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The effect of substrate concentrations on the production of biodiesel by lipase-catalysed transesterification of vegetable oils

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Abstract: All the kinetic studies, found in the literature, on the production of biodiesel (fatty acids methyl esters) considered the esterifications of free fatty acids rather than the transesterification of the vegetable oil itself. The main industrial interest, however, is for the production of biodiesel with the triglyceride (oil) being the substrate. A mathematical model taking into account the mechanism of the methanolysis reaction starting from the vegetable oil as substrate, rather than the free fatty acids, has been developed. From the proposed model equation, the regions where the effect of alcohol inhibition fades, at different substrate concentrations, were identified. The proposed model equation can be used to predict the rate of methanolysis of vegetable oils in a batch or a continuous reactor and to determine the optimal conditions for biodiesel production.
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Keywords: biodiesel; lipase; kinetics; transesterification; vegetable oils

NOTATION

[A] Alcohol concentration ($mol\,cm^{-3}$)
[Bd] Fatty acid ester (i.e. biodiesel) concentration ($mol\,cm^{-3}$)
[E] Free enzyme concentration ($mol\,cm^{-3}$)
[E_o] Total enzyme concentration ($mol\,cm^{-3}$)
[F] Free fatty acid concentration ($mol\,cm^{-3}$)
[G] Glycerol concentration ($mol\,cm^{-3}$)
K_A Binding constants for the alcohol ($mol\,cm^{-3}$)
K_F Binding constants for the fatty acid ($mol\,cm^{-3}$)
K_{IA} Inhibition constant for the alcohol ($mol\,cm^{-3}$)
K_{IF} Inhibition constant for the fatty acid ($mol\,cm^{-3}$)
K_{IS} Apparent inhibition constant for the substrate ($cm^3\,mol^{-1}$)
K_S Binding constants for the substrate ($mol\,cm^{-3}$)
[S] Substrate concentration (i.e. the ester bond on the triglyceride) ($mol\,cm^{-3}$)
V_{max} Maximum reaction rate (min^{-1})
[W] Water concentration ($mol\,cm^{-3}$)
υ: Initial reaction rate ($cm^3\,mol^{-1}\,min^{-1}$)

INTRODUCTION

Alternative fuels for diesel engines are becoming increasingly important due to diminishing petroleum reserves and the environmental consequences of exhaust gases from petroleum-fuelled engines. A number of studies have focused on the possibility of using vegetable oils as starting material for biodiesel production.[1–12] Biodiesel is defined as monoalkyl fatty acid esters (preferentially methyl and ethyl esters) and represents a promising alternative fuel for use in compression-ignition (diesel) engines.[9] Fatty acid esters are formed by transesterification, also called alcoholysis, of vegetable oils. The process is similar to hydrolysis, except that an alcohol is employed instead of water. Suitable alcohols include methanol, ethanol, propanol, butanol, and amyl alcohol. Methanol and ethanol are utilised most frequently, especially methanol because of its low cost and its physical and chemical advantages.[13] This process has been widely used to reduce the viscosity of triglycerides, thereby enhancing the physical properties of renewable fuels to improve engine performance.[13] Fukuda *et al*.[13] compared the properties of biodiesel fuels, produced from various vegetable oils, with those of petroleum diesel. They showed that biodiesel fuels have viscosities close to those of diesel. The volumetric heating values are a little lower, but they have high cetane numbers and flash points. Since the characteristics of biodiesel are generally similar to those of diesel, the former is a strong candidate to replace diesel. Among the attractive features of biodiesel fuel are: it is plant, not petroleum, -derived and as such it is less toxic and comes from renewable sources, it is biodegradable; and relative to conventional diesel, its combustion products have reduced levels of particulates, carbon

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oxides, sulfur oxides and, under some conditions, nitrogen oxides.[13,14]

In conventional chemical processing, synthesis of these esters is achieved by an alkaline transesterification reaction which has several drawbacks: it is energy intensive, recovery of glycerol is difficult, the alkaline catalyst has to be removed from the product, alkaline wastewater requires treatment, and free fatty acids and water interfere with the reaction. In addition, alkaline transesterification has low selectivity, leading to undesirable side reactions.[9,13] As an alternative, lipase has been used as a biocatalyst for the synthesis of biodiesel from natural oils.[9–13] Lipase is able to efficiently catalyse the transesterification of triglycerides in non-aqueous systems to produce biodiesel. Lipolytic transesterification can overcome the problems of conventional chemical processes, mentioned above. In particular, glycerol can be easily recovered without any complex processing, and free fatty acids contained in waste oils can be completely converted to methyl esters.[13]

