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Production and Characterization of Isopropyl Laurate Using Immobilized Lipase

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Abstract

Immobilized *Candida antarctica* lipase (Novozym 435) was used for the synthesis of isopropyl laurate from isopropyl alcohol and lauric acid. A molecular sieve was used to shift the reaction towards the synthesis. The maximum enzyme activity was obtained at 60 °C. The amount of product seemed to be linearly proportional to the enzyme concentration up to 50 mg. A linear increase in the amount of isopropyl laurate was observed throughout the 50 min reaction. Product formation followed a linear increase up to a lauric acid concentration of 100 mg. Then product formation slowed down. The hydrophile-lipophile balance (HLB) value of isopropyl laurate was calculated as 3.5 and its critical micelle concentration (CMC) value was found to be 0.684 mM and 0.687 mM from the conductivity and surface tension curve, respectively.

Key words: *Candida antarctica* lipase, Enzymatic esterification, Isopropyl laurate, Surfactant.

Introduction

Surfactants are amphiphilic molecules that tend to lower the interfacial tension at the interphase. They find applications in an extremely wide variety of industrial processes (Lin, 1996). Recently rapid advances in biotechnology have led to a surge in interest in biosurfactants, that is surface-active agents produced by biological catalysts in the form of isolated enzymes or microorganisms (Kosaric, 1992).

Fatty acid derivatives (fatty acid esters, fatty acid esters of sugars and sugar alcohols etc.) are important surfactants for household, cosmetic and industrial purposes (Maag, 1984). The current manufacture of these esters is realized by reacting a fatty acid and an alcohol at high temperature in the presence of a metal catalyst. This high temperature process can lead to degradation of the ester and the formation of undesired side products; additionally, the resulting energy costs are high (Awang *et al.*, 2000).

Enzyme catalyzed reactions have gained considerable importance in synthetic organic chemistry, pharmaceuticals and food ingredients compared with conventional chemical synthesis (Vulfson, 1993; Sarney and Vulfson, 1995). These enzymatic methods have the advantages of low energy requirements, minimal thermal degradation and high biodegradability and therefore have emerged as promising substitutes for conventional approaches for the production of surfactants (Kosaric, 1992).

One of these enzymes is lipase (triacyl glycerol acyl hydrolase, E.C. 3.1.1.3). Lipase catalyzed reactions operate at mild conditions, which prevent degradation of starting materials and reduce side reactions. The application of lipases in various modifications of fats and oils is well established, and the industrial uses of lipase catalyzed reactions are not limited to nutritional and pharmaceutical applications, but also include the synthesis of surfactants and soaps, and flavor production in the dairy and related industries (Şekeroğlu *et al.*, 2002).

They cover a wide spectrum of molecules and applications. The production of esters can be achieved either by synthesis from free acid and hydroxyl groups or by ester exchange or transesterification. Transesterification includes acidolysis, alcohol-

ysis and interesterification (Coulon and Ghoul, 1998; Yaqoob *et al.*, 1997; Lortie, 1997).

The formation of water presents a problem in the esterification reaction because the equilibria catalyzed by hydrolytic enzymes are in favor of hydrolysis (Ünal, 1998). Activated molecular sieves or salt hydrates can be added to the system to remove the water produced by the reaction (Şekeroğlu *et al.*, 2002).

The use of immobilized enzyme for the production of fatty acid esters improves the quality of the final product but also reduces the cost of the enzyme, and is friendly to the environment (Aran *et al.*, 2000).

The aims of this study were to investigate the enzymatic esterification of lauric acid with isopropyl alcohol by using immobilized *Candida antarctica* lipase (Novozym 435) (Figure 1) and to examine the effects of various reaction parameters. The surface activity of isopropyl laurate was also determined. The lipase-catalyzed alcoholysis of lauric acid could lead to a product from natural products while providing a value-added use for surfactants.

Materials and Methods

Materials

Novozym 435 (Type B lipase from *Candida antarctica* immobilized on an acrylic resin) was a gift from Novo Nordisk A/S (Bagsvaerd, Denmark). Its ester synthesis activity was determined as 7000 propyl laurate units/g immobilized enzyme using lauric acid and 1-propanol as substrates at 60 °C, according to the manufacturer (Novozym 435 Product sheet, 1999).

Lauric acid (99%), molecular sieves (4 °A) and silica gel (60 °A in pore diameter) were supplied by Sigma (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) grade acetone, isopropyl alcohol, acetonitrile, tetrahydrofuran, acetic acid (glacial), methanol and chloroform were supplied by Merck (Darmstadt, Germany). Potassium hydroxide (KOH) and phenolphthalein were from Riedel de Haen (Hannover, Germany).

