CHEMICAL ENGINEERING TRANSACTIONS, Volume 11, 2007
Edited by Sauro Pierucci
 ISBN 978-88-95608-00-6

Enzymatic synthesis of biodiesel

Domenico Pirozzi, Mirella Di Lorenzo, Luca Zuccaro, Guido Greco jr.
Dipartimento di Ingegneria chimica – Università Federico II
P.le Tecchio, 80 – 80125 - Napoli

Lipases offer interesting perspectives as biocatalysts for the biodiesel synthesis, though their application is still limited by the poor stability of the enzyme at the operating conditions of industrial interest. In order to overcome this bottleneck, the inactivating effect of specific components of the methanolysis reacting system has been characterised, to develop a rational stabilization strategy. The experimental results have shown that the inactivating effect of the methanol can be avoided using long-chain alcohols as acyl acceptors, whereas the effect of glycerol is associated with diffusional limitations within the solid support used for the enzyme immobilisation.

1. Introduction

Biodiesel is produced from vegetable oils, i.e. from a renewable resource, and is a potential alternative to traditional, fossil fuels (Wang et al., 2000). In comparison with the latter, it results in similar engine performance, associated with significantly reduced emissions (Cardone et al., 2002).
Further ecological and economical advantages could be achieved using lipases in biodiesel synthesis (Dossat et al., 2002; Du et al., 2003; Fukuda et al, 2001; Hsu et al., 2002; Iso et al., 2001; Soumanou et al., 2003; Shah et al., 2004; Watanabe et al., 2001). As a matter of fact, the enzymatic process avoids the use of basic or acid catalysts, thus reducing separation and waste-disposal costs. Furthermore, thanks to lipase specificity and to the mild operating conditions required (low temperature and pressure), their application results in reduced investment and energy costs, as well as in higher quality products. Finally, the glycerol, a co-product of biodiesel synthesis, is of better quality (and of higher commercial value) when using lipases as catalyst.
A bottleneck in the lipase-catalysed transformation of raw vegetable oils is the enzyme deactivation produced by the methanolysis reaction mixture. This study is aimed at characterising the enzyme inactivation mechanism, to establish a strategy for the stabilisation of lipases at the operating conditions required in the industrial reactors.

2. Materials and methods

2.1 Enzymes and chemicals

Lipase from *Candida antarctica,* immobilized on anion exchange resin beads (Novozyme 435), was a gift of Novo Nordisk (Denmark). All solvents and reactants were from Sigma-Aldrich, and were of the highest available purity.

2.2 Soybean oil methanolysis

A typical reaction mixture contained 10 mg of Novozyme 435 and fixed amounts of soybean oil and methanol (see captions in each figure). When indicated, the reagents were diluted in 2 ml octane. The mixture was incubated in 5-ml test-tubes, using a thermostatic shaker. Enzyme and substrate solutions were separately pre-equilibrated for 24 hr at 37 °C, in the presence of molecular sieves. The concentrations of methyl esters (methanolysis products) were measured by a Shimadzu GC 17/3 gas-chromatograph equipped with a flame ionisation detector.

2.3 Lipase stability tests

The enzyme samples were placed in sealed tubes together with a fixed mixture, and kept at constant temperature (usually 50°C). Samples were drawn after 24 h, and cooled down at room temperature. The mixture was removed by 3 washing cycles, each consisting of addition of hexane (3 ml), centrifugation (10 min at 3000 rpm) and removal of the surnatant. Finally, the residual hexane was removed under vacuum (6 hr). Subsequently, the enzyme was re-equilibrated over silica gel (24 hr), and tested for residual activity. Preliminary tests have shown that the washing cycles and the vacuum treatment do not affect the catalytic activity of the enzyme.

2.4 Residual activity measurement

The residual activity of the enzyme was measured (usually at 37°C) using 100 mM ethyl caproate and 100 mM butanol as reactants, and octane as solvent.

