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Synthesis of Isoamyl Laurate and Isoamyl Stearate in Supercritical Carbon Dioxide

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Abstract

The synthesis of isoamyl laurate and isoamyl stearate was studied in supercritical carbon dioxide with three lipases, Novozym 435, Lipolase 100T, and *Candida rugosa*. The maximum conversion of 37% and 53%, respectively for isoamyl laurate and isoamyl stearate was obtained when Novozym 435 was used. The effect of various parameters such as molar ratio of alcohol to acid, presence of water, time and temperature was investigated. An optimum temperature of 40–45°C was observed for all reactions. The kinetics of reactions was fast and equilibrium was achieved in 2–3 h. Although the presence of excess alcohol did not reduce conversion, excess water reduced conversion significantly.

Index Entries: Isoamyl laurate; Isoamyl stearate; esterification; supercritical carbon dioxide; lipase.

Introduction

Esters of fatty acids are mainly used as flavors in food industry, and are also used as cosmetics, emulsifiers and lubricants. Isoamyl laurate *(1,2)* is used as a flavor in beer and wines whereas isoamyl stearate *(3)* is used in coatings for magnetic recording media. Although esters synthesized chemically are not considered natural, enzymatically synthesized ester are considered natural *(4)* by food regulatory agencies. Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) have been used for hydrolysis, esterification, and transesterification in absence of solvents *(5–7)* in organic solvents *(8–10)* and in supercritical fluids *(11–12)*. Enzymatic reactions are conducted in organic solvents and are heterogeneous *(13)* and are diffusion-controlled *(14)*. Supercritical fluids (SCFs) are defined as fluids above their critical

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pressure and critical temperature whose properties are between that of gas and liquid phase. In SCFs, the solubility of organics is similar to that in liquid and the diffusivity is similar to that in gas. An advantage of SCF is that the solubility of reactants and products greatly depends on pressure *(15)* and temperature, as a result of which SCF solvents gives single step downstream processing by altering the temperature and pressure of system. Among SCFs, supercritical carbon dioxide ($SCCO_2$) is often used because it is cheap, nontoxic, and nonflammable, with an ambient critical temperature (31.1°C) and moderate critical pressure (73.8 bar). Many lipase catalyzed enzymatic reactions are reported in literature *(10,16)*.

Although many lipase catalyzed reactions have been investigated in $SCCO_2$, esterification of lauric acid and stearic acid with isoamyl alcohol in $SCCO_2$ has not been investigated. Therefore, the objective of this study is to investigate the various parameters that influence the enzymatic synthesis of isoamyl laurate and isoamyl stearate by three different lipases, Novozym 435 (Candida Antarctica), Lipolase 100T (T. langinosus), and lipase from Candida rugosa.

Materials and Methods

Materials

The chemicals lauric acid (99%), stearic acid (90%), and isoamyl alcohol (98%), high-performance liquid chromatography (HPLC)-grade methanol (99.8%) were obtained from S.D.Fine Chem. Ltd. (India). Silica gel, Sodium hydrogen carbonate (99.5%), and Sulfuric acid (98%) were purchased for Merck (India). Novozym 435 and Lipolase 100T were gifted by Novo Nordisk (Denmark) and Lipase from Candida rugosa (with an activity of 1410 U/mg) was purchased from Sigma-Aldrich. Among the enzyme employed, Novozym 435 and Lipolase 100T are immobilized, and Lipase from Candida rugosa is free enzyme. Carbon dioxide (98%) from Vinayaka gases (India) was used after dehydrating by passing the gas through a bed of silica gel. Distilled deionized water was filtered thorough micro pore membrane before use.

Methods

All the reactions were performed in a 7-mL stainless steel tubular batch reactor. Each reactor, loaded with reactants and enzyme, was pressurized to an initial pressure of 68 bars at room temperature, 25°C. The pressurized reactors were then immersed in a water bath maintained at the desired temperature, with fluctuations less than ±0.5°C. All the reactions were conducted at a constant density of CO_2 (0.79 g/mL) for various temperatures. Even though the pressure was higher at every temperature increment, the density of system remained constant. The reactors were equipped with a pressure gauge to ensure that the system operated at the required region for pressure throughout reaction. After the desired time,

