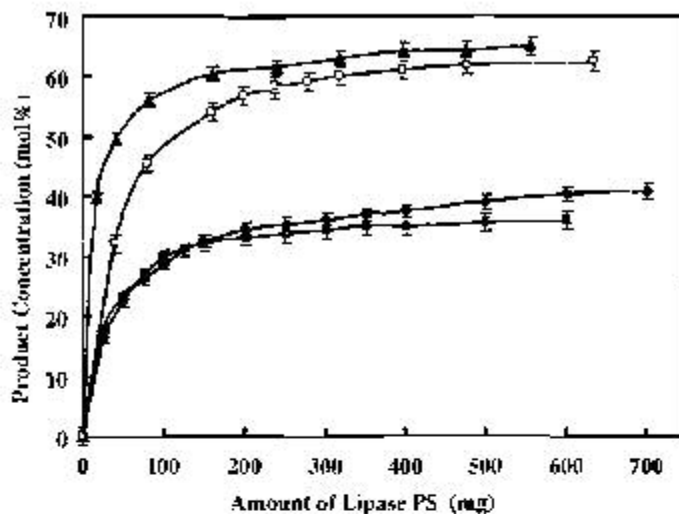


Fig. 2. Effect of enzyme loading on transesterification of soybean oil 0.5g water, 10g oil, a stirring rate of 700rpm and a 1-h reaction at 35°C. (◆) Free lipase PS, 3g of methanol. (▲) Immobilized lipase PS, 3.0g of methanol. (■) Free lipase PS, 5g of ethanol reaction. (□) Immobilized lipase PS, 5g of ethanol.

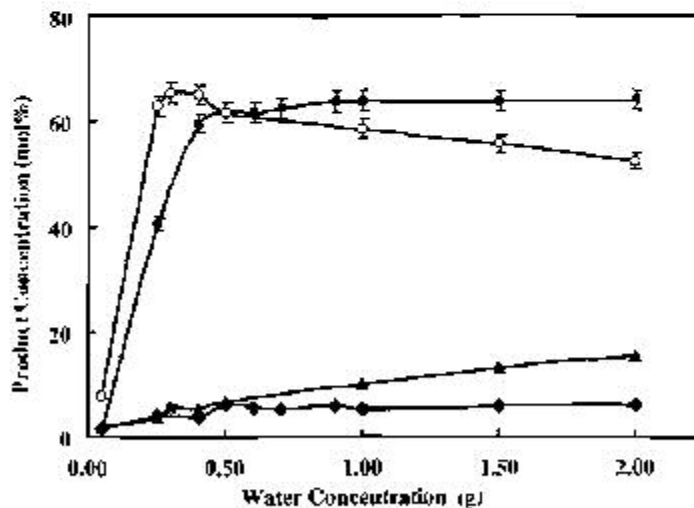


Fig. 3. Effect of water concentration on immobilized lipase catalyzed transesterification of soybean oil, subject to a loading of 3g of gel/10g of oil, a stirring rate of 700rpm and a 1-h reaction at 40°C. (●) Methylesters and (◆) free fatty acids: 3g methanol; (□) methylesters and (▲) free fatty acids: 8g ethanol



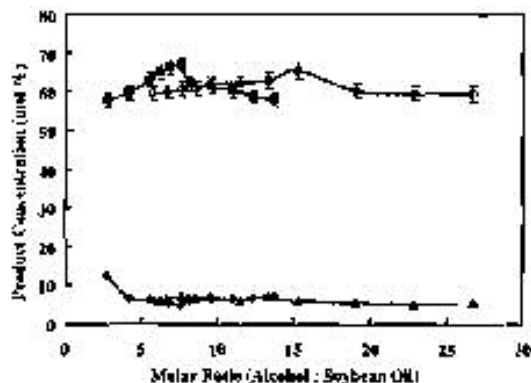


Fig. 4. Effect of alcohol concentration on immobilized lipase catalyzed transesterification of soybean oil, subject to a loading of 3 g of gel/10 g of oil, a stirring rate of 700 rpm and a 1-h reaction at 40°C (■) Methyl esters and (●) free fatty acids; 0.5 g water; (□) ethyl esters and (▲) free fatty acids; 0.3 g water.