In order to identify the optimal conditions for the lipase-catalysed transesterification, it is essential to understand the kinetics of this reaction. Although the application of lipase in the production of biodiesel from vegetable oils has been thoroughly addressed in the literature,[9–11,15–22] these studies were purely experimental. All the kinetic studies found in the literature considered the esterifications of free fatty acids rather than the transesterification of the vegetable oil itself.[12,23–28] It is therefore desired to develop a reliable kinetic model for the transesterification of vegetable oil to produce biodiesel using lipase.

KINETIC MODEL

Most of the earlier kinetic studies on lipase-catalysed esterifications of short-chain alcohols and long-chain fatty acids are described by a Ping-Pong kinetic model[12,23–28] with competitive inhibition by the alcohol, as given below:

$$\upsilon = \frac{V_{\max}}{1 + \dfrac{K_F}{[F]}\left[1 + \dfrac{[A]}{K_{IA}}\right] + \dfrac{K_A}{[A]}} \qquad (1)$$

where υ is the initial reaction rate, $V_{\max}$ is the maximum reaction rate, K_F and K_A are the binding constants for the fatty acid (F) and the alcohol (A), and K_{IA} is the inhibition constant for the alcohol. Krishna and Karanth[15] have modified the above model by incorporating a Ping-Pong Bi-Bi mechanism with inhibition by both the substrates for which the expression for the reaction rate is given as:

$$\upsilon = \frac{V_{\max}[F][A]}{[F][A] + K_A[A]\left[1 + \dfrac{[A]}{K_{IA}}\right] + K_F[F]\left[1 + \dfrac{[F]}{K_{IF}}\right]} \qquad (2)$$

where K_{IF} is the inhibition constant for the acid, other notations being the same as in Eqn (1). However, it was proven experimentally that the substrate (S) (i.e. the ester bond on the triglyceride) does inhibit lipase-catalysed reactions of vegetable oils.[17,32] Therefore, the modification of Krishna and Karanth[15] is not applicable for the transesterifications of vegetable oils.

Equations (1) and (2) describe the initial reaction rate in the absence of any product. Janssen *et al*.[29] derived an equation to be used when the water, [W], is taken as one of the products which inhibits the reaction. This modification is applicable when free fatty acids are considered as the substrate. However, when the substrate is the triglyceride, water is replaced with monoglyceride, diglyceride or glycerol, and, unlike water which is usually present in the reaction medium at time zero, these products are not present at time zero. Therefore, the product inhibition is neglected when considering the initial rate of reaction.

It is important to note the main industrial interest is for the production of biodiesel (fatty acid methyl esters) with the triglyceride (oil) being the substrate. Hence, a liberating step of fatty acids from triglyceride should precede the esterification of free fatty acids. For simplicity, Michaelis–Menten kinetics will be used for the preliminary hydrolysis step to liberate the free fatty acids from the oil as used by Knezevic *et al*.[30] and Mukataka *et al*.[31] And, as described earlier, the most suitable model for esterification of the free fatty acids is the Ping-Pong kinetic model with competitive inhibition by the alcohol. It should, however, be realised that more accurate, but more complex, models for the liberation of free fatty acids from vegetable oils are presented in the literature.[32–35] Figure 1 shows a schematic diagram of the mechanismic steps proposed for the esterification of triglycerides with lipase. As proposed by Malcata *et al*.[36,37] all steps, except deacylation of the lipase, are assumed to be in quasi-steady state. With this proposed mechanism and assumptions, the following equations can be

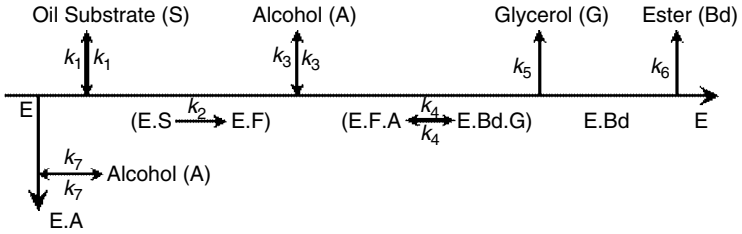

Figure 1. Graphical representation of the mechanismic steps of triglyceride ester-bond transesterification.