the reactor was depressurized, and the contents were eluted in 4 mL of methanol. Enzyme was removed by centrifugation at 4000 rpm for 2 min and supernatant reaction mixture was analyzed by HPLC. The solubilities of lauric acid *(17)* and stearic acid *(18)* are 4×10^{-3} and 1.17×10^{-5} mole fraction at 313 K and 318.15 K at 11.1 MPa, respectively. These solubilities correspond to 116.5 mg and 4.19 mg in a 7-mL reactor for lauric acid and stearic acid, respectively. The solubilities of fatty acids increase in the presence of alcohol by an order of magnitude *(18)*. The amount of the substrate of the reaction were chosen low enough so that they dissolved in $SCCO_2$ but high enough to ensure proper detection in analysis. The influence of water on reaction is investigated by adding water with reactants before pressurizing reactor.

Analysis

The reaction samples were analyzed by a HPLC system consisting of pump (waters 501) a reverse phase column (0.39 cm × 25 cm μBondapak C18) an injector (Rheodyne 7010 with an injection loop of 250 μL) equipped with an ultraviolet (UV) detector (Waters 2484 dual λ absorbance detector). The UV spectra showed a λ_{max} of 212 nm for esters, this wave length is used for all calculations. The sample was analyzed by methanol–water solution 77:23 for isoamyl laurate and 86:14 for isoamyl stearate at a flow rate of 0.5 mL/min. Calibration curve using the area under curve for known concentrations was obtained by using esters synthesized by Fischer's method *(19–20)*. Several experiments were repeated and errors in results were within ±2%.

Results and Discussion

Enzymatic esterification of isoamyl laurate and isoamyl stearate was studied in $SCCO_2$ with three different enzymes, Novozym 435, Lipolase 100T and lipase from Candida rugosa.

Effect of Alcohol Concentration

Figure 1A,B shows the effect of alcohol concentration on the synthesis of isoamyl laurate and isoamyl stearate, respectively. The other parameters are 10 mg acid, 10 mg enzyme, and a varying amount of alcohol up to 88 μL for isoamyl laurate and 60 μL for isoamyl stearate, for 8 h at 50°C. The results indicate a sharp increase in conversion initially, but an excess of alcohol does not reduce the conversion. This is in contrast to the study that shows as inhibitory effect of alcohol for the synthesis of esters in presence of ethanol *(7,20,21)*, geraniol *(22)*, and isopropyl alcohol *(8)*. The results are consistent with the results for reaction of various acids with isoamyl alcohol *(23)* and alcoholysis of methylmethacrylate *(12)*. The highest conversions obtained for Novozym 435, Lipolase 100T, and Candida rugosa were 37%, 28%, 34% and were 46%, 37%, 33% for laurate and stearate, respectively.

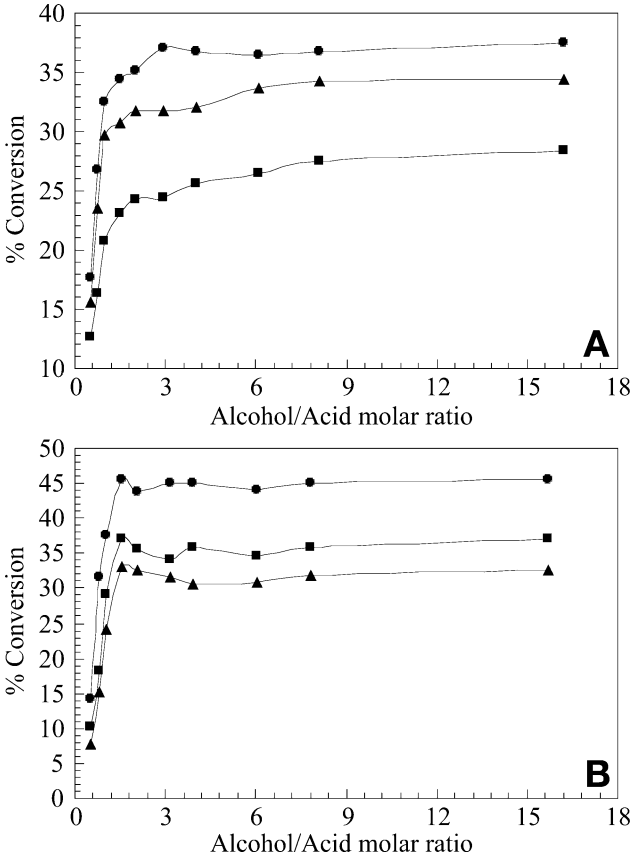

Fig. 1. Effect of alcohol concentration on the esterification reaction with 10 mg acid and 10 mg enzyme at the 50°C for 8 h **(A)** isoamyl laurate and **(B)** isoamyl stearate. (●) Novozym 435; (■) Lipolase 100T; (▲) lipase from *Candida rugosa*.