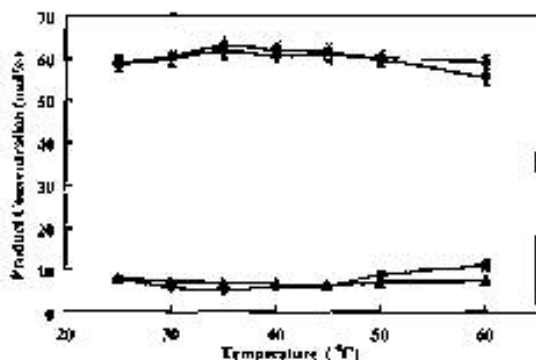


Fig. 5. Effect of temperature on immobilized lipase catalyzed transesterification of soybean oil, subject to a loading of 3 g of gel/10 g of oil, a stirring rate of 700 rpm and a 1-h reaction. (■) Methyl esters and (●) free fatty acids; 3 g methanol, 0.5 g water; (□) ethyl esters and (▲) free fatty acids; 3 g ethanol, 0.3 g water.

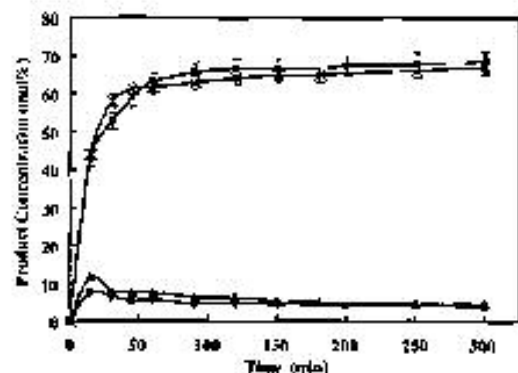


Fig. 6. Time course of the transesterification of soybean oil, subject to a loading of 3 g of gel/10 g of oil, a stirring rate of 700 rpm for at 35°C (■) Methyl esters and (●) free fatty acids; 3 g methanol, 0.5 g water; (□) ethyl esters and (▲) free fatty acids; 3 g ethanol, 0.3 g water.

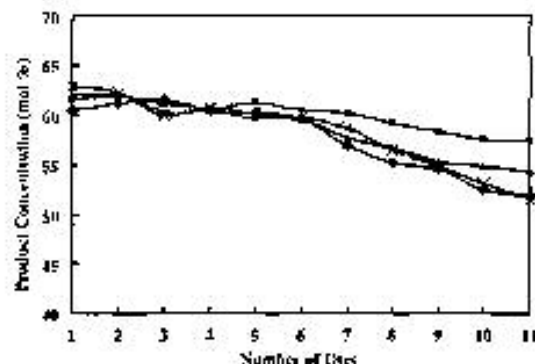


Fig. 7. Reusability of immobilized PS subject to a loading of 3 g of gel/10 g of oil, 0.3 g water, a stirring rate of 700 rpm for and a 1-h reaction at 35°C (●) 4 g EtOH, (■) 5 g EtOH, (▲) 6 g EtOH, (×) 7 g EtOH.

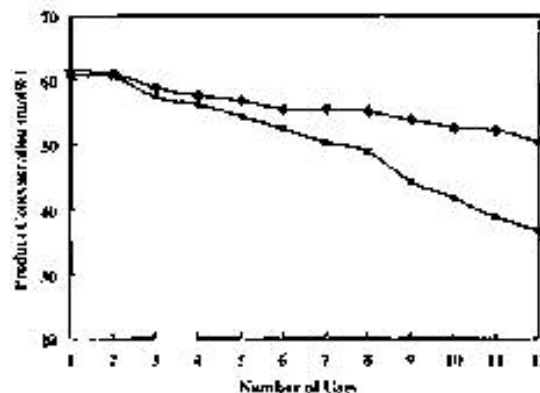


Fig. 8. Reusability of immobilized PS subject to a loading of 3 g of gel/10 g of oil, 0.5 g water, a stirring rate of 700 rpm for and a 1-h reaction at 35°C (●) 1.5 g MeOH, (■) 3.0 g MeOH.

