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A predictive kinetic study of lipase-catalyzed ethanolysis reactions for the optimal reutilization of the biocatalyst

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ABSTRACT

A new application of the kinetics modeling for the optimal reutilization of an immobilized lipase from *Pseudomonas cepacia* in the ethanolysis of vegetable oils is presented. Two different rate expressions were explored to take into account the lipase inactivation. The methodology developed is based on the utilization of the pseudo reaction time that indicates how much longer the reaction mixture must remain in the reactor (actual reaction time) to achieve the conversion that would have been achieved if the enzyme had not been partially deactivated (pseudo reaction time).

An initial batch of lipase was employed in 15 consecutive trials in order to quantitatively characterize the process over a range of lipase activity and to validate the ability of the methodology utilized to describe the kinetics of both ethanolysis and deactivation. Then, the model developed was employed to predict the time necessary to attain a desired conversion in subsequent reaction cycles in both, trials with the same batch of lipase with different degree of inactivation, and trials with a new batch of immobilized lipase with different specific activity.

The reaction times predicted to attain a 38% disappearance of glyceryl ester bonds were experimentally verified by carrying out the corresponding ethanolysis reactions of 100 g of sunflower oil. The agreement between the desired and experimentally attained conversions achieved validates the methodology developed to estimate reaction time in lipase-catalyzed ethanolysis reactions.

1. Introduction

The lipase from *Pseudomonas cepacia* (recently reclassified as *Burkholderia cepacia*) has been widely used in the hydrolysis and transesterification of triglycerides and in the enantioselective synthesis and hydrolysis of a variety of esters [1–5].

Lipase-catalyzed ethanolysis of fats and oils have been widely used for production of pure monoacylglycerols [6], highly purified concentrates of eicosapentaenoic and docosahexaenoic acids [7], structured lipids [8], bio-fuel [9], and selective recovery of minor components found in fats and oils deodorizer distillates [10].

Several kinetic models have been developed to describe lipase-catalyzed hydrolysis and ethanolysis reactions [4,11–13,14]. These models describe the rate of release of fatty acid residues from the precursor acylglycerol and the rate of formation of the different acylglycerol species. Some of the described models incorporate enzyme deactivation term to evaluate the decrease of activity of the lipase during the course of the reaction. The rate expression obtained permit one to evaluate the rates of release of the different fatty acid residues present in the precursor oil, determine the preference of the lipase for *sn*-1,3 versus *sn*-2 fatty acid residues, and also provides mechanistic information concerning lipase-catalyzed reactions.

Loss of enzyme activity with time is the deactivation of the enzyme as time elapses as a consequence of thermal effects and alcohol concentration. Knowledge of the rate law governing the deactivation process is often important in modeling enzyme-catalyzed processes and that would be of interest in process design. One of the problems that arise when one tries to evaluate the loss of enzyme activity in batch reactors is the fact that the time of the reaction and the time at which the set of experiments is started are equivalent. This fact does not permit one to separate the rate of the reaction from the rate at which the enzyme is losing activity because both processes occur simultaneously. One strategy to overcome this problem is to carry out several cycles of reaction with the same batch of lipase. Hence, it is possible to distinguish between the time characteristic of the ethanolysis reaction (reaction time) and that corresponding to lipase deactivation (the time elapsed since the start of the first experiment). Each cycle involves the same elapsed time for the ethanolysis reaction but this elapsed time will differ from the time during which the lipase has been susceptible to deactivation. Thus, the mathematical model used in

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the analysis of the data should account for the effect of deactivation of the enzyme in the combined rate expressions for the rate of consumption of fatty acid residues and the rates of release of the corresponding fatty acid ethyl esters.

In this work, the ethanolysis of sunflower oil catalyzed by *P. cepacia* lipase is studied. The primary thrust of this research was to validate a model for elucidating rate expressions for both the ethanolysis reaction and deactivation of the enzyme, and to develop a methodology to assess the potential for reuse of the immobilized enzyme.

A modified version of the generalized Michaelis–Menten rate expressions, proposed by Torres and Hill [3] for the ethanolysis of menhaden oil, was employed to describe the kinetics of the ethanolysis reaction. The effect of enzyme deactivation was taken into account exploring two different deactivation rate expressions similarly to those proposed by Malcata et al. [14]. By using this methodology, a mathematical model with high correlative capability of the experimental data was developed. In addition, these rate expressions were used to predict how long the reaction mixture must remain in the reactor as a consequence of: (1) the specific activity of the particular batch of lipase employed and (2) its partial inactivation. These predictions result decisive to optimize the reutilization of the biocatalyst and to achieve similar reaction conversions regardless the residual lipase activity or the batch of lipase utilized. The predicted reaction times were utilized several times with the same batch of immobilized lipase to experimentally demonstrate both the feasibility of the methodology developed and the accuracy of kinetic rate expressions utilized.