written:

$$[\mathrm{E.S}] = \frac{k_1[\mathrm{E}][\mathrm{S}]}{k_{-1} + k_2} \quad (3)$$

$$[\mathrm{E.F}] = \frac{k_2[\mathrm{E.S}] + k_{-3}[\mathrm{E.F.A}]}{k_3[\mathrm{A}]} \quad (4)$$

$$[\mathrm{E.F.A}] = \frac{k_3[\mathrm{E.F}][\mathrm{S}] + k_{-4}[\mathrm{E.Bd.G}]}{k_{-3} + k_4} \quad (5)$$

$$[\mathrm{E.Bd.G}] = \frac{k_4[\mathrm{E.F.A}]}{k_{-4} + k_5} \quad (6)$$

$$[\mathrm{E.Bd}] = \frac{k_5[\mathrm{E.Bd.G}]}{k_6} \quad (7)$$

$$[\mathrm{E.A}] = \frac{k_7[\mathrm{E}][\mathrm{A}]}{k_{-7}} \quad (8)$$

$$[\mathrm{E_o}] = [\mathrm{E}] + [\mathrm{E.S}] + [\mathrm{E.F}] + [\mathrm{E.F.A}] + [\mathrm{E.Bd.G}] + [\mathrm{E.Bd}] + [\mathrm{E.A}] \quad (9)$$

where, E_o and E are total and free enzyme, respectively, F is fatty acid, S is the substrate (i.e. the ester bond on the glyceride), A is alcohol, G is glycerol and Bd is the fatty acid ester (i.e. biodiesel).

From the above equations, the rate of transesterification of triglycerides can now be expressed as given in Eqn (10). Table 1 shows the definition of the parameters in Eqn (10).

$$\upsilon = k_6[\mathrm{E.Bd}] = \frac{V_{\max}[\mathrm{S}]}{1 + K_{\mathrm{IS}}[\mathrm{S}] + \frac{K_{\mathrm{S}}}{[\mathrm{S}]}\left(1 + \frac{[\mathrm{A}]}{K_{\mathrm{IA}}}\right) + \frac{K_{\mathrm{A}}}{[\mathrm{A}]}} \quad (10)$$

The above equation describes the rate of transesterification reaction of triglycerides using lipase. The equation was derived from the mechanismic steps of the reaction shown in Fig. 1. Comparison of Eqn (10) and the earlier kinetic rate equation of lipase-catalysed esterifications of free fatty acids[12,23–28] (Eqn (1)) shows that $K_{\mathrm{IS}}[\mathrm{S}]$ is an extra term. This term is associated with the liberation of free fatty acids from the substrate (triglyceride). Therefore, Eqn (10) describes transesterification of triglyceride better than the previous models found in the literature that consider the substrate as the free fatty acids rather than the triglyceride.[12,23–28] The mechanismic steps do not show any substrate inhibition, therefore the constant K_{IS} is considered as an apparent inhibition of the substrate, as it results in reduction of the initial rate of reaction.

MODEL VALIDATION

The significance of the modification in the proposed model equation (Eqn (10)), in comparison with the previous models found in the literature,[3,12,15,23–28] was not evident in the previous works on biodiesel production from vegetable oils by lipase.[9–11,15–22] That is because in all those studies the concentration of substrate (oil) is considered constant, and variation of methanol concentration was the only parameter considered, and, by keeping the concentration of substrate constant, Eqn (10) will have the same general form as Eqn (1). In this study, it is proposed to consider the changes of both methanol and substrate concentrations to

Table 1. Definition of the kinetic parameters found in Eqn (10)