Table 1  
Lipases tested in the transesterification screening

Lipase <sup>a</sup>	Source organism
R	<i>Penicillium roqueforti</i>
AK	<i>Pseudomonas</i> sp.
PS	<i>Pseudomonas</i> sp.
M	<i>Mucor</i> sp.
A	<i>Aspergillus niger</i>
FAP	<i>Rhizopus oryzae</i>
G	<i>Penicillium camembertii</i>
N	<i>Rhizopus oryzae</i>
AY	<i>Candida rugosa</i>

<sup>a</sup> All lipases were donated by Ajinomoto Enzyme (Nagoya, Japan).

### 3. Results and discussion

Although lipases generally catalyze the hydrolysis of carboxylic esters; they bring about a range of bioconversion reactions such as etherification, transesterification; acidolysis, and aminolysis. Lipase screening was performed to find the lipase with the best catalytic activity in the transesterification of soybean oil. The most active lipase was then used in the immobilization studies. Nine lipases, as listed in Table I, were screened for their transesterification activity. The screening experiments were intended for an initial valuation of the activity of the lipases, they were conducted under a preliminary set of reaction conditions which may not have been the optimum set for all the lipases. In a typical reaction, 250mg of dry enzyme was added to the mixture of 3 g methanol (8.2 molar ratio of methanol to soybean oil), 0.5 g of water- and 10g of soybean oil. The reactions were carried out at 35°C and according to the reaction setup which was described earlier. The screening results for the tested lipase are presented in Fig. 1. Reaction products are presented as mol% of methyl esters of fatty acids in the reaction mixture. The formation of free fatty acids is also included in this figure since the presence of water in the reaction medium naturally promotes the competing hydrolysis reaction. As this figure shows, among the tested lipases, lipase PS from *Pseudomonas cepacia* showed the highest activity toward the transesterification of soybean oil with methanol. Other lipases showed very little or no activity toward the transesterification reaction. After 1 h of reaction with lipase PS, the product contained 34mol% methyl esters, 91mol% of fatty acids, 6mol% of monoglycerides, 44mol% of diglyceride and 7 mol% of triglycerides.

### 3.2 Enzyme Loading:

Experiments were performed to determine the effect of enzyme loading on the extent of the transesterification reaction. Enzyme loading in the range of 0-700mg of Free enzyme and 0-3.5g of the immobilized enzyme were examined in the transesterification of soybean oil with methanol and ethanol. One gram of immobilized enzyme corresponds to 158mg free enzyme in these reactions. Based on the concentration of soybean oil, the alcohol concentration was at 8.2 molar equivalents for methanol and 9.5 molar equivalents for ethanol (3.0g methanol and 5.02 ethanol). Other reaction parameters were as was stated earlier in the reaction setup and optimization section. Reaction results for the formation of methyl and ethyl esters of fatty acids are presented in Fig. 2. For all cases studied, as the enzyme loading was increased there was a sudden surge in the formation of alkyl esters, followed by a slower rate at higher enzyme loadings. This surge was steeper and the formation of alkyl esters was significantly higher for the immobilized enzymes compared with the free enzymes. For the reactions with methanol, the concentration of methyl esters reached 11 and 65mol% for the free and the immobilized lipase, respectively. At the end of the reaction period, the concentration of triglyceride in both systems reached negligible levels, while, the formation of fatty acid, mono- and di-glyceride were 8, 9, and 42mol% for the free enzyme reaction and 7, 22, and 6mol% for the immobilized enzyme. Similarly; for the reaction with ethanol the level of ethyl esters reached 36 and 63mol% for the free and the immobilized lipase. At the end of the reaction period, the concentration of triglyceride in both systems reached negligible levels, while, the formation of fatty acid, mono- and diglyceride were 9, 6, 43mol% for the free enzyme reaction and 9, 22, and 6mol% for the immobilized. This behavior of the immobilized lipase was consistent with those of other researchers (e.g. Reetz, 1997) and has been attributed to lipophilic nature of the alkyl group of the sol-gel. Free alkyl groups in the sol-gel create a lipophilic microenvironment that subsequently

interacts with the lipase, triggering a phenomenon similar to a classical interfacial interaction. However, unlike the interfacial activation, which is an interactive process, the alkyl effect is believed to be due to a more favorable lipase conformation caused during the sol-gel process. The lipophilic environments are also believed to facilitate the transport of the organic substrate to the biocatalyst sites in the outer surface of the support and possibly in and out of the matrix.

