Enzymatic Synthesis of Sugar Fatty Acid Esters in Supercritical Carbon Dioxide and their Antibacterial Activity

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To overcome the drawbacks of enzymatic synthesis in organic solvents, synthesis of different applicable sugar fatty acid esters, such as sucrose laurate, sucrose palmitate, fructose laurate and fructose palmitate was performed in SC CO_2 in high yields. Antibacterial activity of commercial and enzymatically synthesized sugar fatty acid esters against food pathogenic bacteria *Bacillus cereus* was determined. Sucrose laurate, commercial and enzymatically synthesized, almost completely inhibited the growth of *Bacillus cereus* in concentration of 9.375 mg/ml.

INTRODUCTION

Sugar esters of long-chain fatty acids are nonionic surfactants which are widely used as emulsifiers in food industry [1, 2]. Furthermore, their antimicrobial properties [3-6] may open new possibilities for their use, especially as food preservatives.
Applications of commercial, chemically synthesized sorbitan and sucrose esters are limited because they are produced at high temperatures in toxic solvents which leave traces in the products [7, 8]; therefore biocatalysis is researched as an alternative. Enzymatic synthesis can be performed under mild reaction conditions, which compared to the chemical synthesis minimize side reactions and browning of the products [9, 10]. Catalytic performance of the enzymes in terms of conversion and enantioselectivity strictly depends on the characteristics of the media, therefore suitable solvents for lipase-catalyzed synthesis have to be chosen.
Supercritical carbon dioxide (SC CO_2) may provide an interesting alternative medium for enzymatic synthesis since it exhibits properties similar to those of some organic solvents and due to its high selectivity it can be considered as a medium for the reaction and the simultaneous separation of the products [11]. Due to its low critical temperature which prevents thermal denaturation when processing biological compounds it presents the most suitable supercritical fluid. Being nontoxic and nonflammable, carbon dioxide provides an interesting solvent for enzymatic synthesis leaving no residues in the products, which has a great importance for the production of foodstuff [12-14].
Esterification of different sugar fatty acid esters, catalyzed by immobilized lipase B from *Candida antarctica* was performed in SC CO_2 at 10 MPa.
Growth inhibitory effect of sucrose and fructose fatty acid esters, enzymatically synthesized in SC CO_2 on Gram-positive bacteria *Bacillus cereus*, was tested. In addition, antibacterial activities of enzymatically synthesized fructose and sucrose fatty acid esters were compared to antibacterial activities of commercial sucrose fatty acid esters.

MATERIALS AND METHODS

Enzyme, chemicals and microorganisms

Immobilized lipase B from *Candida antarctica* (Novozym 435) was a gift from Novo Nordisk AS (Copenhagen, Denmark).

D-(-) fructose (≥ 98%), molecular sieves (3 Å) and sucrose monolaurate (≥ 97%) were obtained from Fluka (Buchs, Switzerland). Palmitic acid (min 98%) was obtained from Riedel de Haën (Seelze, Germany). Sucrose (99+%), D-(+)-glucose (min 99% GC) and agar were supplied from Sigma (Deisenhofen, Germany). Lauric acid (for synthesis), sodium hydroxide solution (0.1 N), ethanol (absolute), ethanol (96%), sodium chloride (GR for analysis), meat extract (dry, granulated, for microbiology), peptone from meat (pancreatically digested, granulated, for microbiology) and phenolphthalein were purchased from Merck (Darmstadt, Germany). Carbon dioxide 4.5 (purity 99.995 vol. %) was supplied from Messer MG (Ruše, Slovenia).

Gram-positive bacteria *Bacillus cereus* (ATCC 11778) was obtained from stock cultures at the Department of Agricultural Chemical Technology (Budapest University of Technology and Economics, Hungary).

