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Kinetics of enzymatic esterification of glycerol and free fatty acids in crude Jatropha oil by immobilized lipase from *Rhizomucor miehei*

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ABSTRACT

Enzymatic neutralization is a recent research focus due to an increasing awareness of environmental problems caused by conventional oil refining. This study investigated the kinetics of enzymatic neutralization in crude Jatropha oil utilizing an immobilized lipase from *Rhizomucor miehei*. Free fatty acids, in particular oleic acid were esterified with glycerol. The reaction seems to follow a multisubstrate Ping Pong mechanism with competitive inhibition by the acyl acceptors (mono-, diacylglycerides and glycerol). Free fatty acid content did not affect lipase activity within the ranges investigated. The kinetic parameters were determined and showed that enzyme affinity is much higher to glycerol than to free fatty acids. These observations were supported by the fact that the optimum glycerol level is rather small while the reaction rate increases with increasing free fatty acid concentration.

1. Introduction

Jatropha curcas L. is a tropical plant of the genus *Euphorbiaceae*. The plant is rather non-demanding – growing under various climatic conditions and able to survive in poor and stony soils, requesting only low amounts of water [1,2]. Jatropha seeds contain about 35% of oil. Due to several toxic constituents comprised, such as phorbol esters, lectin and phytate, Jatropha is not directly competing with food production [3]. Therefore, the plant is an alternative oil source for biodiesel production [4].

In order to prepare the crude oil for technical or power generation applications, oil purification by refining is essential. The conventional refining process includes degumming, neutralization, bleaching and deodorization and seeks for the removal of non-triglyceride impurities from the oil [5]. All these steps are linked to disadvantages concerning environmental, economic and energetic aspects including high oil losses and the consumption of high amounts of chemicals and water [6]. Thus, in particular for the sustainable application of Jatropha oil aligned alternatives to conventional pathways such as enzymatic neutralization with lipases are desired.

In presence of alcohols acting as acyl acceptor, lipases (triacylglycerol hydrolases E.C.3.1.1.3) are able to catalyze the esterification of free fatty acids (FFA) and thus the neutralization of crude oils. This reaction is commonly assumed to follow a Ping Pong mechanism [7–9]. A certain number of publications also suggest an ordered mechanism [10,11]. Since lipases catalyze not only the esterification reaction but also and even preferably the hydrolysis of triacylglycerides the reaction equilibrium has to be shifted toward the synthesis by the removal of water produced in the process [12,13].

Previous research activities focussed on the investigation of enzymatic esterification reactions in model systems, utilizing different acids and alcohols. Usually, esterification reactions were performed in organic solvents, such as *n*-hexane, *n*-heptane or *tert*-butanol [8,14,15]. Only a few studies also considered solvent-free esterification of free fatty acids e.g. [7,16,17].

This study deals with a solvent-free neutralization of crude Jatropha oil via esterification of FFAs (mainly oleic acid) with glycerol catalyzed by a carrier-bound lipase from *Rhizomucor miehei*. Glycerol level in the reaction medium is known to be a crucial process parameter: on the one hand certain amounts of glycerol are needed to push the reaction equilibrium from hydrolysis toward esterification reaction, on the other hand alcohols are known to inhibit lipase activity [18,19].

Up to now, most of the studies investigating solvent-free systems dealt with a model reaction examining the esterification of

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two distinct substrates. For example, Wang et al. [20] studied the kinetics of enzymatic esterification of palmitic acid and starch in a solvent free system concluding that the reaction follows a Ping Pong Bi Bi mechanism.

Phuah et al. [21] investigated the hydrolysis of palm oil catalyzed by *R. miehei* lipase in solvent-free system reporting that the reaction follows a Ping Pong Bi Bi mechanism with competitive inhibition by water. However, there is no data available concerning the kinetics of solvent-free lipase-catalyzed esterification reactions carried out in crude oil displaying a rather complex reaction system.

For any design of large scale enzymatic neutralization processes it is necessary to generate deep knowledge of the reaction system, especially with regard to kinetic parameters. Besides, the evaluation of the process will only be realistic and economically feasible if performed in the later matrix, namely crude oil. Therefore, the objectives of this study comprise the investigation of these parameters for *R. miehei* lipase in esterification reaction of glycerol with FFAs in crude Jatropha oil. Based on these findings a simplified kinetic model describing the acid value reduction should be established. It is of profound interest to investigate lipase inhibition by the alcohol and to determine if there is also an influence of the second substrate, namely the FFAs, on the reaction rate.

2. Materials and methods

2.1. Raw materials and chemicals

Jatropha seeds were obtained from Rajasthan (India). The seeds were stored at 14 °C until further processing. Crude Jatropha oil was obtained by screw-pressing of the seeds and was stored at 0 °C until use.