### 3.3. Effect of water concentration:

Lipases possess the unique feature of acting at the interface between an aqueous and an organic phase. Lipase interfacial action is due to the fact that their catalytic activity generally depends on the aggregation of the substrates. Activation of the enzyme involves unmasking and restructuring of the active site through conformational changes of the lipase molecule, which requires the presence of oil-water interface. Lipase activity generally depends on the available interfacial area. With the increased addition of water, the amount of water available for oil to form oil-water droplets increases, thereby increasing the available interfacial area. However, since lipases usually catalyze hydrolysis in aqueous media, excess water may stimulate the competing hydrolysis reaction. The optimum water content is a compromise between minimizing hydrolysis and maximizing enzyme activity for the transesterification reaction. The effect of water concentration in the range of 0.05-2.0s and at constant alcohol concentrations of 8.2 and 15.2 molar ratios of methanol and ethanol with respect to soybean oil were examined (3.9 g methanol and 8.0g ethanol). The reactions were carried out according to the reaction setup and optimization section described earlier. Results presented in Fig. 3 indicate very little enzyme activity at low water concentrations which supports the fact that a minimum amount of water is required to activate the enzyme. With the increased addition of water there was a considerable increase in the ester production showing the enhancement in the activity of the enzyme. In the case of methanol, the ester production reached about 62molW at 0.5g water after which there was very little increase in the production of ester but also a slight increase in the formation of fatty acid. In the case of ethanol, the ester production reached a maximum of about 65molK at 0.3 water. At water concentrations beyond 0.3g, there was a considerable decrease in the production of esters and accordingly an increase in the formation of fatty acid.

### 3.4. Effect of alcohol concentration

In the immobilized enzyme transesterification reaction, the reactants initially form a three-phase system (triglyceride/alcohol/suppliment). The reaction is diffusion-controlled and poor diffusion between the phases exists. As alkyl esters are formed, they act as a mutual solvent for the reactants and a two phase liquid solid system results (Nouredini and Zhu, 1997). However, as the reaction progresses toward completion and the glycerol concentration is increased, a small alcohol and glycerol phase separates from the rich alkyl ester phase and a three phase system forms again. This is more likely at lower initial alcohol concentrations and higher extent of reaction and in the range of alcohol concentration that was investigated this separation did not occur. Alcohol in excess

H. Nouredini, X. Gao, R.S. Philkana, Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil, © Bioresource Technology 96 (2005) 769-777, © 2004 Elsevier Ltd.

of the stoichiometric molar ratio of 3:1, was used to ensure higher reaction rates as the transesterification of triglycerides with alcohols consist of three stepwise and reversible reactions and (2) minimize the diffusion limitations. However, excess alcohol levels may inhibit the enzyme activity and thereby decrease its catalytic activity toward the transesterification reaction. Experiments were performed to optimize the amount of ester production by varying the alcohol concentration. Optimum alcohol requirements were determined for both methanol and ethanol. The amount of alcohol added was varied from 2.7 to 13.7 molar equivalents for methanol and from 5.7 to 26.7 molar equivalents for ethanol, based on the moles of triglycerides. Water concentration was kept constant at the optimum level of 0.5 g for the methanol reactions and at 0.3g for the ethanol reactions. Results are summarized in Fig. 4. As expected, an increase in the number of moles of alcohol with respect to the triglycerides resulted in an increase in the production of esters. Ultimately, the formation of esters reached a maximum level and further increases in the alcohol concentrations resulted in a decrease in the formation of esters. The optimum alcohol concentration was determined at 7.5 molar ratio of methanol to soybean oil where about 65 mol% of methyl esters was formed. For the reaction of ethanol and soybean oil this ratio was at 15.25 for which about 65 mol% of ethyl esters was formed.