Synthesis in SC CO_2

Lipase-catalyzed synthesis of sugar fatty acid esters was performed in a 100 mL high-pressure batch stirred-tank reactor at temperature of 60 °C and stirring rate of 600 rpm. Reaction mixture consisted of 20 mmol of sugar and 20 mmol of fatty acid. 10 % (w/w of substrates) lipase was added to the reaction mixture. Cooled liquid carbon dioxide was pumped into the reactor 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.

Analytical methods

The ester content was quantified by calculating the residual fatty acid amount in the reaction mixture, which was analyzed by high-pressure liquid chromatography (HPLC) [15] and by volumetric titration [16].

Purification of sugar esters

After the synthesis was performed in SC CO_2, separation of sugar esters from reaction mixture was accomplished through liquid-liquid extraction with ethanol. Then, immobilized lipase, molecular sieves and unreacted sugar were separated by centrifugation and ethanol was evaporated under vacuum.

Antimicrobial tests

Antimicrobial activity of sugar fatty acid esters against bacteria *B. cereus* was determined [17, 18].

Analyses of antimicrobial activity of commercial and enzymatically synthesized sugar fatty acid esters were performed using broth containing 5 g/l meat peptone, 3 g/l meat extract, 5 g/l glucose and 0.5 g/l sodium chloride. The medium was sterilized for 10 min at 121 °C. Liquid medium was inoculated with bacteria and incubated for 24 h at 30 °C. Prepared solution of *B. cereus* (1 ml of a solution was containing approximately 10^8 cells/ml) was used to inoculate the test mediums (50 ml) containing 1 ml solution of sugar fatty acid esters.

Antimicrobial activity was determined by measuring the turbidity of microorganism suspension on UV/VIS spectrophotometer at 550 nm at defined time intervals. The growth curves of bacteria in the sample containing sugar fatty acid ester were compared with those obtained in a medium without sugar fatty acid ester, containing only 2% (w/v) of solvent (alcohol control). A bacteria control without addition of sugar fatty acid ester was measured, as well. The inhibition was calculated as the optical density of the sample containing bacteria and sugar fatty acid ester at defined time of measurement (ODx) compared to the optical density of alcoholic control at defined time of measurement (OD_{AC}).

$$\text{Inhibition (\%)} = [(OD_{AC} - OD_x) / OD_{AC}] \times 100 \quad (1)$$

All the results were calculated as a mean value of three parallel measurements.

RESULTS

Due to drawbacks of enzymatic synthesis in organic solvents at atmospheric pressure, lipase-catalyzed synthesis of various sucrose and fructose fatty acid esters, applicable as emulsifiers in foodstuff and for personal care products, was performed in SC CO_2, which presents a safe reaction media leaving no residues in the products.

Furthermore, antibacterial activity of various sugar fatty acid esters, enzymatically synthesized in SC CO_2 against foodborne bacteria *B. cereus* was investigated.

Synthesis of different sugar fatty acid esters in SC CO_2

Different applicable sugar fatty acid esters such as fructose palmitate, fructose laurate, sucrose laurate and sucrose palmitate were synthesized in SC CO_2 at 60 °C and 10 MPa. Enzymatic synthesis was performed with addition of 46 % (w/w of substrates) molecular sieves for absorption of water, generated during esterification reaction and compared to the reaction performed without any addition of molecular sieves (Figure 1).