Effect of Enzyme Loading

The effect of enzyme loading on the conversion of isoamyl laurate and isoamyl stearate were studied with 10 mg acid, 8.1 µL alcohol for laurate and 6 µL alcohol for stearate, and a varying amount of enzyme from 2 mg to 30 mg, and kept 50°C for 8 h. Figure 2 indicate that enzyme loading beyond 10 mg has no influence on the conversion. These results are consistent with earlier studies for the synthesis of ethyl palmitate *(21)*, octyl palmitate *(24)*, and various flavor ester of isoamyl alcohol *(23)*.

Effect of Temperature

The effect of temperature in the synthesis of isoamyl laurate and isoamyl stearate in $SCCO_2$ is shown in Fig. 3. The reactions were investigated with 10 mg acid, 10 mg enzyme, and 8.1 µL alcohol for laurate and 6 µL for stearate for 8 h for 35–65°C. The highest conversion was obtained when the temperatures ranged between 40–45°C.

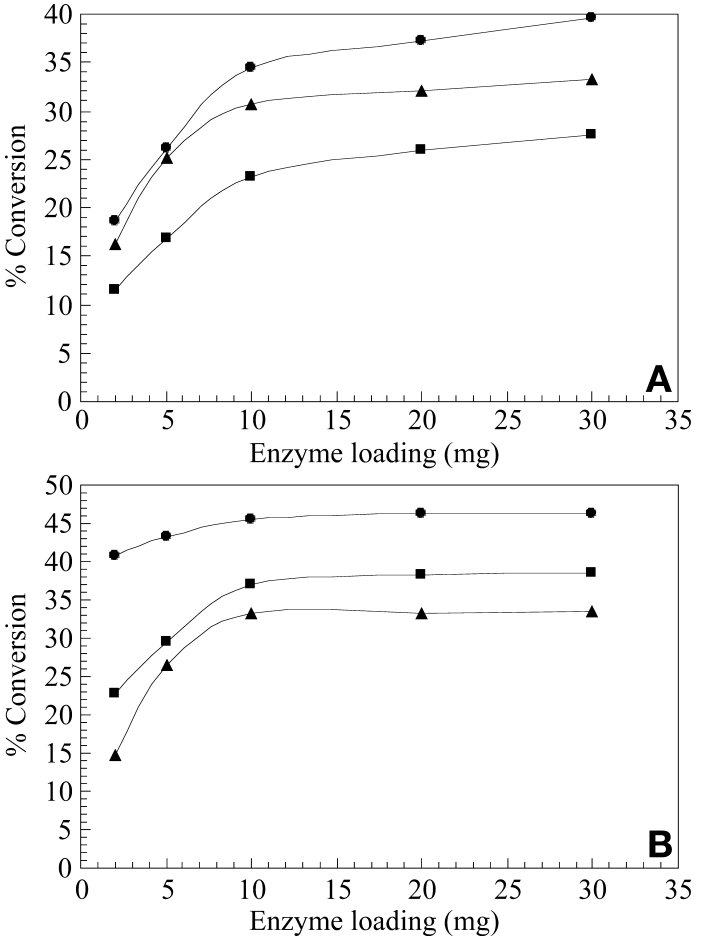

Fig. 2. Effect of enzyme concentration on the esterification reaction with 10 mg acid at the 50°C for 8 h, 8.1 µL alcohol for laurate and 6 µL for stearate. **(A)** Isoamyl laurate and **(B)** Isoamyl stearate. See Fig. 1. for legend.

Previous studies, on the enzymatic synthesis of esters in $SCCO_2$ *(21,23–24)* indicate that the optimum temperature can vary between 45 and 55°C.

Kinetics of Reactions

The kinetics of reactions was studied with 10 mg acid, 10 mg enzyme, and 8.1 µL alcohol for laurate and 6 µL for stearate at 50°C. The results obtained are plotted in Fig. 4, and the time required to achieve 95% of the equilibrium value was 2, 2.4, and 4 h to conversion for isoamyl laurate for Novozym 435, Lipolase 100T, and lipase for *Candida rugosa*, respectively, whereas corresponding values for isoamyl stearate are 2, 1.9, and 2.5 h. These values are significantly lower than 12 h required for esterification of myristic acid *(9)* and for the synthesis of various esters of isoamyl alcohol with carboxylic acids *(23)*.

