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In situ monitoring of turbid immobilized lipase-catalyzed esterification of oleic acid using fiber-optic Raman spectroscopy

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ABSTRACT

Raman spectroscopy with a fiber-optic probe was used to monitor the immobilized *Candida antarctica lipase* B (Novozym 435)-catalyzed esterification of oleic acid with ethanol in iso-octane as an organic solvent. The effects of reaction temperature and molar ratio of substrates were studied. An optimal reaction rate was found at 60 °C, beyond which deactivation due to denaturing of the lipase was observed. The variation in molar ratio of substrates suggests that the esterification of oleic acid and ethanol proceeds via a *Ping-Pong Bi-Bi* mechanism with ethanol exhibiting an inhibitory effect. A good fit was obtained between the experimental results and the best-fit *Ping-Pong Bi-Bi* mechanism. This current work shows that fiber-optic Raman spectroscopy is indeed a suitable instrument to monitor immobilized lipase-catalyzed reaction in turbid organic systems *in situ*. Since this approach is reliable, simple to use, allows automatic data acquisition, and accurate, it should be applicable to the detailed kinetic analysis on other immobilized enzymatic reactions as well.

1. Introduction

Owing to the advantages offered by enzymes as catalysts in organic solvent, the interest in lipase-catalyzed reactions, such as esterification, inter-esterification, and trans-esterification, has increased significantly [1]. It has also prompted chemical industries to exploit the catalytic characteristics of lipases isolated from various microbial sources. Off-line chromatography is one of the most common analytical techniques employed to investigate the kinetics of enzymatic reactions. However, this conventional technique generally requires more sample preparation, longer analysis time, and it is also invasive.

Alternative analytical techniques, such as *in situ* spectroscopy are certainly preferred, since the progress of the reaction can be monitored in real time. In recent years, various *in situ* spectroscopies have been used to monitor enzymatic reactions. These included Fourier transform infrared spectroscopy (FTIR) [2], ultraviolet (UV) spectroscopy [3,4], nuclear magnetic resonance (NMR) spectroscopy [5,6], and Raman spectroscopy [7,8]. The use of Raman spectroscopy to monitor reactions in the aqueous phase is advantageous, since water is a poor Raman scatterer, and hence the vibrations of the organic functional groups are pronounced. Consequently, Raman spectroscopy readily allows detection of other constituents present in the aqueous system [9]. This is in contrast to infrared spectroscopy which suffers from the intense infrared bands of water, and thus identification and quantification of other constituents observed in aqueous system can be rather difficult.

In the present study, we investigate the use of fiber-optic Raman spectroscopy to monitor *in situ* the lipase-catalyzed synthesis of ethyl oleate by the esterification of oleic acid and ethanol in iso-octane. Since water is being formed as one of the products from this esterification reaction, Raman spectroscopy is probably the preferable choice. Since this is a multi-phase system, involving suspended immobilized enzymes, a flow-through Raman cell may not be appropriate. Instead, a Raman fiber-optic probe immersed into the reaction system may provide a better approach. The present contribution attempts to demonstrate the usefulness and the reliability of this approach to monitor turbid enzymatic systems and obtain quantitative information. The effects of reaction temperature and molar ratio of substrates are also studied and reported.

2. Materials and methods

2.1. Materials

All substrates (oleic acid and ethanol), solvent (iso-octane), and immobilized *Candida antarctica lipase* B (Novozym 435) were

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purchased from Sigma–Aldrich. All chemicals were of analytical grade and used as received.

2.2. Reaction temperature study

The standard reaction mixture consisted of 10 g of oleic acid, 1.95 g of ethanol and 0.4 g of Novozym 435 in 10 ml of iso-octane as an organic solvent. The molar ratio (mol ethanol/mol oleic acid) was fixed at 1.2 for all runs. The reaction mixtures were incubated at various different temperatures (25, 30, 35, 40, 50, 60, and 65 °C). The mixture was then agitated continuously at the speed of 200 rpm throughout the reaction course.

2.3. Molar ratio study

The standard reaction mixture consisted of 0.4 g *C. antarctica lipase* in 10 ml of iso-octane as the organic solvent at 40 °C. The reaction mixtures were reacted under different molar ratio of substrates. Two sets of molar ratio experiments were performed. The first set (A) was carried out by varying the amount of ethanol while keeping the amount of oleic acid fixed at 10 g (mol ethanol/mol oleic acid = 1, 1.2, 1.5, 2, 2.5 and 3). The second set (B) was carried out by varying amount of oleic acid while keeping the amount of ethanol fixed at 1.95 g (mol oleic acid/mol ethanol = 1, 1.5, 1.7, 2, and 2.5).

