LIPASE-CATALYZED ESTERIFICATION OF SUGAR FATTY ACID ESTERS IN SUPERCRITICAL CARBON DIOXIDE

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ABSTRACT

Lipase-catalyzed esterification of different applicable sugar fatty acid esters, fructose palmitate, fructose laurate and sucrose laurate was performed in high yields in supercritical carbon dioxide (SC CO_2). Additionally, thermal stability of immobilized lipase from *Candida antarctica B* at atmospheric pressure and in SC CO_2 was determined. Effect of temperature on enzyme activity was studied in 2-methyl-2-butanol at atmospheric pressure and in SC CO_2 at 10 MPa.

INTRODUCTION

Sugar fatty acid esters, synthesized from renewable and inexpensive substances, are biodegradable, nonionic surfactants. Their surfactant and emulsification properties vary with the nature of the fatty acid and the sugar. Being nontoxic, they have broad applications in food and cosmetic industries, detergents, oral-care products and medical supplies [1-4]. Antibacterial activity of fructose monolaurate against *Streptococcus mutans* [5] and sucrose monolaurate against *Streptococus sobrinus* [6] was determined. Antibacterial effect of sucrose monolaurate against *Escherichia coli* [7] was reported, as well.

Applications of most of sugar fatty acid esters are limited by problems associated with the methods used in their preparation. Commercially they are produced by chemical synthesis in toxic solvents and inorganic catalysts that leave traces in the products, therefore a difficult and expensive purification steps are required [4, 8].

Product of chemical synthesis is a mixture of esters, hence research is steered towards biosynthesis. Selective synthesis catalyzed by enzymes can be performed under mild reaction conditions, thereby minimizing side reactions and browning of the products [9-11].

SC CO_2 may provide an interesting medium for biocatalysis, due to its tunable solvation ability and the possibility to eliminate solvent residues. Compared to the organic solvents is nontoxic, nonflammable and inexpensive [12-16].

Synthesis of sugar fatty acid esters in SC CO_2 does not require any addition of molecular sieves for the removal of water generated during esterification compared to the synthesis performed in organic media. On a larger scale, molecular sieves increase the reactor volume and mass transfer limitations may occur due to difficult stirring [1]. Furthermore, whereas only monoester is produced without molecular sieves, the addition of molecular sieves leads to the synthesis of diester [17].

Because carbon dioxide is nontoxic and therefore suitable solvent in the preparation of food additives, lipase-catalyzed synthesis of fructose palmitate, fructose laurate and sucrose laurate was performed in this medium.

I - MATERIALS AND METHODS

Enzymes and chemicals

Immobilized lipase Novozym 435 from *Candida antarctica B* (EC 3.1.1.3) was a gift from Novo Nordisk AS (Copenhagen, Denmark). D-(-) fructose (≥ 98%), 2-methyl-2-butanol (≥ 99%) and molecular sieves (3 Å) were purchased from Fluka (Buchs, Switzerland). Palmitic acid (min 98%) was obtained from Riedel de Haën (Seelze, Germany). Sucrose (99+%) was supplied from Sigma (Deisenhofen, Germany). Lauric acid (for synthesis) and sodium hydroxide solution (0.1 N) was from Merck (Darmstadt, Germany) and phenolphthalein was from Kemika (Zagreb, Croatia). Carbon dioxide 4.5 (purity 99.995 vol. %) was supplied from Messer MG Ruše, Slovenia).

Determination of lipase thermal stability at atmospheric pressure

Lipase from *Candida antarctica B* was incubated at different temperatures at atmospheric pressure for 24 hours. After 24 hours of incubation, lipase was used for synthesis of fructose palmitate in 2-methyl-2-butanol at atmospheric pressure.

Determination of lipase thermal stability in SC CO_2

Lipase from *Candida antarctica B* was incubated in SC CO_2 in the high-pressure batch stirred-tank reactor at different temperatures for 24 hours. After slow depressurization, the preincubated lipase was used as biocatalyst for the synthesis of fructose palmitate in 2-methyl-2-butanol at atmospheric pressure.

Synthesis of fructose palmitate in 2-methyl-2-butanol at atmospheric pressure

The reaction mixture consisted of equimolar (20 mmol) fructose and palmitic acid and 59% (w/w of reaction mixture) 2-methyl-2-butanol. Molecular sieves (12% w/w of reaction mixture) were added for the absorption of water, generated during esterification reaction. Synthesis of fructose palmitate was performed in a 100 mL round bottom flask, thermostated to the desired operating temperature and stirred by a magnetic stirrer (600 rpm). Reaction was started by addition of the lipase (10% w/w of substrates). Samples from the reaction mixture were taken at certain intervals and the level of free fatty acid (FFA) was determined.

Synthesis of fatty acid sugar esters in SC CO_2

The reaction mixture consisted of equimolar (20 mmol) fructose and palmitic acid. 10% (w/w of substrates) lipase was added to the reaction mixture. Esterification was performed in a 78 mL high-pressure batch stirred-tank reactor at a defined temperature with stirring rate 600 rpm. Cooled liquid carbon dioxide was pumped into the reactor up to 10 MPa. The start of the reaction was assumed to be when both temperature and pressure of the system were reached. The reaction was terminated by depressurisation of SC CO_2 and the reaction mixture was analyzed.