The water used in the analyses was deionized and triple-distilled. All reactions were carried out in triplicate and repeated twice. The results presented here are the average of these multiple determinations.

Esterification reaction

Unless otherwise stated, the reactions were carried out by mixing 100 mg of lauric acid, 200 mg of molecular sieves and 50 mg of Novozym 435. After the addition of 4 ml of isopropyl alcohol, the reaction was allowed to occur in a 100 ml stoppered glass Erlenmeyer flask shaken at 120 strokes/min in a thermo-constant water bath shaker (Nuve, Sanayi ve Malzemeleri İmalat ve Tic. AS, İstanbul, Turkey) for 15 min at 60 °C. To enhance the reaction towards synthesis, molecular sieves were used (Arcos et al., 1998a). The quantity of the molecular sieve was greater than that necessary to completely absorb the amount of water that would be produced from the complete esterification of the fatty acids present, considering the high capacity of molecular sieves for absorption of water (20% w/w). Optimum substrate and enzyme concentrations, and the effects of temperature and time on the enzyme activity were investigated for the production of isopropyl laurate.

Assay of enzyme activity

The reaction was terminated by removing the enzyme and molecular sieves by centrifugation. Ester formation was calculated based on the acid values of the reaction mixture measured before and after the incubation time with a procedure suggested by Novo Nordisk A/S (Novozym 435 Product sheet, 1999). The acid values were determined by titration with 0.05 M KOH using phenolphthalein (1%) as an indicator. The reaction mixture operated without the enzyme was titrated in the same way and used as a blank (Fadiloğlu and Söylemez, 1998).

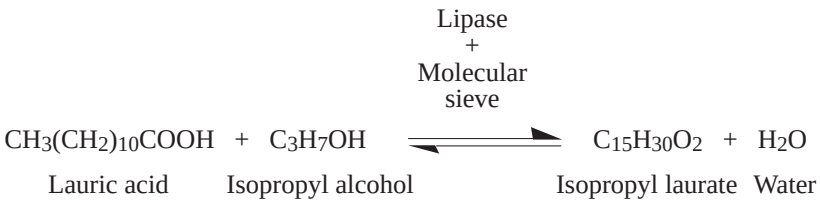

Figure 1. Esterification reaction of lauric acid with isopropyl alcohol.

Enzyme separated by centrifugation was immediately introduced into a new reaction mixture for repetitive assays.

Purification of fatty acid ester

After the removal of the enzyme and molecular sieves, the remaining solvent was evaporated in a vacuum oven for 3 h at 40 °C. Purification of the monoester was accomplished by column chromatography (Arcos *et al.*, 1998a). A silica gel column (1.6 cm in diameter, 50 cm in height) equilibrated with chloroform was used to purify the monoester. The solvent-free reaction mixture was dissolved in chloroform and introduced into the column. The excess acid was first eluted with chloroform. Elution of monoester was accomplished with a 93:7 (v/v) mixture of chloroform and methanol (Arcos *et al.*, 1998a).

HPLC analysis of the product

The HPLC system consisted of an HP series 1100 variable wavelength detector at 214 nm, a Jasco PU-980 model isocratic pump (Tokyo, Japan) and a Waters Nova-Pak C-18 (3.9 x 150 mm) column (Minnesota, USA), and a software program (Borwin version 1.21) was used to identify the product. The flow rate was 1.0 ml/min. The mobile phase used was a mixture of acetonitrile, water, tetrahydrofuran and acetic acid (70:25:5:0.1) (v/v) (Ducret *et al.*, 1995).

Stability of enzyme

Novozym 435 (50 mg) was incubated in 4 ml of isopropyl alcohol containing 100 mg of lauric acid in a thermo-constant water bath shaker at 40 °C for 6 days. Following the incubation period, the remaining activity of enzyme at 60 °C was calculated. The residual activity of lipase was expressed as a percentage relative to the activity of fresh lipase for the same reaction (Arcos *et al.,* 1998b).

Statistical analysis

Statistical analysis (ANOVA) was carried out using the Statgraphics (Rockville, MD, USA) computer program. ANOVA testing was performed for all experimental runs to determine significance at the 95% confidence level.

Surfactant properties of ester

HLB The HLB value of isopropyl laurate was obtained using the Griffin equation (Griffin, 1949).

$$HLB = 20 \times [1 - (S/A)] \quad (1)$$

where S is the saponification index of the ester group and A is the acidity index of the fatty acid.