Parameter	Definition
$V_{\max}$	$k_6[\mathrm{E_o}]$
K_{m}	$\frac{K_{\mathrm{ES}} + K_{\mathrm{ES}}K_{\mathrm{EF}} - K^*_{\mathrm{EFA}}K_{\mathrm{ES}} - K^*_{\mathrm{EFA}}K_{\mathrm{ES}}K_{\mathrm{EF}} - K^*_{\mathrm{EF}}K_{\mathrm{EFA}}}{K_{\mathrm{Bd}}K_{\mathrm{EBdG}}K_{\mathrm{EFA}}K_{\mathrm{EF}}K_{\mathrm{ES}}}$
K_{IS}	$\frac{[K_{\mathrm{BD}} + K_{\mathrm{EBdG}}(K^*_{\mathrm{EF}} + K_{\mathrm{Bd}}K_{\mathrm{EBdG}}K_{\mathrm{EF}} + K_{\mathrm{EBdG}} + 1)] \cdot (K_{\mathrm{ES}} + K_{\mathrm{ES}}K_{\mathrm{EF}} - K^*_{\mathrm{EFA}}K_{\mathrm{ES}} - K^*_{\mathrm{EFA}}K_{\mathrm{ES}}K_{\mathrm{EF}} - K^*_{\mathrm{EF}}K_{\mathrm{EFA}})}{K_{\mathrm{ES}} + K_{\mathrm{ES}}K_{\mathrm{EF}} - K^*_{\mathrm{EFA}}K_{\mathrm{ES}} - K^*_{\mathrm{EFA}}K_{\mathrm{ES}}K_{\mathrm{EF}} - K^*_{\mathrm{EF}}K_{\mathrm{EFA}}}$ $- \frac{K_{\mathrm{EFA}}K^*_{\mathrm{EF}}(K_{\mathrm{ES}} + K_{\mathrm{ES}}K_{\mathrm{EF}})}{K_{\mathrm{ES}} + K_{\mathrm{ES}}K_{\mathrm{EF}} - K^*_{\mathrm{EFA}}K_{\mathrm{ES}} - K^*_{\mathrm{EFA}}K_{\mathrm{ES}}K_{\mathrm{EF}} - K^*_{\mathrm{EF}}K_{\mathrm{EFA}}}$
K_{S}	$\frac{1 - K^*_{\mathrm{EFA}}}{K_{\mathrm{ES}} + K_{\mathrm{ES}}K_{\mathrm{EF}} - K^*_{\mathrm{EFA}}K_{\mathrm{ES}} - K^*_{\mathrm{EFA}}K_{\mathrm{ES}}K_{\mathrm{EF}} - K^*_{\mathrm{EF}}K_{\mathrm{EFA}}}$
K_{IA}	K_{EA}
K_{A}	$\frac{K_{\mathrm{EA}}K^*_{\mathrm{EF}}K_{\mathrm{EFA}}}{K_{\mathrm{ES}} + K_{\mathrm{ES}}K_{\mathrm{EF}} - K^*_{\mathrm{EFA}}K_{\mathrm{ES}} - K^*_{\mathrm{EFA}}K_{\mathrm{ES}}K_{\mathrm{EF}} - K^*_{\mathrm{EF}}K_{\mathrm{EFA}}}$
K_{ES}	$\frac{k_1}{k_{-1} + k_2}$
K_{EBdG}	$\frac{k_4}{k_{-4} + k_5}$
K_{Bd}	$\frac{k_5}{k_6}$
K_{EA}	$\frac{k_{-7}}{k_7}$
K_{EFA}	$\frac{k_3}{k_{-3} + k_4}$
K^*_{EFA}	$\frac{k_{-4}}{k_{-3} + k_4}$
K_{EF}	$\frac{k_2}{k_3}$
K^*_{EF}	$\frac{k_{-3}}{k_3}$

demonstrate the significance of our proposed model (Eqn (10)).

Soumanou and Bornscheuer[9] studied the effect of methanol content on the methanolysis of sunflower oil in organic solvent systems using two types of commercial immobilised lipases, namely *Rhizomucor miehei* lipase (RM) immobilised on ion-exchange resins and *Thermomyces lanuginosa* lipase (TL) immobilised on silica gel. The activity of RM and TL lipases were 0.63 and 0.16 kLU g^{-1}, respectively, where 1 LU is defined as the amount of enzyme which liberates 1 μmol methyloleate per minute at 30 °C. The reactions were performed as batch processes in 2 mL of *n*-hexane organic solvent in glass tubes containing different mixtures of sunflower oil/methanol and immobilised lipase. The reaction mixtures were incubated at 40 °C and agitated with magnetic stirring at 200 rpm. Samples were withdrawn at specific intervals and analysed using a gas chromatograph, equipped with a polar column. The initial rate of reaction was determined from the progressive production of methyl esters of fatty acids. The results of this study is the only one that shows the effect of oil content in addition to methanol content, since the mixture of oil/methanol was changed, rather than keeping the oil concentration constant and changing the amount of methanol, as has been done in other studies.