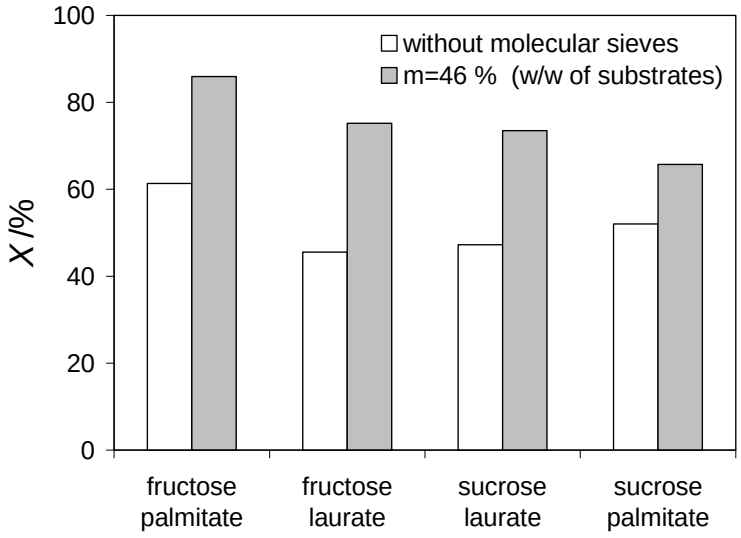

Figure 1 : Sugar fatty acid ester synthesis in SC CO_2.
Reaction conditions: equimolar mixture of sugar and fatty acid (20 mmol), 10% (w/w of substrates) lipase, 60 °C, 10 MPa, 600 rpm, 24 h.

When synthesis was performed in the presence of molecular sieves, up to 30 % higher conversion was obtained compared to the synthesis without addition of molecular sieves. The highest conversion (86 %) after 24 h of reaction was obtained for fructose palmitate synthesis in the presence of 46 % (w/w of substrates) of molecular sieves.

Antimicrobial activity of sugar fatty acid esters against bacteria *B. cereus*

Since there are only few studies of antimicrobial activity of lipase-synthesized sugar fatty acid esters against food borne bacteria [6] and against bacteria causing dental caries [4, 5], the effect of enzymatically synthesized sugar fatty acid esters on the growth of bacteria *B. cereus* was determined and compared to the antimicrobial activity of commercial sugar fatty acid esters.

Antibacterial activitity of commercial sucrose monolaurate, as well as fructose laurate and sucrose laurate (mixture of mono- and diesters), enzymatically synthesized in SC CO_2 for 24 h, in concentration of 9.375 mg/mL EtOH in comparison with an alcoholic control against *B. cereus* is shown in Figure 2.

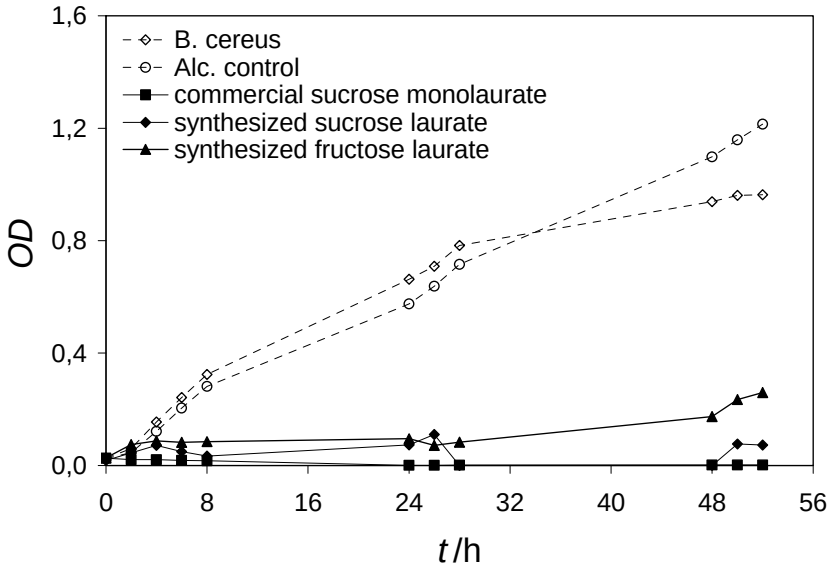

Figure 2 : Antimicrobial activity of enzymatically synthesized sucrose and fructose fatty acid esters in comparison with antimicrobial activity of commercial sucrose fatty acid ester.

