SAŠA ŠABEDER
MAJA HABULIN
ŽELJKO KNEZ

University of Maribor,
Faculty of Chemistry and
Chemical Engineering,
Laboratory for Separation
Processes,
Maribor, Slovenia

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612.015.1+665.12:664.165+
+66.061.547

THE LIPASE–CATALYZED SYNTHESIS OF FATTY ACID FRUCTOSE ESTERS IN ORGANIC MEDIA AND IN SUPERCRITICAL CARBON DIOXIDE

Sugar fatty acid esters are biodegradable surface active compounds in foodstuffs and cosmetics or pharmaceuticals. They have potential in replacing pollutant chemically synthesized surfactants. The enzymatic synthesis of fructose palmitate catalyzed by Candida antarctica B lipase was performed in different organic media in a batch reactor at atmospheric pressure. The influence of the organic solvent and temperature on the esterification was studied. Since supercritical carbon dioxide (SC CO_2) has several advantages over organic solvents, such as high reaction rate, high mass transfer, non–toxicity, non–flammability and low price, it was also chosen as a reaction medium for fructose palmitate production. The influence of temperature on immobilized lipase activity was studied at 10 MPa and the results were compared to the results obtained from reactions performed at atmospheric pressure under the same reaction conditions. The highest conversion (67%) was obtained after 24 hours of reaction in SC CO_2 at 80°C. A change of the particle size distribution and morphology of the untreated lipase and lipase treated with 2–methyl 2–butanol and SC CO_2 was observed.

Key words: Fructose palmitate, Surfactant, Candida antarctica lipase, Esterification, Organic solvent, Supercritical carbon dioxide.

Sugar fatty acid esters are non–ionic surfactants, which are mostly used for personal care products, cosmetic applications and as emulsifiers for foodstuffs [1]. Fructose monoesters also have antibacterial properties and suppress the cell growth of *Streptococcus mutans* [2], while sucrose esters inhibit the growth of *Escherichia coli* [3].

The large–scale chemical esterification between fatty acids and sugars is performed at high temperatures in the presence of an alkaline catalyst, which is accompanied by high energy consumption, low selectivity toward the various hydroxyl groups in sugars and caramelization of the product [4].

In recent years, the use of enzymes as an alternative for the synthesis of sugar fatty acid esters has been investigated, emphasizing the synthesis of regioselective products. Carbohydrate fatty acid esters can be enzymatically synthesized from renewable substrates under mild reaction conditions, which compared to the chemical process, minimizes side reactions. Furthermore, the undesirable browning of the products may be avoided. Moreover, the enzymatic synthesis can be performed in less toxic solvents compared to those used in the chemical synthesis [5].

Supercritical fluids (SCF) can offer many advantages over organic solvents and are therefore attracting much interest as possible media for enzymatic reactions. Supercritical carbon dioxide (SC CO_2) is the most suitable SCF because of its relatively low critical pressure and temperature (7.3 MPa and 31°C) and its low cost. The solubility of solutes can easily be controlled by regulating temperature and pressure. Being non–toxic and non–flammable, carbon dioxide is interesting to the food and pharmaceutical industries as a safe SCF medium [6–10]. Since it is a gas at room temperature, the solvent can be easily removed without leaving any residues in the product. SC CO_2 has a high diffusivity, low viscosity and low surface tension which allow easy penetration into macro and microporous materials.

Because the enzymatic syntheses of fatty acid sugar esters are mostly catalyzed by immobilized lipases, these enhanced transport properties of SC CO_2 make it attractive when mass transfer in the immobilization matrix is rate limiting. Another advantage of SC CO_2 compared to organic media is that the addition of molecular sieves, which are necessary for the removal of water generated during the esterification, is not required. The addition of molecular sieves is not practical on a larger scale, because it increases the reactor volume and mass transfer is limited due to difficult stirring [1]. Another disadvantage of using molecular sieves is that their addition to the reaction mixture leads to the synthesis of fructose dipalmitate, while only fructose monopalmitate is produced without molecular sieves [11].

The synthesis of fructose palmitate catalyzed by immobilized lipase from *Candida antarctica* B was performed in SC CO_2. The stability of immobilized enzymes in SC CO_2 has proven to be good and similar to that obtained in liquid organic solvents [7].

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Author address: Ž. Knez, University of Maribor, Faculty of Chemistry and Chemical Engineering, Laboratory for Separation Processes, Smetanova 17, SI–2000 Maribor, Slovenia
E-mail: zeljko.knez@uni–mb.si
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Furthermore, easy separation of the unreacted fatty acid from the sugar ester seems to be feasible in this system [12]. However, the solubility of apolar compounds, such as fructose, in carbon dioxide is limited and consequently, various methods have been suggested, such as the addition of co–solvents or preadsorption of the polar compound onto an inert material with high internal surface [13–15].

The aim of this study was to optimize the temperature of the lipase–catalyzed synthesis of fructose palmitate and to find an appropriate organic solvent for the reaction performed at atmospheric pressure. The synthesis of fructose palmitate was also performed in SC CO_2. The influence of temperature on enzyme activity and the effect of co–solvent on the reaction rate were investigated.

MATERIALS AND METHODS

Enzymes and chemicals

Immobilized lipase Novozym 435 from *Candida antarctica* B (EC 3.1.1.3) was kindly donated from Novo Nordisk AS (Copenhagen, Denmark). D–(–) Fructose (≥ 98%), 2–methyl 2–butanol (98%), ethyl methyl ketone (≥ 99.5% (GC)) and molecular sieve (3 Å) were purchased from Fluka (Buchs, Switzerland). Palmitic acid (min 98%) was obtained from Riedel de Haën (Seelze, Germany). Acetone (absolute) and *t*–butanol (99%) were supplied from Aldrich (Deisenhofen, Germany). The sodium hydroxide solution (0.1 N) was obtained from Merck (Darmstadt, Germany) and phenolphthalein from Kemika (Zagreb, Croatia). Carbon dioxide 4.5 (purity 99.995 vol. %) was supplied from Messer MG Ruše, Slovenia).