CMC The CMC values of isopropyl laurate were determined by the conductivity method (Rosen and Goldsmith, 1972) and the capillary rise method (Halpern, 1997). The apparatus used for the conductivity method produces a pulsed, bipolar voltage having a frequency of 1 kHz and a peak-to-peak amplitude of 0.5 V. This waveform was generated from an integrated circuit powered by a 9-V power supply (Leader-Lag. 1265-Audio Signal generator). A digital ammeter (ITT MX 20 type avometer) was placed in series between one electrode and the pulsed voltage supply (Halpern, 1997). The currents (I in milliamperes) were recorded at different concentrations (C in molarity) of isopropyl laurate. Then a plot of I/C versus $C^{1/2}$ was drawn. Data points of the exponential curve were connected by straight lines, and the intersection point of the 2 lines gave the CMC value (Halpern, 1997). The apparatus used for the capillary rise method consists of a graduated scale marked from 0 to 10 cm in 1 mm increments. At different concentrations of surfactant solution the height of the liquid in the capillary tube was noted. A plot of height versus concentration was drawn. The inflexion point of this curve gives the CMC value of the ester.

Results and Discussion

Effect of lauric acid concentration

Lauric acid concentrations of 25 to 3500 mg were used to see the effect of substrate concentration on product formation (Figure 2). The amount of product was increased linearly up to a lauric acid concentration of approximately 1000 mg. Then product formation slowed down and reached 30 μmol $ml^{-1}min^{-1}$ at a lauric acid concentration of 2500 mg. There was no significant ($P > 0.05$) change in product formation after this point.

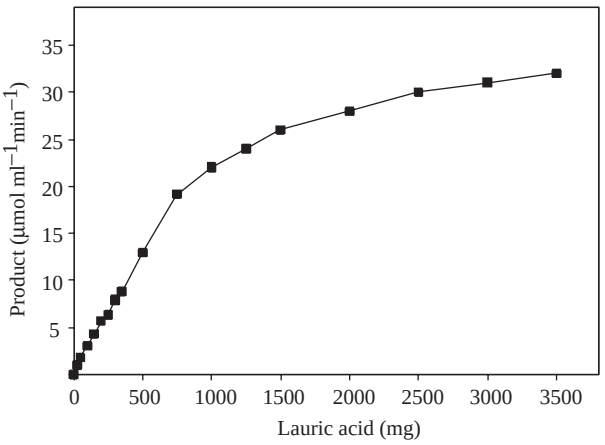

Figure 2. Effect of lauric acid concentration on product formation.
Reaction conditions: Lauric acid, 50 mg of Novozym 435, 200 mg of molecular sieve and 4 ml of isopropyl alcohol, reaction temperature 60 °C, reaction time 15 min.

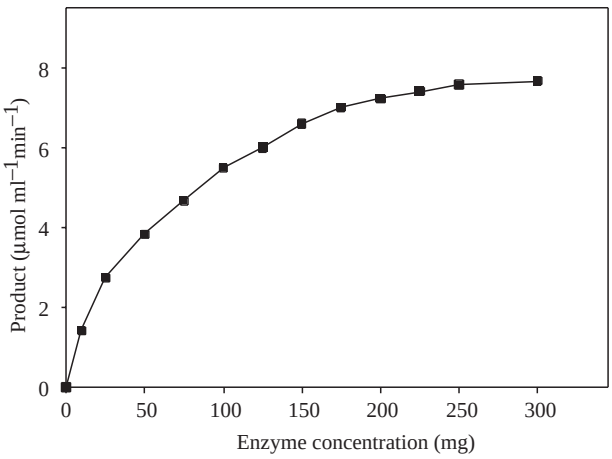

Figure 3. Effect of enzyme concentration on product formation.
Reaction conditions: Novozym 435, 100 mg of lauric acid, 200 mg of molecular sieve and 4 ml of isopropyl alcohol, reaction temperature 60 °C, reaction time 15 min.

Awang *et al.* (2000) obtained a similar plot for the esterification of dihydroxystearic acid using Novozym 435. They reported that further increases in the substrate had no effect on the conversion.

Effect of enzyme concentration

Novozym 435 concentrations of 10 to 300 mg were used in isopropyl alcohol to catalyze the esterification of lauric acid into isopropyl laurate at 60 °C for 15 min. Figure 3 shows the effect of enzyme concentration on product formation. The amount of isopropyl laurate produced seemed to be linearly proportional to the enzyme concentration up to 50 mg, showing that the system was in steady state. After this point, linearity deviates and product formation was slowed down. The amount of isopropyl laurate reached 7.67 μmol ml^{-1} min^{-1} at an enzyme concentration of 300 mg. Therefore the decision was made to take 50 mg of immobilized enzyme per 100 mg lauric acid for further experiments. The progress curve of the lipase catalyzed esterification reaction was the same as that of general esterification reactions (Segel, 1975).