The results of Soumanou and Bornscheuer[9] presented in terms of conversion (%) versus methanol/oil (mol/mol) were represented as initial rate of reaction, υ (mol cm^{-3} min^{-1}), versus substrate concentration [S] (i.e. ester bond in the oil) and methanol concentration [A] (mol cm^{-3}) being the two variables considered. The fatty acids composition of sunflower oil[38] was used to determine the molecular weight of the substrate, found to be 278 g mol^{-1}. A multiple regression method, using MATLAB computer package, was applied to the modified experimental results to validate the model equation proposed in this study (Eqn (10)) and determine the reaction rate constant. The numerical values of the rate constants found in the proposed model equation (Eqn (10)) using immobilised lipase (RM) and lipase (TL) are presented in Table 2. The model equations, with the estimated rate constants, for the lipases RM and TL are given in Eqns (11) and (12), respectively.

$$\upsilon = \frac{0.414 \times [\mathrm{S}]}{1 + 0.13 \times [\mathrm{S}] + \dfrac{0.16}{[\mathrm{S}]}} \pm 0.00283 \times \left(1 + \frac{[\mathrm{A}]}{1.9}\right) + \frac{0.98 \times 10^{-4}}{[\mathrm{A}]} \quad (11)$$

$$\upsilon = \frac{0.197 \times [\mathrm{S}]}{1 + 0.13 \times [\mathrm{S}] + \dfrac{0.44}{[\mathrm{S}]}} \pm 0.00262 \times \left(1 + \frac{[\mathrm{A}]}{0.9}\right) + \frac{4.8 \times 10^{-4}}{[\mathrm{A}]} \quad (12)$$

Equations (11) and (12) are appropriate for predicting the initial rate of methanolysis of sunflower oil at any substrate and alcohol concentrations and can be used for the design of batch and continuous bioreactors and to determine the optimum operating conditions.

Table 2. Numerical values of the kinetic parameters found in Eqn (10)

Parameter	RM lipase	TL lipase
V_{max} (min^{-1})	0.414	0.197
K_{IS} (cm^3 mol^{-1})	0.13	0.13
K_S (mol cm^{-3})	0.16	0.44
K_A (mol cm^{-3})	0.98×10^{-4}	4.82×10^{-4}
K_{IA} (mol cm^{-3})	1.9	0.9

It was found that V_{max} for RM lipase is larger than that for TL lipase, therefore, RM lipase has higher methanolysis activity per kg of enzyme used. This result is expected as the activity of RM is higher than that of TL. Further, it can be noted that K_{IA} for RM lipase is larger than that for TL lipase and K_A for RM lipase is smaller than that for TL lipase. This proves that the inhibition of RM lipase with alcohol is stronger than that of TL lipase. These findings agree with the experimental results of Soumanou and Bornscheuer.[9]

To validate the derived reaction rate expressions, the experimental values of the initial rate of methanolysis

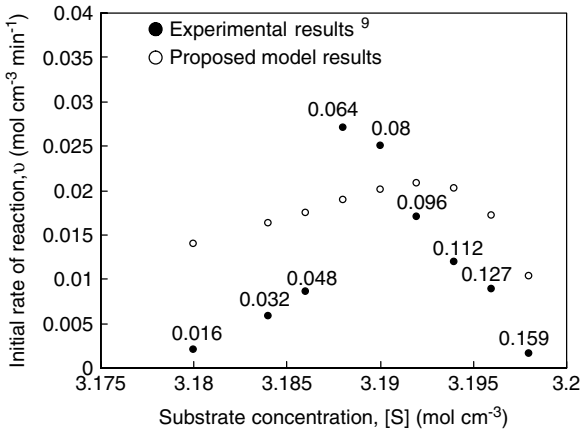

Figure 2. Comparison between the experimental values of the initial rate of methanolysis of sunflower oil using *Rhizomucor miehei* lipase immobilised on ion-exchange resins[9] and those predicted by Eqn (10) at different substrate concentrations.