3. Results and discussion

The enzymatic methanolysis of soybean oil was first carried out in the presence of octane. As a matter of facts, hydrophobic solvents are known to preserve the enzyme activity in organic medium (Reslow et al., 1987; Velivety et al., 1992). As shown in the Figure 1, very high conversion degrees were obtained in the presence of octane at various methanol/oil ratios.

Similar tests were carried out in the absence of solvents. Reaction in no-solvent systems leads to several advantages, namely: (a) the cost of solvents, as well as the cost of their final separation, are avoided; (b) the space time yield of the reactor is reduced; (c) the methanolysis kinetics is faster, due to the higher concentration of oil and methanol. Unfortunately, when carrying out methanolysis in the absence of solvents, the degrees of conversion obtained after a 24 h reaction were less satisfactory, as shown in the Figure 2. In particular, the conversion decreases dramatically as the initial concentration of methanol increases, and drops to less 5% when a stechiometric molar ratio (3:1) is adopted.

In principle, the slower kinetics observed at higher concentrations of methanol may be originated by inhibition phenomena, or, alternatively, by the lipase inactivation. In order to discriminate between these two effects, we have evaluated the residual activity of lipases after each of the reaction tests described in the Figures 1-2. The data reported in the Table 1 demonstrate that, whatever the experimental conditions adopted, lipases undergo significant activity losses. In particular, inactivation phenomena are more significant when carrying out methanolysis in the absence of solvent, and when higher

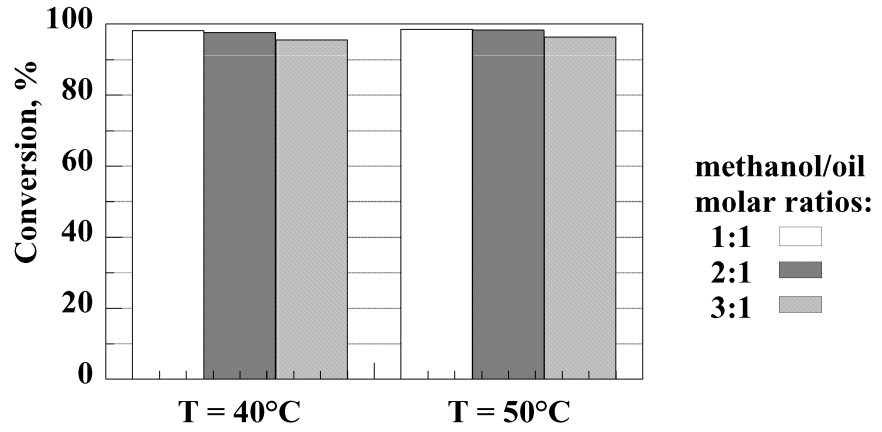

Figure 1. Methanolysis of soybean oil, using octane as a solvent. A reaction mixture of soybean oil (100 mM) and methanol in 2 ml of octane was incubated for 24 h and agitated with an orbital shaker.

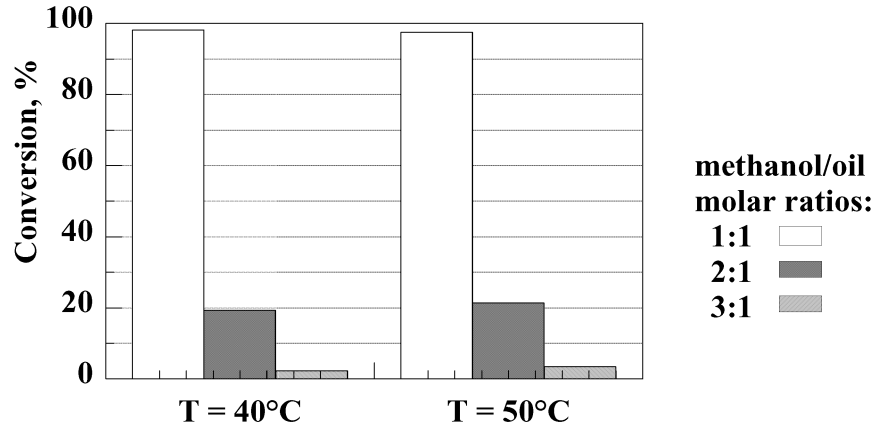

Figure 2. Methanolysis of soybean oil in the absence of solvent. A reaction mixture (2 ml) of soybean oil and methanol was incubated for 24 h and agitated with an orbital shaker.