2.4. Experimental set up

The reactions were carried out in a 3-neck 50-ml jacketed and thermostated glass reactor. Stirring was achieved with a magnetic stirrer controlled at 200 rpm. The enzyme-catalyzed reaction was then monitored *in situ* throughout the reaction course, using a dispersive fiber-optic probe attached to an inVia Reflex Raman microscope, Renishaw, UK. The laser source employed for Raman scattering was a near infrared laser (785 nm) with a power setting of 100%. The spectral range was 1300–1800 cm^{-1} with a resolution of circa 1.1 cm^{-1}. Each Raman spectrum was measured with a 10 s acquisition time and the interval between each scan was 60 s.

2.5. Spectral analysis

The collected *in situ* Raman spectra were processed and analyzed to determine the percentage conversion of substrates to ethyl oleate ester. Since the raw spectra acquired from the reaction mixture were a combination of Raman scattering signals, spikes due to cosmic rays, and some autofluorescence background, some spectral preprocessing was needed to generate the Raman spectral alone. This spectral preprocessing includes noise removal by adjacent 3-point averaging, spikes removal due to cosmic rays, and baseline correction using third order modified polynomial fitting.

Fig. 1 shows the pure component Raman reference spectra of iso-octane, ethanol, oleic acid and ethyl oleate.

As shown in Fig. 1, there is considerable similarity between the Raman spectra of pure oleic acid and pure ethyl oleate, although the latter has a band around 1743 cm^{-1} due to the ester group (see small circle in Fig. 1). The spectral peaks enclosed by the large ellipse indicate the presence of carbonyl groups in both ethyl oleate and oleic acid. Therefore, in order to evaluate the reaction progress, the ratio of the integrated ester group band area to the total integrated carbonyl band area was used.

A calibration was performed in order to obtain the relationship between ratio of integrated areas and conversion. The calibration data were generated with known amounts of the substrates (ethanol and oleic acid) and product (ethyl oleate). Fig. 2 provides the calibration curve used for evaluating conversions with the standard initial reaction conditions (see Sections 2.2 and 2.3 experiment A). A similar curve was generated for the other series of experiments (Section 2.3 experiment B).

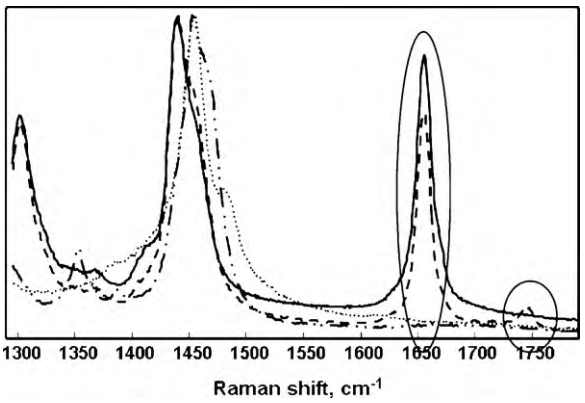

Fig. 1. Pure component Raman reference spectra of pure substances (dashed double dotted curve refers to iso-octane; dotted curve refers to ethanol, solid black curve refers to oleic acid, and dashed curve refers to ethyl oleate).

2.6. Effect of turbidity on spectral quality

The presence of suspended immobilized enzyme results in turbidity of the reaction mixture. The Raman measurements of the reaction mixture are also effected by this increased light scattering. This results in a slightly reduced intensity for the constituents present, but more importantly, to a decrease in the signal-to-noise of the signals. Fig. 3 shows a Raman measurement of one of the calibration solutions and a representative measurement of a reaction mixture. This figure confirms that rather good quality Raman probe measurements can be made of the turbid reaction solution, however, at a reduced signal-to-noise ratio.

3. Results and discussion

3.1. Effect of reaction temperature

The time-dependent concentration profiles for ethyl oleate at different reaction temperatures are shown in Figs. 4 and 5. The results are separated in order to more clearly show the effect of temperature. The maximum concentrations of ethyl oleate shown in Figs. 3 and 4 correspond to circa 85–90% yield. The "noisy" concentration profiles are due in part to the turbidity of the reaction mixtures and the resulting decrease in signal-to-noise ratio in the spectra.