Lipozyme® RM IM (275 IUN/g) was purchased from Novozymes A/S (Bagsvaerd, Denmark). The enzyme is immobilized by adsorption on macroporous anion exchange resin. Analytical grade glycerol (free of water) was obtained from Merck KGaA (Grafing, Germany). Oleic acid added to the crude oil was retrieved from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

2.2. Enzymatic neutralization

Prior to the neutralization experiments, the acid value of the oil was adjusted. The crude oil contained a FFA content of 0.17 mol/l. Higher acid values of 0.23 and 0.33 mol/l were obtained by the addition of oleic acid. To decrease the acid value a caustic neutralization was performed (Table 1). Neutralization reactions were performed in heated double-wall reactors. 100 g of crude Jatropha oil exhibiting different free fatty acid contents were mixed with various amounts of glycerol (Table 1) by a magnetic stirrer at 300 rpm (optimum stirring rate found in a previous study [22]) in order to obtain a homogenous mixture of the components. The reaction mixture was heated to 60 °C. To start the esterification reaction 1% (w/w) of immobilisate (Lipozyme® RM IM) was added. Water was eliminated from the reaction system by constant nitrogen stripping (300 l_n/h) in order to suppress reverse reaction. Lipase from *R. miehei* remains highly active at water activity below 0.0001 [23]. Initial water activity of crude Jatropha oil was 0.370 and was decreased to 0.159 after 8 h, thus high lipase activity throughout the whole reaction time could be expected.

Samples were taken after a reaction time of 4, 5, 6, 7 and 8 h. After sampling the acid value was determined following the procedures described in Section 2.4. Experiments were performed in duplicate. The initial reaction rate was calculated from the initial slope of the curve.

Table 1
Parameters varied in order to investigate lipase inhibition in enzymatic neutralization of crude Jatropha oil in dependence of the initial free fatty acid and glycerol content in the reaction system.

Parameter	Levels investigated
Free fatty acid content (M)	0.07-0.10-0.13-0.17-0.20-0.23-0.33
Glycerol content (M)	0.08-0.1-0.2-0.3-0.4-0.5

2.3. Kinetic investigation of the inhibition of lipase activity by the acyl group acceptors and free fatty acids

Kinetic experiments were carried out following the procedure described in Section 2.2. Experiments were conducted by maintaining the concentration of one of the substrates constant and varying the concentration of the other and vice versa (Table 1). The plots shown in the present work were constructed from all the experimentally determined reaction rates utilizing Lineweaver–Burk reciprocal analysis.

Kinetic parameters were determined graphically. The general rate equation for the Ping Pong Bi Bi mechanism with inhibition by one substrate is [24]:

$$V = \frac{V_{max}[A][B]}{K_{m(COOH)}[B](1 + ([B]/(K_{i(OH)}))) + K_{m(OH)}[A] + [A][B]} \quad (1)$$

and for the Ordered Bi Bi mechanism [24]:

$$V = \frac{V_{max}[A][B]}{K_{i(OH)}K_{m(OH)} + K_{m(COOH)}[B] + K_{m(OH)}[A] + [A][B]} \quad (2)$$

where V is the initial reaction rate; V_{max} is the maximum reaction rate; $[A]$ is the acid concentration and $[B]$ the alcohol concentration; $K_{m(COOH)}$ and $K_{m(OH)}$ are the Michaelis Menten constants of acids and alcohol; $K_{i(OH)}$ is the inhibition coefficient of the alcohol. However, the enzymatic esterification of fatty acids and glycerol is a much more complex process, being a multi-substrate reaction. The reaction follows a multi-substrate multi-product mechanism, since it involves the formation of mono-, di- and triacylglycerides [7]. Therein, the product of one step can also react as the substrate for the subsequent step.

To obtain a simplified kinetic model appropriate only for the kinetic description of free fatty acid reduction in crude Jatropha oil the kinetic constants $K_{m(OH)}$ and $K_{i(OH)}$ do not only refer to the glycerol, but constitute more general constants comprising the combined influence of glycerol and also mono- and diacylglycerides on the reaction rate. V_{max} was determined as the intercept of the lines obtained for the higher substrate concentrations. K_m values were calculated by the determination of the apparent values of V_{max} and K_m according to:

$$V_{max(app)} = -\left[\frac{K_{m(OH)} * V_{max(app)}}{c_{(OH)}}\right] + V_{max} \quad (3)$$

$$K_{m(app)} = K_{m(COOH)}\frac{c_{(OH)}(1 + c_{(OH)}/K_{i(OH)})}{K_{m(OH)} + c_{(OH)}} \quad (4)$$

The apparent values of V_{max} and K_m are the values they appear to have when measured in the presence of an inhibitor.

2.4. Evaluation of the neutralization reaction

To evaluate the enzymatic neutralization of crude Jatropha oil the acid value was determined according to the DGF-Einheitsmethode C-V2 (06) [25] by titration of the oil sample with 0.1 M KOH. A definite amount of oil (app. 1 g) was dissolved in 50 ml ethanol:diethyl ether mixture (1:1 vol.%). Phenolphthalein was added as an indicator and the sample was titrated to the transition point with 0.1 M KOH.