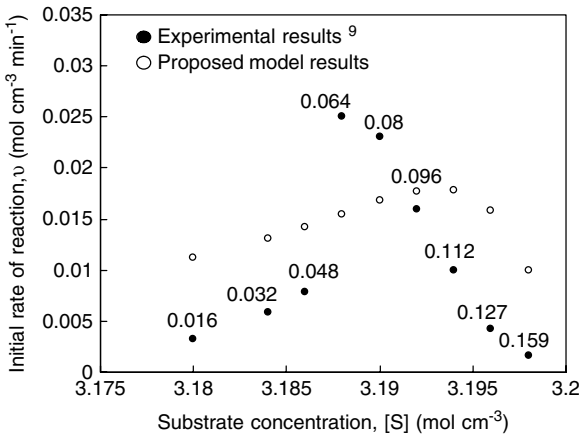

Figure 3. Comparison between the experimental values of the initial rate of methanolysis of sunflower oil using *Thermomyces lanuginosa* lipase immobilised on silica gel[9] and those predicted by Eqn (10) at different substrate concentrations.

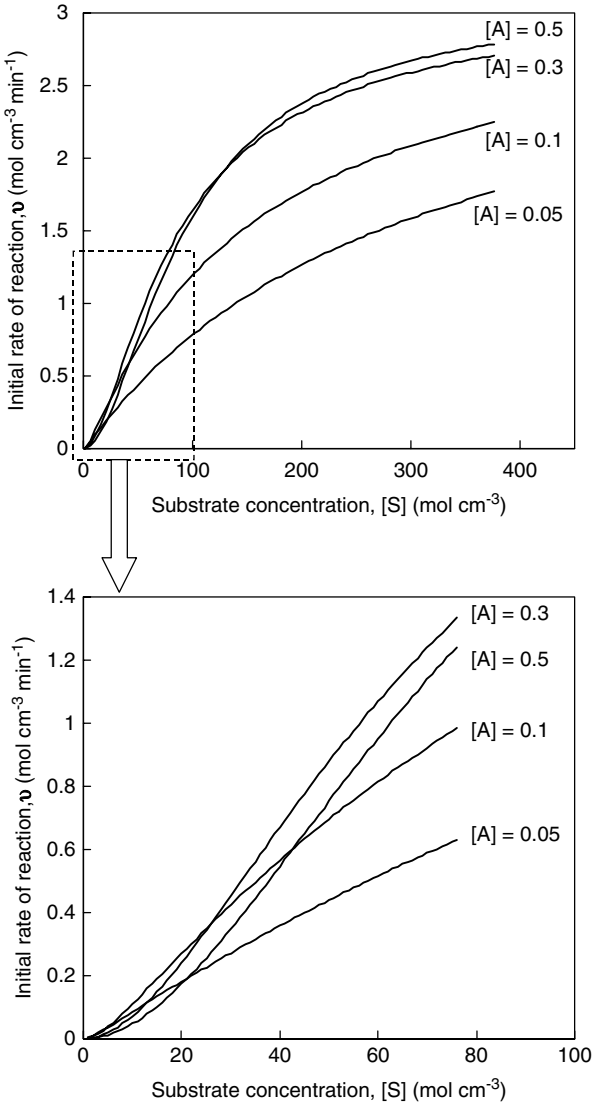

Figure 4. Effect of substrate concentration, [S], on the initial rate of methanolysis of sunflower oil using *Rhizomucor miehei* lipase immobilised on ion-exchange resins at different alcohol concentrations.