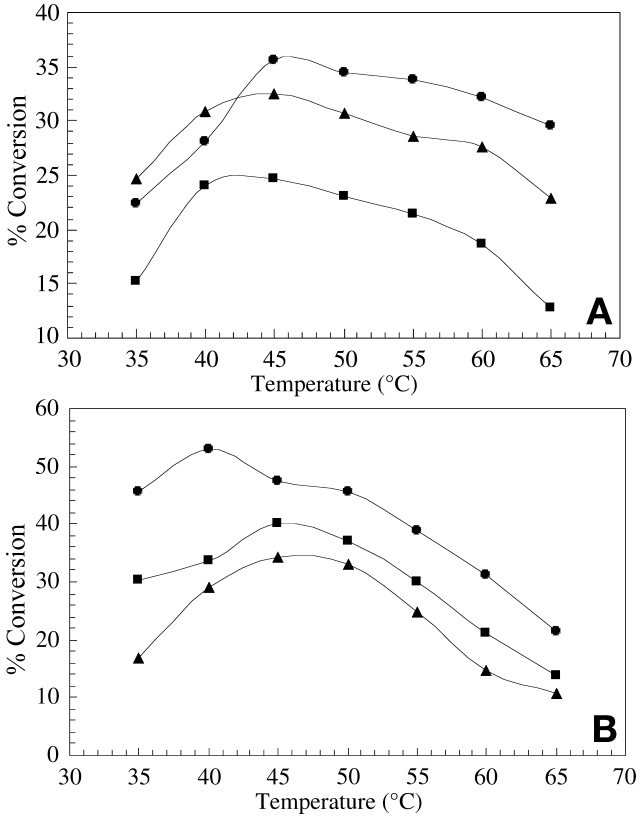

Fig. 3. Effect of temperature on the esterification reaction with, 10 mg acid and 10 mg enzymes for 8 h, 8.1 µL alcohols for laurate and 6 µL for stearate (a) Isoamyl laurate, (b) Isoamyl stearate. (●) Novozym 435; (■) Lipolase 100T; (▲) lipase from *Candida rugosa*.

Effect of Water Addition

The presence of water plays an important role for enzymatic reactions. Although water is essential for all enzymatic reactions *(13)*, excess of water would shift the equilibrium conversion towards reactant side leading to lower conversion. Therefore, the effect of water was studied by adding water to reaction system of 10 mg acid, 10 mg enzyme, and 8.1 µL alcohol for laurate and 6 µL for stearate for 8 h, at 50°C. To the study effect of water, 0–10 µL of water was added to reactor along with other reactants. Results plotted in Fig. 5 shows that the conversion decreases continuously for both of the products, indicating that water present with enzyme is enough for reaction. The conversion decreases trends as the reactions are shifted toward reactant side with addition of water. An optimum water concentration was observed for isoamyl acetate *(10)*, ethyl palmitate *(21)*, octyl palmitate *(24)*, glycidyl butyrate *(16)*, and enzymatic transesterification *(25)* of