The time-dependent concentration profiles of ethyl oleate were then used to calculate the initial reaction velocity. Fig. 6 shows the

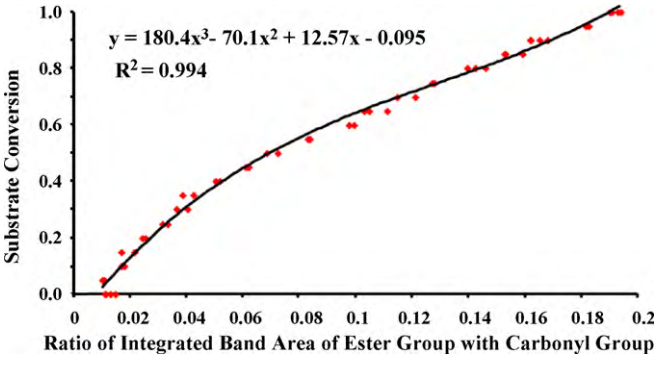

Fig. 2. A calibration curve for experiment A.

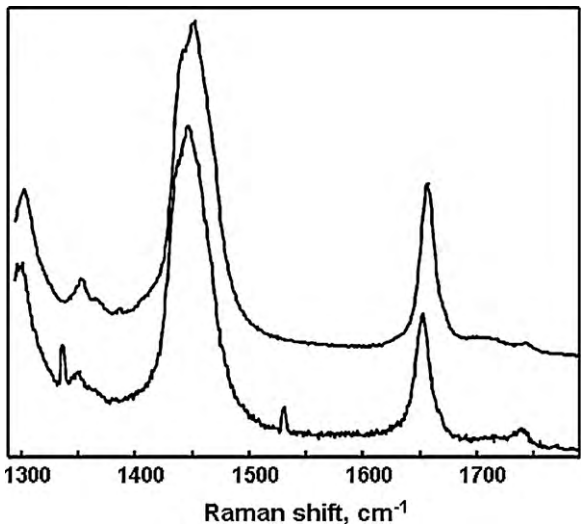

Fig. 3. A Raman spectrum of one the calibration solutions (top) and a representative Raman spectrum from one of the reaction mixtures (bottom) with lipase added.

plot of initial reaction velocity obtained at various reaction temperatures. The initial velocity is observed to increase steadily with respect to increasing temperature till around 60 °C. This effect of reaction temperature suggests that the optimal reaction temperature is circa 60 °C. Beyond this temperature, the initial reaction velocity starts to decrease due to thermal denaturing of the lipase.

3.2. Effect of molar ratio

Similar experiments were conducted by varying the molar ratios of reactants (see Section 2.3). The initial velocity of the reactions for different molar ratio of substrates (mol ethanol/mol oleic acid) is shown in Fig. 7.

Fig. 7 indicates that the initial reaction velocity reaches a maximum at a ratio of 1.2 (mol ethanol/mol oleic acid), after which it decreases. The decrease in reaction velocity at higher ratios indicates that increasing ethanol indeed inhibits Novozym 435 enzyme activity. This was further verified by plotting the reciprocal of initial reaction rate against reciprocal of ethanol concentration (shown in Fig. 8). Since the plot shown in Fig. 8 is not a straight line, but instead hyperbolic, the result indicates substrate inhibition. This plot is indeed quite similar to the Lineweaver-Burk plot in the presence of substrate inhibitor. This result is also in agreement

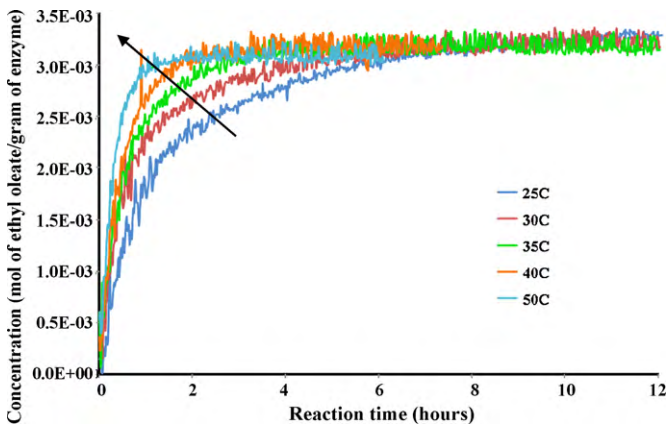

Fig. 4. Concentration of ethyl oleate at various reaction temperatures. Arrow indicates increasing temperatures 25, 30, 35, 40 and 50 °C.