### 3.4. Effect of Temperature

Experiments were performed to examine the effect of temperature on the catalytic activity of the immobilized lipase PS in the transesterification of soybean oil with methanol and ethanol. Reactions were carried out as was described earlier in the reaction setup and optimization section. Temperatures in the range of 25-40°C at 5°C increments and at constant alcohol concentrations of 8.2 and 9.5 molar ratios of methanol and ethanol with respect to oil were examined (3.0g methanol and 5.0g ethanol). Results, presented in Fig. 5, show slight changes in the transesterification activity of the immobilized lipase PS with variations in temperature. Transesterification activity of lipase PS reached a maximum at 35°C with methanol, where about 63 mol% of methyl esters was formed. As the reaction temperature was further increased, a decrease in the ester production and an increase in the fatty acid production were observed. This behavior was consistent with our previous study (Nouredini et al., 2002) which revealed more favorable hydrolysis reactions at higher temperatures. The reactions with ethanol also showed a similar trend and an optimum temperature of 35°C at which about 62 mol% of ethyl esters were formed. Experiments were also performed to examine thermal stability of the immobilized lipase PS in the transesterification of soybean oil with methanol at 35°C. In these experiments, the immobilized enzyme (33) was initially incubated at 35°C in 10g of oil for up to 72h. The incubated immobilized enzyme was then used in the transesterification reaction. Results showed no change in the catalytic activity of the incubated enzyme. For the enzymes which were subjected to 24, 48, and 72h of incubation the formation of methyl esters was practically identical to the formation of methyl esters for the enzymes with no prior incubation. The observed enhancement in thermal stability of the immobilized enzymes was consistent with our previous study on the hydrolysis of soybean oil (Nouredini et al.; 2002) and also the work of Kauakami and Yoshida (1996), and may be attributed to interaction between lipase and sol-gel support. It appears that stable lipase conformations similar to the native activation occur due to the interaction of enzyme with the polymer surface. Both physical and chemical interactions such as hydrogen bonding and ionic interactions are believed to be responsible for the enhanced thermal stability of the immobilized enzyme (Reetz et al., 1996).

### 3.6. Effect of Temperature:

The kinetics of the reaction plays an important role in the process scale up and design. The kinetics of the transesterification reaction was further investigated for both methanol and ethanol reactions with lipase PS. The reaction conditions for the methanol reaction were log soybean oil, 8.2 molar ratio of methanol to soybean oil (13.0g methanol), 0.5% water, 35°C, and 3g immobilized lipase PS; and the reaction setup described earlier in the reaction setup and optimization section. The conditions for the ethanol reaction were identical except for 9.5 molar ratio of ethanol to soybean oil (5.0% ethanol) and 0.3 g water. The reaction results for the formation of alkyl esters and free fatty acids for the initial 300min of the reaction are presented in Fig. 6. In the case of methanol transesterification reaction, results showed an initial surge in the formation of methyl esters during the first 30min of the reaction which was followed by a slower rate as the reaction progresses and ultimately a state of equilibrium was approached. More than 65% of methyl esters are formed during the first 90min of the reaction. Similar trends were observed for the transesterification reaction with ethanol where 63% of reaction was completed over the first 90min of the reaction and only 4% of additional conversion was observed over the next 210min of the reaction. The formation of the free fatty acids was below 1% in both cases.