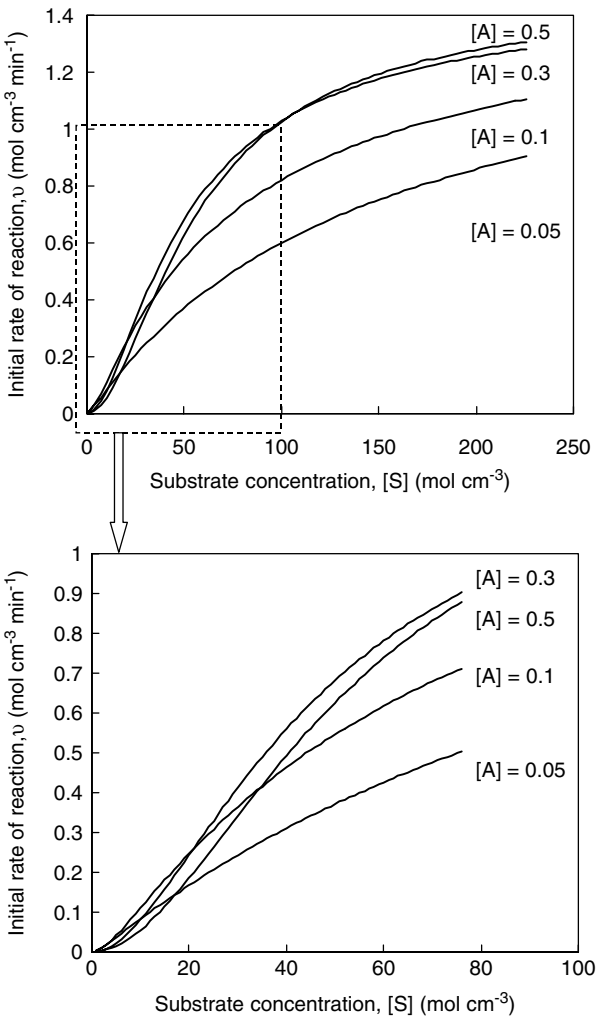

Figure 5. Effect of substrate concentration, [S], on the initial rate of methanolysis of sunflower oil using *Thermomyces lanuginosa* lipase immobilised on silica gel at different alcohol concentrations.

of sunflower oil using RM lipase[9] and those predicted by Eqn (11) at different substrate concentrations have been compared, and the results are shown in Fig. 2. Figure 3 shows the comparison between the experimental values of the initial rate of using TL lipase[9] and those predicted by Eqn (12). The experimental results shown in Figs 2 and 3 were deduced from different mole fractions of alcohol, which resulted in different concentrations of both alcohol and substrate. Therefore, it should be noted that each point in Figs 2 and 3 is taken at different concentrations of alcohol and substrate. The numerical values of the alcohol concentration ($\mathrm{mol\,cm^{-3}}$) at each point are shown above their respective data point in both figures.

It is clearly seen that Figs 2 and 3 do not show the sole effect of substrate concentration on the initial rate of reaction, which is the objective of our study. Therefore, Eqns (11) and (12) were used to show this effect at constant alcohol concentrations, and the results are shown in Figs 4 and 5 for RM lipase and TL lipase, respectively. It can be seen from Figs 4 and 5 that at high substrate concentrations, above 150 and 100 $\mathrm{mol\,cm^{-3}}$ for lipases RM and TL, respectively, the initial rate of reaction increases with substrate concentration and/or alcohol concentration. Hence, much higher alcohol concentrations are required for inhibition to take place at high substrate concentrations. This is explained by realising that the inhibition by alcohol is competitive, and increasing the substrate concentration reduces the probability of attachment of enzyme to the alcohol. Therefore, at high substrate concentrations, effectively all the enzyme is attached to the substrate, which is available in abundance. However, increasing the alcohol concentration to higher values may result in the onset of significant inhibition. At substrate concentrations below 30 and 20 $\mathrm{mol\,cm^{-3}}$ for lipases RM and TL, respectively, the optimum alcohol concentration is 0.1 $\mathrm{mol\,cm^{-3}}$. However, alcohol concentrations of 0.3 $\mathrm{mol\,cm^{-3}}$ become more effective at substrate concentrations of 30 and 20 $\mathrm{mol\,cm^{-3}}$ for lipases RM and TL, respectively. It is clear that the proposed model equation, Eqn (10), can be used to determine

the optimal substrate and alcohol concentrations and to design a batch or a continuous bioreactor.

CONCLUSION

A kinetic model based on the mechanism of the reaction for the lipase-catalysed methanolysis of vegetable oils has been proposed. The proposed model was verified with experimental results of the reaction of sunflower oil activated by two types of lipases, namely *Rhizomucor miehei* lipase (RM) immobilised on ion-exchange resins and *Thermomyces lanuginosa* lipase (TL) immobilised on silica gel. The rate constants in the mathematical model were determined numerically from the experimental results. There was a good agreement between the experimental results of the initial rate of reaction and those predicted by the model equations, for both enzymes. It was found that RM lipase has higher methanolysis activity per kg of enzyme in comparison with TL lipase. But the inhibition of RM lipase with alcohol was found to be stronger than that of TL lipase. The effect of changing substrate and alcohol concentrations on the initial rate of reaction has also been investigated. It was shown that at higher substrate concentration, the effect of alcohol inhibition tends to fade. The proposed model equation can be used to predict the rate of methanolysis of vegetable oils in a batch reactor or a continuous reactor and to determine the optimal conditions for biodiesel production.

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