2. Materials and methods

2.1. Materials

Sunflower oil, with less of <0.5% (w/w) of humidity, according to the vendor specifications, was utilized as source of triacylglycerols in the present study. *P. cepacia* (PS) was obtained from Amano (Lombard, IL). All solvents used were HPLC grade from Lab scan (Dublin, Ireland). Ethanol absolute (water content <0.1%, w/w) was obtained from Panreac (Barcelona, Spain) and was dried with molecular sieves 4 Å from Sigma–Aldrich (St. Louis, MO, USA). The rest of the materials were used without further purification.

2.2. Methods

2.2.1. Lipase immobilization

500 mg of lipase PS were solved in 5 ml of sodium phosphate buffer 0.2 M, pH 7. This mixture was added dropwise to 500 mg of celite 545 coarse from Fluka (St. Louis, MO, USA) in a 120-ml flask and the final mixture was placed in a orbital shaker 30 min at 200 rpm. Then, 20 ml of chilled acetone were added. The suspension was allowed to stand in the mentioned orbital shaker for another 5 min at 200 rpm and was vacuum filtered. Finally, the immobilized lipase was washed with 5 ml of chilled acetone and dried by vacuum filtration. During the washing procedure null loss of lipase activity was observed.

2.2.2. Ethanolysis reaction

10 g of a mixture of sunflower oil containing 8% (w/w) of hexadecane (internal standard) and 1.5 g of ethanol were placed in a 120-ml flask and mixed by swirling. After 0.5 g of the immobilized lipase PS was added, the flask was stoppered and placed in an orbital shaker (200 rpm) at 40 °C. Samples (100 μL) were withdrawn periodically, and the flasks were resealed after each sampling. Unless other thing is stated, trials were allowed to proceed for 6 h.

Four identical batches were simultaneously prepared and allowed to react concurrently under identical conditions to ensure that sufficient lipase would always be available for use in subsequent cycles. Only one of the batches was used for sampling and the other batches were utilized as reservoir of lipase with identical reaction and recovery history. Hence, any lipase that might have been lost in the procedural steps involved in a particular cycle (removing aliquots for analysis, recovery by filtration, washing, etc.,) could be easily replaced with the mentioned three batches that had been processed simultaneously in an identical manner. Thus, identical amounts of lipase could be utilized in all cycles.

In order to obtain a broad range of experimental data to fit adequately the proposed kinetics model, the same batch of lipase was employed in 15 consecutive reaction cycles. Each experimental reaction cycle was carried out up to a desired time; the immobilized lipase was then recovered by filtration under vacuum and utilized as a biocatalyst in the subsequent reaction cycle. This procedure permits one to carry out numerous ethanolysis reactions with the same batch of lipase but with different degree of inactivation, and thus provide appropriate information to fit the parameters of the kinetic model.

2.2.3. Study of lipase stability

In order to study the stability of the immobilized lipase PS under the experimental reaction conditions, several cycles of the ethanolysis reaction were carried out with the same initial mixture of sunflower oil and ethanol. The lipase was first submerged in a fresh reaction mixture for 5 min to allow the liquid to wet the sample and then dried at room temperature under vacuum for 2 min. One washing with acetone was effected to eliminate the reaction mixture from the biocatalyst. Then, the lipase was again dried under vacuum for 1 min. Finally, the lipase was weighed to ensure that no traces of either reaction mixture or acetone were present. This lipase was subsequently used as a catalyst for a total of 15 charges of reactants according to the procedure described in the previous section. For each charge, the lipase was used to catalyze the reaction for 6 h. Then, it was recovered at room temperature using the washing procedure described above and was reused with the next charge to the batch reactor. The lipase recovery process took approximately 10 min.

2.2.4. Analysis of reaction products (gas chromatography)

The samples (100 μL) were mixed with 2 ml of chloroform ethanol 2:1 (v/v) and immediately filtered with a 0.45 μm Sartorius (Goettingen, Germany) nylon syringe filter. Samples were then dried with sodium sulfate. Aliquots of the final transparent solution (250 μL) were diluted with 750 μL of hexane. 1 μL of the diluted sample was injected into an Agilent (Avondale, PA) gas chromatograph (6890N Network GC System) coupled to an autosampler (Agilent 7683B). The capillary column was a 30 m HP-88 (Avondale, PA) (0.25 mm i.d.). The temperatures of the injector and detector were both 220 and 250 °C, respectively. The temperature program was as follows: starting at 100 °C and then heating to 180 °C at 20 °C/min; followed by heating from 180 to 220 °C at 15 °C/min. The final temperature (220 °C) was held for 30 min. Identification of the various free fatty acids was based on a PUFA No 3 standard (#4-7085) obtained from Supelco.

3. Mathematical modeling

3.1. Reaction rate for the ethanolysis reaction: uniresponse model

Rate expressions based on a generalized Michaelis–Menten mechanism for the ethanolysis reaction were utilized (Fig. 1). A ping-pong mechanism controlled by the rate of deacylation of the