### 3.7. Immobilized enzyme: stability and reusability.

One of the most important characteristics of an immobilized enzyme is its stability and reusability over an extended period of time. Experiments were performed to examine the recyclability and the stability of the immobilized lipase PS. After each standard 1 h transesterification reaction, lipase-containing gel was recovered by filtration and subsequently reused. This procedure was repeated several times to examine the extent of the stability of the immobilized enzyme. Experiments were performed with 10g soybean oil, 1g immobilized lipase PS, 35°C, 0.5 and 0.32 water for methanol and ethanol reactions, respectively. The concentration of alcohols was also varied to examine its long-term effect on the activity of the immobilized lipase. Other reaction conditions were as described earlier in the reaction setup and optimization section. To facilitate the alteration of the immobilized gel after each run, 1.0g of diatomaceous earth was mixed to the reaction medium prior to the first alteration procedure. The diatomaceous earth was recycled along with the immobilized gel throughout the replications. Reactions are conducted at 4.0, 5.0, 6.9, and 7.0% ethanol and 10% soybean oil which corresponded to about 7.6, 9.5, 11.4, and 13.5 molar ratios of ethanol to soybean oil, respectively. Experimental results summarized in Fig. 7 show a slight decreasing trend in the activity of the immobilized lipase upon repeated uses in all cases studied. The decrease in the activity was smaller at 9.5 molar ratio of alcohol to the soybean oil where only about 5% loss in the activity was observed after 11 replicates. It appears that at this level of alcohol there is a good balance between the availability of the alcohol as a reaction substrate and its inhibitory effects to the lipase. In general, the loss of activity may be attributed to the deactivation of lipase and to the gradual loss of the immobilized lipase during the processing procedures. The transesterification reactions with ethanol were conducted at 2.5 and 3.0g alcohol and 10g of soybean oil which corresponded to 6.9 and 8.2 molar ratios of alcohol to soybean oil, respectively. Results summarized in Fig. 8 demonstrate a similar but more drastic decreasing trend in the immobilized lipase when subjected to repeated uses. This may be explained by the fact that methanol exhibits a stronger inhibition toward lipase PS than ethanol.

H. Nouredini<sup>1</sup>, X. Gao, R.S. Philkana, Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil, © Bioresource Technology 96 (2005) 769-777, © 2004 Elsevier Ltd.

#### 4. Conclusions

Enzymatic transesterification of triglycerides offers an environmentally more attractive option to the conventional physicochemical process. The key step in enzymatic processes lies in successful immobilization of the enzyme which will allow for its recovery and reuse. In this study the immobilized-enzyme transesterification of soybean oil with methanol and ethanol was investigated. Lipase PS from *Pseudomonas cepacia* was immobilized by entrapment within a sol-gel structure which was prepared by polycondensation of hydrolyzed TMOS and tri-BTMS. The immobilized lipase so prepared was consistently more active than the free lipase toward the transesterification of soybean oil. The immobilized lipase also proved to be stable and lost little activity when was subjected to repeated uses.

#### Acknowledgement:

The authors express their gratitude to the Nebraska Research Initiative and the Consortium for Plant Biotechnology research for their support of this work.

#### References

- Abigor, R.D., Uadia, P.O., Foglia, T.A., Haas, M.J., Jones, K.C., Okpefa, E., Obibuzor, J.U., Bafor, M.E., 2000. Lipase-catalyzed production of biodiesel fuel from some Nigerian lauric oils. *Biochemical Society Transactions* 28 (6), 979-981.
  - Ali, Y., Hanna, M.A., Leviticus, L.L., 1995. Emissions and power characteristics of diesel engines on methyl soyate and diesel fuel blends. *Bioresource Technology* 52 (2), 185-195.
  - Ayub, D., Braun, S., Lev, O., Ottolenghi, M., 1994. Enzymes and other proteins entrapped in sol-gel materials. *Chemistry of Materials* 6 (10), 1605-1614.
  - Balcao, V.M., Paiva, A.L., Malcata, F.X., 1996. Bioreactors with immobilized lipases: state of the art. *Enzyme and Microbial Technology* 18 (3), 392-416.
  - Rickerstaff, G.F., 1997. *Immobilization of Enzymes and Cells*. Humana Press, Totowa, NJ.
  - Busley, A., Pielow, A., 1997. Immobilization of lipases on porous polypropylene: reduction in esterification efficiency at low loading. *Journal of American Oil Chemists' Society* 74 (1), 107-111.
  - Cao, S.G., Liu, Z.B., Feng, Y., Ma, L., Ding, Z.T., Cheng, Y.H., 1992. Esterification and transesterification with immobilized lipase in organic solvent. *Applied Biochemistry and Biotechnology* 32 (1), 1-6.
-