Mutua and Akoh (1993) reported that any further increase in the enzyme concentration did not result in an increase in the amount of desired product, because the substrate concentration became a limiting factor in the face of an excess of active enzyme sites.

Effect of temperature

Temperature was changed from 40 °C to 80 °C to observe the effect of temperature on product formation. As seen from Figure 4, Novozym 435 exhibited its maximum activity at 60 °C. The manufacturer also suggested an optimum temperature of 60 °C for Novozym 435 (Novozym 435 Product sheet, 1999). This observation was similar to those by Arcos *et al.* (1998a) and Compton *et al.* (2000). At temperatures higher than 60 °C, thermal deactivation of the enzyme occurs. As observed in the work of Habulin *et al.* (1996), this temperature was 50 °C for Lipozyme IM, which was another important enzyme for esterification reactions. It was reported that the reaction rate decreased when the temperature decreased (Habulin *et al.*, 1996).

Effect of incubation time

The effect of incubation time on isopropyl laurate formation was studied from 5 min to 520 min. As shown in Figure 5, at a fixed substrate and enzyme concentration, there was a linear increase in the amount of isopropyl laurate up to 50 min. After that point, the amount of product formation slowed down. No significant ($P > 0.05$) change was observed in the amount of product after 150 min. A similar plot was obtained by Habulin *et al.* (1996) in their work on the lipase-catalyzed synthesis of oleic acid esters.

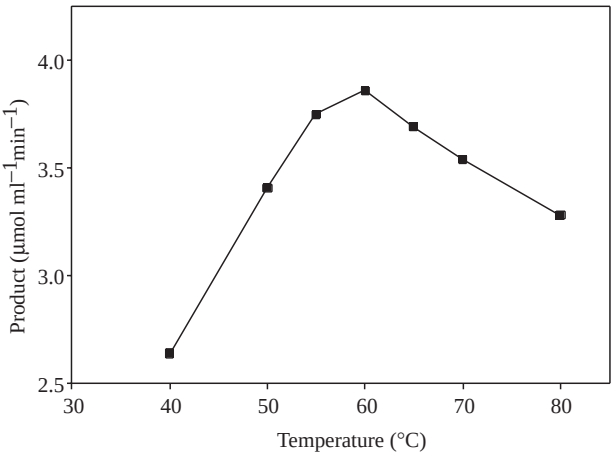

Figure 4. Effect of temperature on product formation. Reaction conditions: 100 mg of lauric acid, 50 mg of Novozym 435, 200 mg of molecular sieve and 4 ml of isopropyl alcohol, reaction time 15 min.

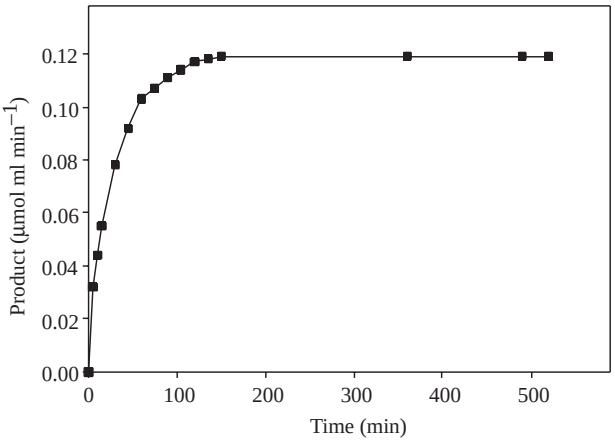

Figure 5. Effect of incubation time on product formation.
Reaction conditions: 100 mg of lauric acid, 50 mg of Novozym 435, 200 mg of molecular sieve and 4 ml of isopropyl alcohol, reaction temperature 60 °C.

Stability of enzyme

After 6 days of use at 40 °C in isopropyl alcohol containing lauric acid, the activity of Novozym 435 was determined. After this incubation period, the activity of Novozym 435 was reduced to 70% of the initial value.

Arcos *et al.* (1998b) reported a similar finding in their work on the enzymatic production of sugar esters. They reported that after 18 days of incubation at 40 °C in acetone containing lauric acid, the activity of Novozym 435 was reduced to 50% of its initial value. The stability of Novozym 435 is relatively high in the reaction medium, considering the reaction time required (typically 2-3 days).