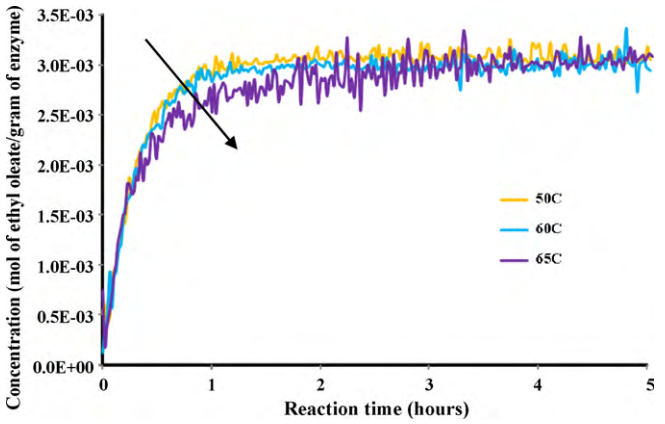

Fig. 5. Concentration of ethyl oleate at various reaction temperatures. Arrow indicates increasing temperatures (50, 60 and 65 °C).

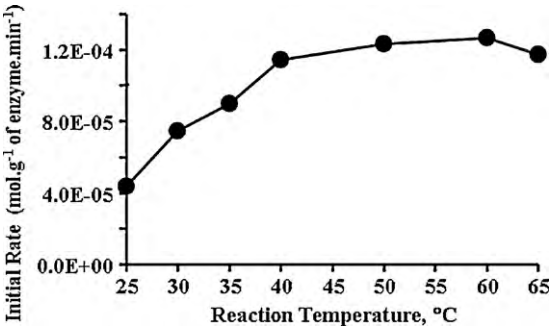

Fig. 6. Effect of reaction temperature on initial reaction velocity.

with the result found by Chulalaksananukul et al. [10] that ethanol acts as substrate inhibitor to lipase enzyme activity at higher concentration.

The effect of the excess of oleic acid on the initial velocity of the esterification reaction is shown in Fig. 9. As seen, an increase in the ratio of oleic acid to ethanol increases the initial reaction velocity. This result indicates that oleic acid does not exhibit any inhibitory behavior on the present reaction.

Since the inhibition effect of ethanol was only found at low concentrations of oleic acid, i.e. higher molar ratio (mol ethanol/mol oleic acid) of 2, 2.5 and 3, as shown in Fig. 7, it appears that a *Ping-Pong Bi-Bi* mechanism is underlying the kinetics. No ternary complex is expected to be formed. In this manner, lipase reacts

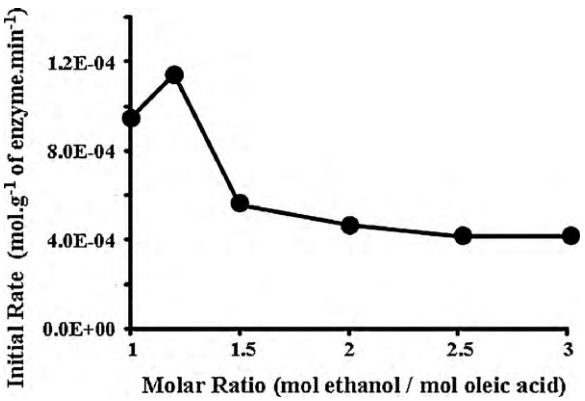

Fig. 7. The effect of molar ratio (mol ethanol/mol oleic acid) on the initial reaction velocity (varying amount of ethanol at fixed amount of oleic acid).

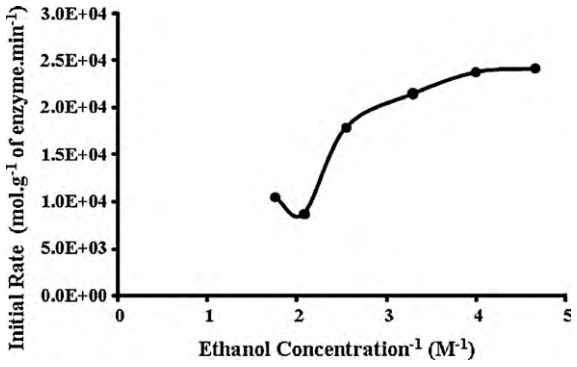

Fig. 8. The reciprocal initial velocity of the reaction versus reciprocal ethanol concentration.