Table 1. Residual activity of lipase (Novozyme 435) used for the methanolysis of soybean oil. After a 24 h methanolysis reaction, the enzyme was washed in ethanol, dried and tested for residual activity (see Methods paragraph).

Temperature, °C	Methanol/oil ratio	Residual activity, %
40	1:1	54,2
40	2:1	45,6
40	3:1	31,3
50	1:1	29,3
50	2:1	19,1
50	3:1	2,4

Table 2. Residual activity of lipase (Novozyme 435) incubated in the presence of specific components of the methanolysis reaction mixture. After a 24 h incubation at 50 °C, the enzyme was washed in ethanol, dried and tested for residual activity (see Methods paragraph).

Reaction component	Residual activity, %
methanol	1,5
soybean oil	94,5
methyl palmitate	92,6
glycerol	2,4

methanol/oil ratios are adopted. These results suggest that the variations observed in the conversion degree (Figures 1-2) is mainly due to the activity loss of the lipases (Table 1).

Consequently, we measured the effect produced by each component of the methanolysis reaction system on the lipase stability. The experimental data in the Table 2 show that the activity loss of the lipase was mainly produced by the methanol and the glycerol. On the contrary, the initial enzyme activity was almost entirely kept when performing the stability tests in the presence of soybean oil and of methyl palmitate (chosen as a representative of the methyl esters produced by methanolysis).

The inactivating effect of methanol and glycerol was also characterised measuring the residual activity of the enzyme at different temperatures. The results are described in the Figure 3 in the form of an Arrhenius diagram. The corresponding values of the activation energy, obtained by nonlinear regression, are reported in the Table 3. Lipases incubated in methanol show a reduced catalytic activity, though the activation energy of the reaction is unaffected. On the contrary, the incubation of the enzyme in the presence of glycerol leads to a reduction of both the catalytic activity and the activation energy. The reduction is more significant as the glycerol concentration is increased.

In conclusion, the experimental results obtained show that methanol and glycerol produce losses in the biocatalyst activity by different effects. The changes in the activation energy produced by the glycerol suggest that glycerol affects the internal resistances to mass transport within the solid support used for the lipase immobilization.

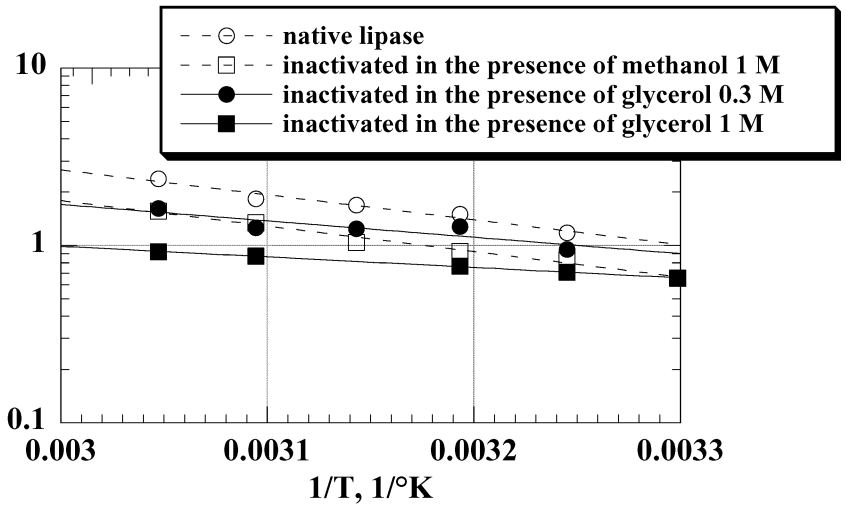

Figure 3. Residual activity of lipase (Novozyme 435) incubated in the presence of methanol and glycerol, as a function of the temperature of the kinetic test. After a 24 h incubation at 50 °C, the enzyme was washed in ethanol, dried and tested for residual activity (see Methods paragraph).