Repetitive use of enzyme

The results of repeated use of Novozym 435 are shown in Table. After the third use of the same enzyme at 60 °C, for 15 min, the retained activity was 59%.

Table. Repetitive use of Novozym 435.

Use	Isopropyl laurate producd (μmol)	Retained activity (%)
1st	179	100
2nd	122	68
3rd	106	59

One important factor limiting the use of lipase catalyzed reactions on an industrial scale is the cost of the enzyme. The cost efficiency of the reaction could be greatly improved by reusing the lipase for several reactions (Compton *et al.,* 2000). When the stability and repetitive use of immobilized enzyme were considered Novozym 435 remains active at 60 °C for weeks and is able to catalyze multiple esterification reactions.

HPLC HPLC was used to determine the reaction product. Isopropyl laurate corresponds to a retention time of 9.71 min.

Surfactant properties of ester

HLB value The HLB value of isopropyl laurate was calculated as 3.5 according to the Griffin equation. This result indicated that it is an emulsifier helping to stabilize the water-in-oil type emulsion (Coulon and Ghoul, 1998).

CMC Figure 6 illustrates the conductivity measurements of isopropyl laurate. From the intersection point of these 2 straight lines the CMC value of isopropyl laurate was calculated as 0.684 mM. Figure 7 illustrates the potential of enzymatically prepared isopropyl laurate to reduce the surface tension. The CMC value obtained from this curve was 0.687 mM. This value is closer to that obtained from the conductivity curve. After a certain concentration value, no change was observed in surface tension or conductivity measurements.

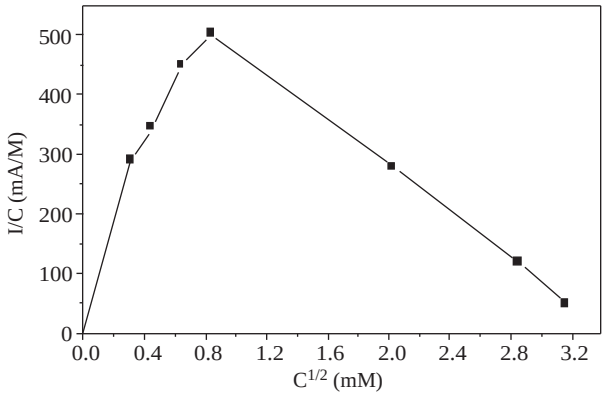

Figure 6. CMC curve of isopropyl laurate.

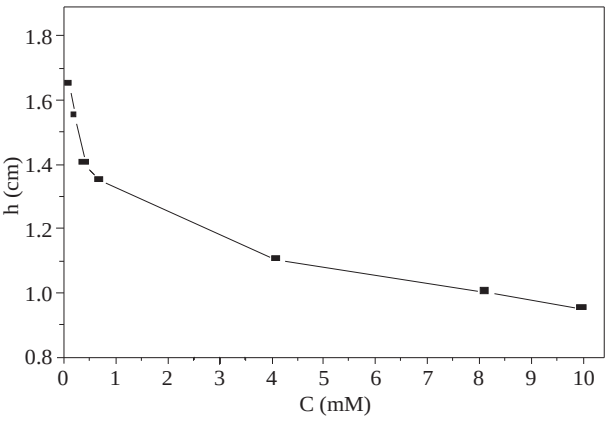

Figure 7. The curve of reduction of surface tension of isopropyl laurate.

These results showed that isopropyl laurate has the ability to reduce the surface tension greatly. The values obtained for isopropyl laurate were close to those obtained for the lauric acid ester of sorbitol and the lauric acid ester of glucose (Ducret *et al.*, 1996).

Ducret *et al.* (1996) reported that the CMC value of a surfactant in an aqueous medium generally decreases as the number of carbon atoms in the hydrophobic group increases to about 16, while above 18 carbons CMC remains unchanged with an increase in the chain length.

The data presented here showed that Novozym 435 is an effective biocatalyst for the esterification of isopropyl laurate from lauric acid and isopropyl alcohol. Enzymatically prepared isopropyl laurate also has good surfactant properties.

Conclusions

A lipase catalyzed esterification process was developed for the synthesis of isopropyl laurate from lauric acid and isopropyl alcohol. By considering the reverse direction, a molecular sieve was used to hold the forming water during the reaction. A study on finding the surfactant properties of ester was performed. It was a suitable emulsifier to stabilize water-in-oil type emulsions. Novozym 435 was found to be highly economical and effective for catalyzing esterification reactions.

Further research is needed to compare this enzymatically produced surfactant with chemically synthesized counterparts, especially for the determination of side products and in terms of biodegradability.

Acknowledgments

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