- Clark, D.S., 1994. Can immobilization be exploited to modify enzyme activity? *TIBTECH* 12 (11), 433-442.
- Cowan, D., 1996. Industrial enzyme technology. *TIBTECH* 14 (5), 177-178.
- Hartman, W., 1985. Immobilized biocatalysts—from simple to complex systems. *Trends in Biotechnology* 3 (6), 149-153.
- Hou, A.F., Jones, K., Manner, W.N., Foglia, T.A., 2001. Production of ethyl esters from tallow and grease using lipase immobilized in a poly (sulfate) sol-gel. *Journal of American Oil Chemists Society* 78 (6), 582-588.
- Kameda, M., Samukawa, T., Kondo, A., Fukuda, H., 2001. Effect of methanol and water contents on production of biodiesel fuel from plant oil catalyzed by various lipases in a solvent-free system. *Journal of Bioscience and Bioengineering* 91 (1), 12-16.
- Kawakami, K., Yoshida, S., 1996. Thermal stabilization of lipase by sol-gel entrapment in organically modified silicates. *Journal of Biotechnology* 42 (1), 239-243.
- Kennedy, J.F., Melo, F.H.M., 1999. Immobilized enzymes and cells. *Chemical Engineering Progress* 86 (7), 81-89.
- Mirrezaei, M., Taheri, P., 1998. Diesel fuel derived from vegetable oils. III. Emulsion tests using methyl ester of seed troying oil. *Journal of American Oil Chemists Society* 65 (7), 1185-1187.
- Nelson, L.A., Foglia, T.A., Manner, W.N., 1996. Lipase catalyzed production of biodiesel. *Journal of American Oil Chemists Society* 73 (5), 191-195.
- Nouredini, H., Zhu, D., 1997. Kinetics of transesterification of soybean oil. *Journal of American Oil Chemists Society* 74 (11), 1457-1463.
- Nouredini, H., Gao, X., Joshi, S., Wagner, P.R., 2002. Immobilization of *Pseudomonas cepacia* lipase by sol-gel entrapment and its application in the hydrolysis of soybean oil. *Journal of American Oil Chemists Society* 79 (10), 35-39.
- Pizzuto, C., Fernandez-Torres, M., Benito, G., Gonzalez-Solis, J., 1997. Optimization by experimental design of polyurethane gel composition as support for enzyme immobilization by entrapment. *Biotechnology and Bioengineering* 55 (2), 497-506.
- Reza, M.T., 1997. Entrapment of biocatalysts in hydrophobic sol-gel materials for use in organic chemistry. *Advanced Materials* 9 (7), 943-954.
- Reza, M.T., Zama, A., Sripalakamp, J., 1996. Efficient immobilization of lipases by entrapment in hydrophobic Sol-Gel materials. *Biotechnology and Bioengineering* 49 (5), 527-534.
- Samukawa, T., Kameda, M., Matsumoto, T., Ban, K., Kondo, A., Shimada, Y., Noda, H., Fukuda, H., 2000. Pretreatment of immobilized *Candida antarctica* lipase for biodiesel fuel production from plant oil. *Journal of Bioscience and Bioengineering* 90 (2), 159-163.
- Schumacher, L.G., Jorgell, S.C., Fossler, J., Goetz, W., Hines, W.G., 1996. Heavy duty engine exhaust emission tests using methyl ester system. *Biodiesel Fuel blends*. *Bioresource Technology* 57 (1), 3-15.
- Shimada, Y., Watanabe, Y., Samukawa, T., Sumitani, A., Noda, H., Fukuda, H., Tomimaga, Y., 1999. Conversion of vegetable oil to biodiesel using immobilized *Candida antarctica* lipase. *Journal of American Oil Chemists Society* 76 (7), 785-793.
- Watanabe, Y., Shimada, Y., Sugihara, A., Noda, H., Fukuda, H., Tomimaga, Y., 2000. Continuous production of biodiesel fuel from vegetable oil using immobilized *Candida antarctica* lipase. *Journal of American Oil Chemists Society* 77 (4), 355-360.
- Wagner, P., 1999. Sol-gel immobilization of lipase for the hydrolysis of soybean oil. MS Thesis, Faculty of Chemical Engineering, University of Nebraska-Lincoln.
- Wu, W.C., Foglia, T.A., Manner, W.N., Phillips, J.G., 1998. Optimizing production of ethyl esters of grease using 95% ethanol by response surface methodology. *Journal of American Oil Chemists Society* 75 (4), 317-321.
- Wu, D.D., Aguin, V., 1994. The chemistry of enzyme and protein immobilization with glutaraldehyde. *Trends in Analytical Chemistry* 13 (9), 494-497.