Analyses

Samples were analyzed qualitatively by thin layer chromatography (TLC) [18].

The ester content was quantified by calculating the residual fatty acid amount in the reaction mixture, which was determined by volumetric titration [19].

II - RESULTS AND DISCUSSION

Thermal stability of lipase from *Candida antarctica B* at atmospheric pressure

Before the synthesis of fatty acid sugar esters was performed in 2-methyl-2-butanol at atmospheric pressure, thermal stability of Novozym 435 was tested at atmospheric pressure. Lipase from *Candida antarctica B* was incubated at temperatures between 40 and 70 °C at atmospheric pressure for 24 hours. After 24 hours of incubation, the preincubated lipase was used for synthesis of fructose palmitate at previously optimized reaction conditions [20] at atmospheric pressure and 40 °C. The activity of the biocatalyst was determined from the difference in conversion after 72 hours of the reaction, catalyzed by untreated lipase and in conversion after 72 hours of the reactions, catalyzed by lipases treated at different temperatures at atmospheric pressure, as shown in Figure 1.

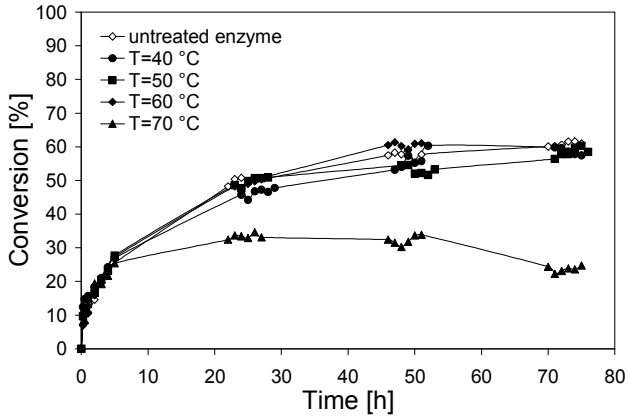

Figure 1 : Conversion of palmitic acid in 2-methyl-2-butanol at 40°C and atmospheric pressure of fructose palmitate synthesis, catalyzed by untreated lipase and lipase that was exposed to different temperatures at atmospheric pressure for 24 h.
Reaction conditions: equimolar (20 mmol) fructose and palmitic acid, 59% (w/w of reaction mixture) 2-methyl-2-butanol, 10% (w/w of substrates) lipase, 12% (w/w of reaction mixture)molecular sieves, 40 °C, 600 rpm.

The residual activity of Novozym 435 increased with incubation temperature from 40 to 60 °C after 24 h of reaction performance. The results show that there is no residual activity change in initial reaction rates. With further increase of incubation temperature a decrease in residual activity was observed at 70 °C after 24 h of reaction performance which was connected with a thermal deactivation of the lipase and with water distribution in the system. The water content of the immobilized lipase was measured by Karl Fisher titration method. Untreated lipase contained 1.44% water, while at atmospheric pressure and at 70 °C pre-incubated lipase contained only 0.58% water.

Thermal stability of lipase from *Candida antarctica B* in SC CO_2

To determine the stability of lipase Novozym 435 in SC CO_2, lipase was exposed to carbon dioxide at 10 MPa and temperatures between 40 and 60 °C for 24 h. After slow

depressurization, the preincubated lipase was used as biocatalyst for the synthesis of fructose palmitate in 2-methyl-2-butanol at atmospheric pressure and 40 °C (Figure 2).

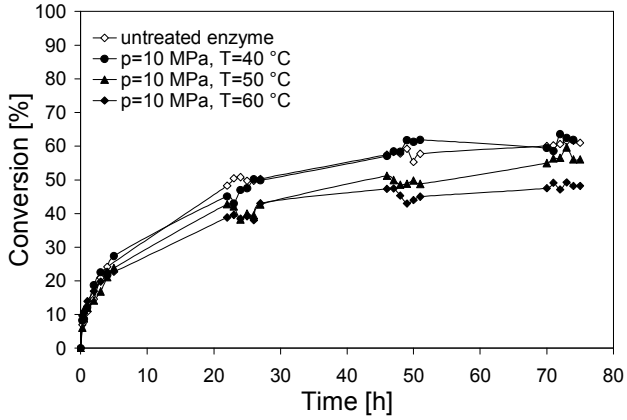

Figure 2 : Conversion of palmitic acid in 2-methyl-2-butanol at 40°C and atmospheric pressure of fructose palmitate synthesis, catalyzed by untreated lipase and lipase that was exposed to different temperatures in SC CO_2 at 10 MPa for 24 h.
Reaction conditions: 20 mmol fructose, 20 mmol palmitic acid, 59% (w/w of reaction mixture) 2-methyl-2-butanol, 10% (w/w of substrates) lipase, 12% (w/w of reaction mixture) molecular sieves, 40 °C, 600 rpm.