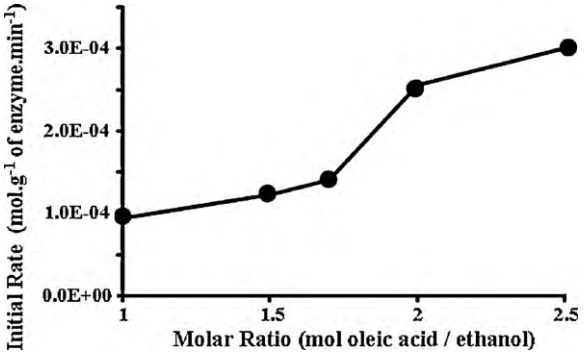

Fig. 9. The effect of molar ratio (mol ethanol/mol oleic acid) on the initial reaction velocity (varying amount of oleic acid at fixed amount of ethanol).

with oleic acid to form a lipase-oleic acid complex. The lipase–oleic acid complex then transforms to a carboxylic-lipase intermediate releasing water. This is then followed by the binding of carboxylic-lipase intermediate to ethanol to form another binary complex, and subsequently yielding ethyl oleate and free lipase. A similar deduction on this reaction sequence has been suggested by Chulalaksananukul et al. [10].

Since the present esterification reaction follows a *Ping-Pong Bi-Bi* mechanism with the dead-end inhibition by ethanol, the equation for initial velocity of reaction can be written as follows:

$$\upsilon_o = \frac{Vab}{K_{mB}a + K_{mA}b(1 + b/K_{siB}) + ab} \quad (1)$$

where b refers to the inhibitory substrate (ethanol) concentration, a refers to oleic acid concentration, V is the maximum possible of reaction rate when A (oleic acid) and B (ethanol) are both saturating; υ_o is the initial reaction velocity; K_{mA}, known as the limiting Michaelis constant for A, is the concentration of A which gives 1/2 V when B is saturating; K_{mB}, known as the limiting Michaelis constant for B, is the concentration of B which gives 1/2 V when A is saturating; K_{siB} is the dissociation constant of EB, with E referring to enzyme. Further details of the ping-pong bi-bi mechanism can be found in the detailed article by Chulalaksananukul et al. [10], as well as [11,12].

In order to determine the kinetic parameters (K_{mA}, K_{mB}, K_{siB}, and V) of the above equation, a global optimization approach was employed to minimize the differences between the initial reaction velocity υ_o obtained from equation 1 and from the spectroscopic measurements. Corana's simulated annealing [13] was chosen as the global optimizer for present problem, since it has been proven to be quite robust in obtaining global solutions for highly nonlinear optimization cases. The major advantage of this optimization approach over the conservative graphical method is that less experimental data is generally needed, but the parameters modeled prove to be quite accurate. Using the experimental results obtained from 10 different experiment runs, the values of K_{mA}, K_{mB}, K_{siB}, and V were calculated. These values are $2.51E5 \pm 4.07E4$ M, $1.53E{-}2 \pm 3.04E{-}2$ M, 0.13 ± 0.03 M, and 224 ± 111 mol g^{-1} min^{-1}, respectively. The sum of squared error between predicted and actual experimental data is circa 7.68E−9.

4. Conclusion

In the present study, it has been demonstrated that fiber-optic Raman spectroscopy can be used to facilitate the kinetic investigation of immobilized enzymatic reaction in turbid systems. Although the signal-to-noise ratio of the Raman measurements were reduced somewhat due to the turbidity, spectral measurements in the range of 1300–1800 cm^{-1} permitted quantitative assessment of the carbonyl and ester vibrational bands associated with oleic acid and ethyl oleate. This information was subsequently used to obtain the concentration profiles of ethyl oleate as a function of time and the values of initial reaction velocity. The experimental runs conducted at various reaction temperatures suggested an optimal reaction temperature of 60 °C. The experimental runs with varying substrates ratios suggest that this reaction undergoes a *Ping-Pong Bi-Bi* mechanism with an ethanol exhibiting inhibitory effect. The present analytical approach to monitor turbid immobilized enzymatic reaction is rather general and not restricted to the present reaction alone. It appears applicable to a wide range of enzymatic reactions conducted in either aqueous or organic solvents.

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