Table 3. Activation energy of transesterification (butyl caproate + geraniol), catalysed by a lipase (Novozyme 435) incubated in the presence of specific components of the methanolysis reaction mixture (see Methods paragraph).

Incubation medium	Activation energy, cal/mol
octane	6466
methanol (1 M)	6616
glycerol (0,3 M)	4248
glycerol (1 M)	2734

Should this be the case, improvements in the enzyme stability could be obtained by a suitable choice of the diameter and the pore size of the solid support.
On the contrary, the inactivating effect of the methanol is likely to be produced by the direct interaction between the alcohol and the external aminoacidic residuals of the enzyme. This effect can be reduced using alcohols with higher chain-length as acyl receptors (data not shown). Alternatively, further enhancements of the process could be obtained by suitable changes in the biocatalyst structure and conformation, to increase the chemical stability of the enzyme.

4. References

Cardone M., M.V. Prati, V. Rocco, M. Seggiani, A. Senatore and S. Vitolo, 2002, Brassica carinata as an alternative oil crop for the production of biodiesel in Italy: engine performance and regulated and unregulated exhaust emissions, Environ. Sci. Technol., 36, 4656.

Dossat V., D. Combes, and A. Marty, 2002, Lipase-catalysed transesterification of high oleic sunflower oil, Enz. Microb. Technol., 30, 90-94.

Du W., Y. Xu and D. Liu, 2003, Lipase-catalysed transesterification of soya bean oil for biodiesel production during continuous batch operation, Biotechnol. Appl. Biochem., 38, 103.

Fukuda H., A. Condo, and H. Noda, 2001, Biodiesel fuel production by transesterification of oils, J. Biosci. Bioeng., 92(5), 405.

Hsu A.F., K. Jones, T.A. Foglia and W.N. Marner, 2002, Immobilized lipase-catalysed production of alkyl esters of restaurant grease as biodiesel, Biotechnol. Appl. Biochem., 36, 181.

Iso M., B. Chen, M. Eguchi, T. Kudo and S. Shrestha, 2001, Production of biodiesel fuel from triglycerides and alcohol using immobilized lipase, J. Mol. Cat. B: Enzymatic, 16, 53.

Reslow, M., P. Adlercreutz and B. Mattiasson, 1987, Organic solvent for bioorganic synthesis. 1. Optimization of parameters for a chymotrypsin-catalized process. Appl. Microbiol. Biotechnol., 26, 1.

Soumanou M.M., and U.T. Bornscheuer, 2003, Improvement in lipase-catalyzed synthesis of fatty acid methyl esters from sunflower oil, J. Biosci. Bioeng., 92(5), 405.

Shah S., S. Sharma and M.N. Gupta, 2004, Biodiesel preparation by lipase-catalysed transesterification of Jatropha oil, Energy and Fuels, 18, 154.

Velivety, R.H., P.J. Halling and A.R. Macrae, 1992, Reaction rate with lipase catalyst shows similar dependence on water activity in different organic solvents. Biochim. Biophys. Acta, 1118, 218.

Wang, W.G., D.W. Lyons, N.N. Clark and M. Gautam, 2000, Emissions from nine heavy trucks fuelled by diesel and biodiesel blend without engine modification, Environ. Sci. Technol., 34, 933.

Watanabe, Y, Y. Shimada, A. Sugihara and Y. Tominaga, 2001, Enzymatric conversion of waste edible oil to biodiesel fuel in a fixed-bed bioreactor, JAOCS, 78, 7.