Results showed that the residual activity of Novozym 435 decreased with incubation temperature from 40 to 60 °C after 24 h of reaction performance. Decreased lipase residual activity at temperatures higher than 40 °C can be explained by removing of essential water for enzyme from its vicinity. At higher temperatures water was extracted from the enzyme microenvironment by the SC CO_2, therefore lower temperature optimum (40 °C) was determined compared to the atmospheric pressure (60 °C). SC CO_2 can dissolve 0.3 to 0.5% (w/w) water, depending on the temperature and pressure of the system [21].
Untreated lipase contained 1.44% water, while the lipase which was previously exposed in SC CO_2 at 60 °C contained only 0.88% water, as it was measured by the Karl-Fischer method.
Obtained results are in agreement with published results of activity of proteinase from *Carica papaya* latex treated with SC CO_2 at 30 MPa for 24 h, where temperature optimum was 40 °C [22].

Temperature effect on the lipase activity in 2-methyl-2-butanol at atmospheric pressure and in SC CO_2
Because Novozym 435 was found to be stable in 2-methyl-2-butanol at atmospheric pressure and in SC CO_2, enzymatic synthesis of fructose palmitate was performed in these two media. Effect of temperature on enzyme activity was studied in 2-methyl-2-butanol at temperatures from 30 to 70 °C at atmospheric pressure and in SC CO_2 at temperatures from 60 to 110 °C at 10 MPa. Reactions were performed for 24 hours. The results are shown in Figure 3.

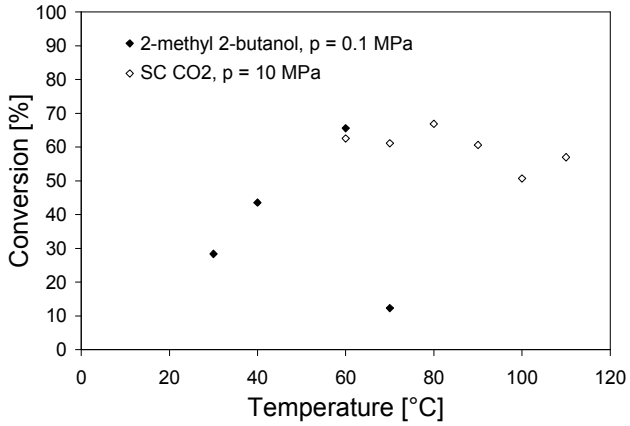

Figure 3 : Temperature effect on conversion of palmitic acid in 2-methyl-2-butanol at atmospheric pressure and in SC CO_2 at 10 MPa after 24 h.

Conversion of palmitic acid in 2-methyl-2-butanol at atmospheric pressure increased with increasing temperature to 60 °C what resulted in 65% conversion after 24 hours. At higher temperature (70 °C) enzyme deactivation occurred. Optimal temperature in SC CO_2 at 10 MPa was found to be 80 °C what resulted in 67 % conversion after 24 h.
High conversion yields of palmitic acid obtained in SC CO_2 at higher temperatures could be due to increased solubility of palmitic acid at high pressures [23] and increased solubility of D-(-) fructose at high temperatures and 10 MPa [24].

Synthesis of fructose palmitate, fructose laurate and sucrose laurate in SC CO_2
Although optimal temperature for fructose palmitate production in SC CO_2 was found to be 80 °C, coloring of the product occurred at high temperatures. Therefore, the synthesis of fructose and sucrose laurate in SC CO_2 was performed at 60 °C and 10 MPa (Figure 4).

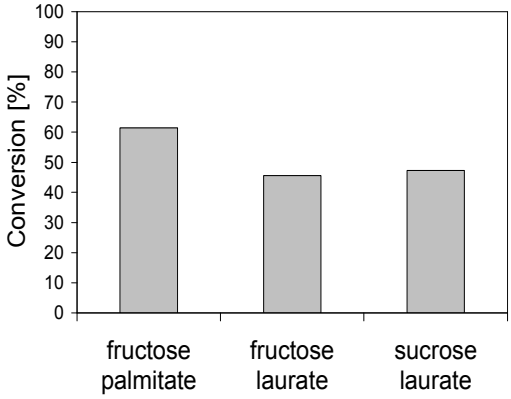

Figure 4 : Synthesis of different fatty acid sugar esters in SC CO_2.
Reaction conditions: equimolar (20 mmol) sugar and fatty acid, 10% (w/w of substrates) lipase, 60 °C, 10 MPa, 600 rpm, 24 h.

The highest conversion (61%) was obtained for fructose palmitate synthesis. Lower conversions were obtained for fructose laurate synthesis (46%) and sucrose laurate synthesis (47%).

CONCLUSION

Lipase-catalyzed synthesis of different sugar fatty acid esters was performed in high yields in relatively short times in SC CO_2, which is recognized as a safe reaction medium due to its nontoxibility and nonflammability. Synthesized sugar esters are not of interest only to food industry, but also to cosmetic and pharmaceutical industries.

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