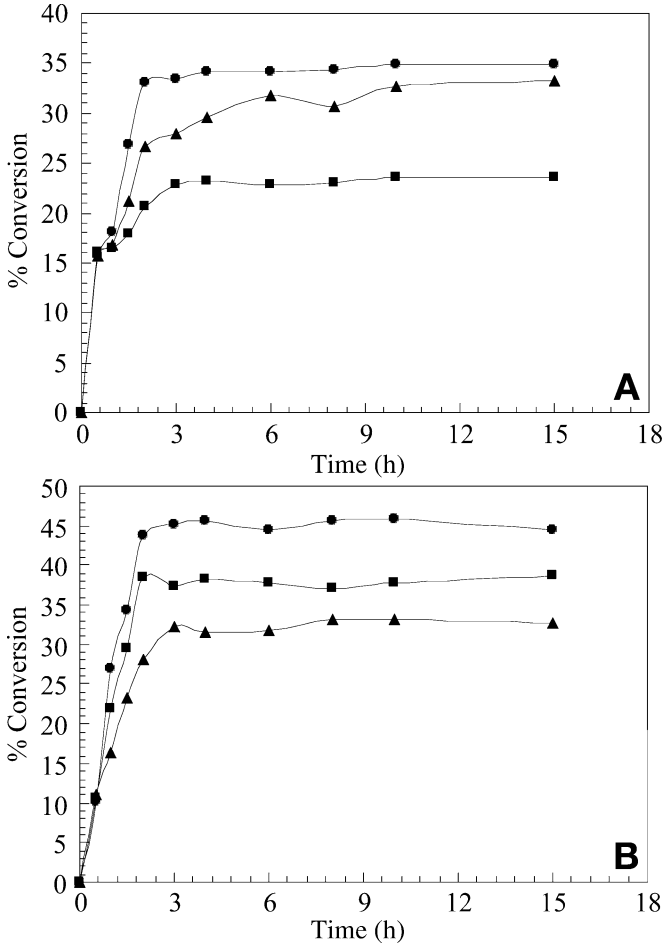

Fig. 4. Kinetics of the esterification reaction with 10 mg acid and 10 mg of enzymes at 50°C, 8.1 µL alcohol for laurate and 6 µL for stearate. **(A)** isoamyl laurate and **(B)** isoamyl stearate. (●) Novozym 435;(■) Lipolase 100T;(▲) lipase from *Candida rugosa*.

benzyl alcohol with vinyl butyrate and geranyl acetate. Conversion decreases on addition of water in the case of isoamyl butyrate *(23)* and ethyl oleate *(20)*.

Conclusions

The synthesis of isoamyl laurate and isoamyl stearate in $SCCO_2$ was investigated in presence of three enzymes Novozym 435, Lipolase 100T and lipase from Candida rugosa. The effect of temperature, time, enzyme loading, isoamyl alcohol concentration and water addition on the conversion was determined. The optimum temperature was found to be 40–45°C. Addition of water reduces conversion significantly but no inhibitory effect was found for excess of isoamyl alcohol.

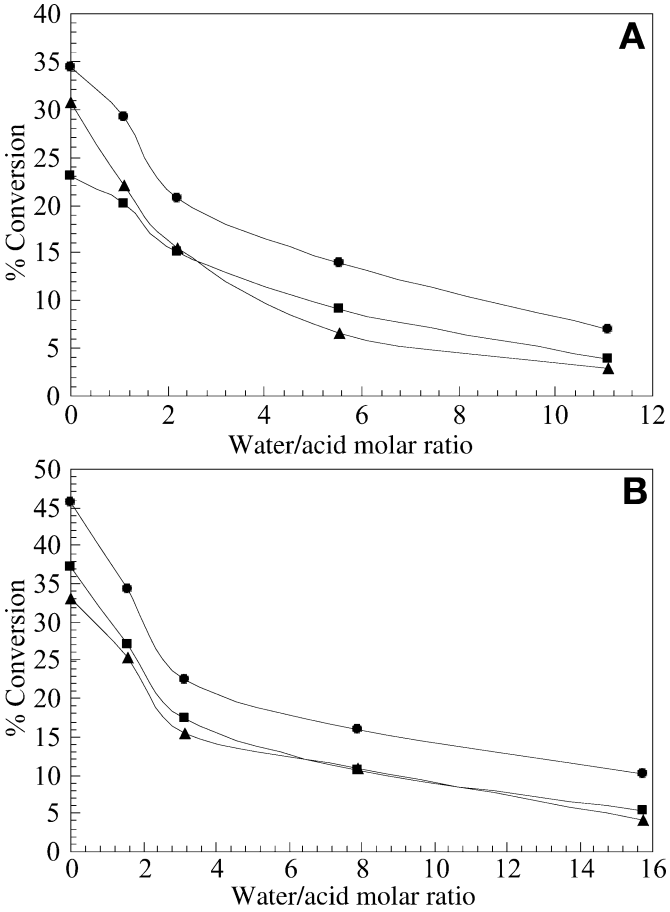

Fig. 5. Effect of water addition concentration on the esterification with 10 mg acid and 10 mg of enzymes at 50°C for 8 h, 8.1 µL alcohol for laurate and 6 µL for stearate. **(A)** Isoamyl laurate and **(B)** isoamyl stearate. (●) Novozym 435; (■) Lipolase 100T; (▲) lipase from *Candida rugosa*.

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