Commercial sucrose monolaurate and enzymatically synthesized sucrose laurate (mixture of mono- and diesters) are highly efficient against bacteria *B. cereus* (99.8 % and 93.4 % inhibition after 2 days of growth, respectively), whereas lower inhibition (86 % after 2 days of growth) was detected with enzymatically synthesized fructose laurate (mixture of mono- and diesters).

To decrease the production costs of the products, suitable as inhibitors of potentially pathogen microorganisms in foodstuff, synthesis was performed for 5 h and antimicrobial activity of the products was compared to the antimicrobial activity of the products synthesized for 24 h. Antibacterial activitity of sucrose laurate (mixture of mono- and diesters), enzymatically synthesized in SC CO_2 for 5 h and 24 h in concentration of 9.375 mg/mL EtOH in comparison with an alcoholic control against *B. cereus* is shown in Figure 3.

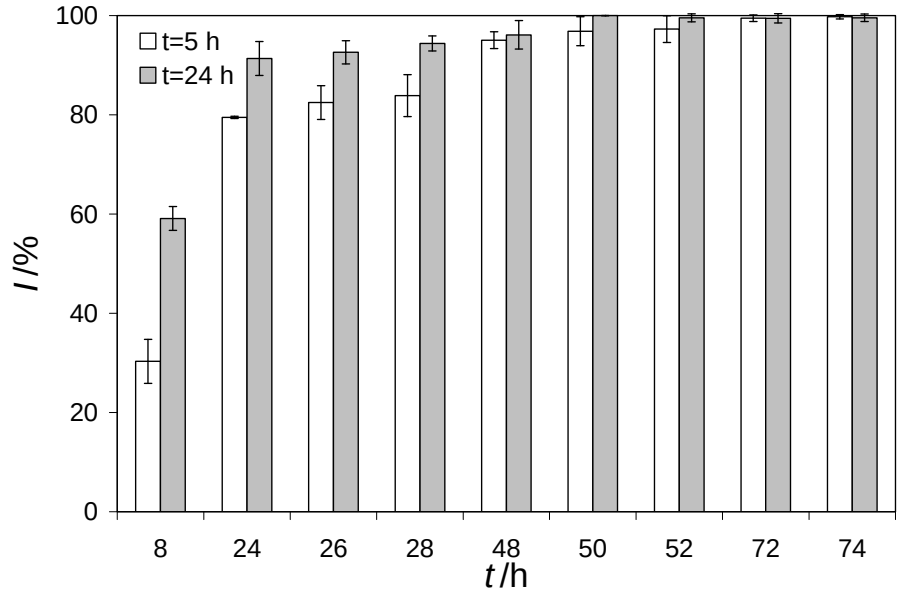

Figure 3 : The inhibitory effect of enzymatically synthesized sucrose laurate (mixture of mono- and diesters) after 5 h and 24 h of reaction performance in SC CO_2 against bacteria *Bacillus cereus* in comparison with ethanol control.

In the first 28 h of incubation, 10 % higher inhibition was obtained with sucrose laurate (mixture of mono- and diesters), which was enzymatically synthesized in SC CO_2 for 24 h. The difference in inhibition between products, enzymatically synthesized for 5 h and 24 h was smaller in the second day of incubation (about 3 %). After 52 h of incubation, there was almost no difference in obtained inhibition against *B. cereus* for both products, synthesized for 5 h or 24 h.

CONCLUSION

The results showed that SC CO_2 represents a promising alternative solvent for the synthesis of sugar fatty acid esters, especially sucrose esters, which can't be synthesized in high yields in organic solvents due to a low solubility of sucrose in tertiary alcohols and ketones. Carbon dioxide is nontoxic and nonflammable, therefore it presents a suitable solvent for the preparation of food additives.
Since a demand for production of novel compounds able to inhibit the growth of microorganisms cousing food spoilage is rising, sugar fatty acid esters, enzymatically synthesized in SC CO_2 present a promising potential in food preservation. Sucrose esters and fructose esters of fatty acids were both very effective against Gram-positive foodborne bacteria *B. cereus*.

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