Synthesis of fructose palmitate in organic solvent at atmospheric pressure

The reaction mixture consisted of 20 mmol fructose and 20 mmol palmitic acid and 59% (w/w of reaction mixture) organic solvent as adjuvant. Molecular sieves were added for the absorption of water, generated during the esterification reaction. The 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). The reaction was started by addition of the lipase. Samples from the reaction mixture were taken at intervals and the level of free fatty acid (FFA) was determined. A control reaction without lipase addition was also considered for the reaction mixtures and it was treated the same way as the reaction mixture.

Synthesis of fructose palmitate in SC CO_2

The reaction mixture consisted of 10 mmol fructose and 10 mmol 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 at a stirring rate of 600 rpm. Cooled liquid carbon dioxide was pumped into the reactor up to 10 MPa. The reaction was terminated by depressurisation of SC CO_2 and the reaction mixture was analyzed. A schematic diagram of the high pressure device is shown in Figure 1.

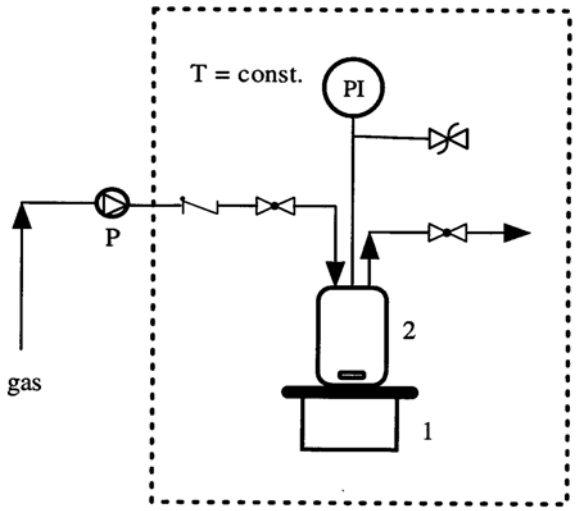

Figure 1. Schematic diagram of the SC CO_2 apparatus: 1–magnetic stirrer and heater; 2–reactor; P–high pressure pump; PI–pressure indicator.

Analyses

Samples were analyzed qualitatively by thin layer chromatography (TLC) [16]. TLC analysis was performed on silica gel plates $60F_{254}$ (Merck, Germany).

The ester content was quantified by calculating the residual fatty acid amount in the reaction mixture, which was determined by volumetric titration [17]. 0.1 g of sample of the reaction mixture was diluted in 20 mL of 0.1 wt% phenolphthalein solution in absolute ethanol and then titrated with standardized sodium hydroxide solution (0.1 N).

The particle size distribution of untreated and treated lipase was determined by laser diffraction using a Malvern Mastersizer 2000 (Malvern Instruments Ltd, Malvern, UK) [21]. All analyses were performed in triplicate.

The morphology of the untreated and treated lipase was analyzed by a LEO Gemini 1530 Scanning Electron Microscope (SEM).

RESULTS AND DISCUSSION

Synthesis in organic solvents at atmospheric pressure

In order to select a solvent in which the substrates will be dissolved in a yield high enough to react and at the same time will not influence the lipase activity and stability, different organic solvents were tested. The synthesis of fructose palmitate was performed in ethyl methyl ketone (EMK), acetone, 2–methyl 2–butanol and

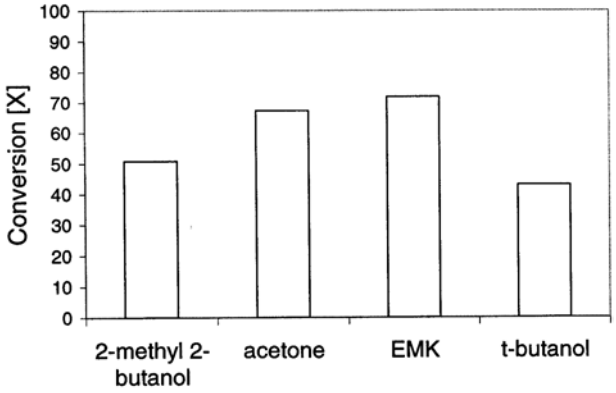

Figure 2. Influence of organic solvents on the production of fructose palmitate, reaction conditions: 20 mmol fructose, 20 mmol palmitic acid, 24 mL organic solvent, 10% lipase, 12% molecular sieve, 40 °C, 600 rpm, 24 h

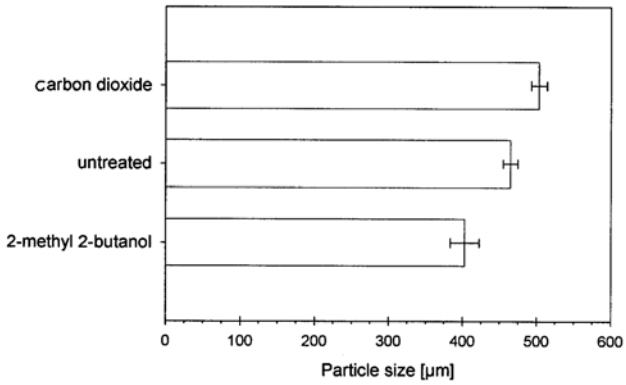

Figure 3. Mean particle size of Candida antarctica lipase (P=0.95, n=3)

t–butanol for 24 hours at 40 °C. The influence of organic solvent on fructose palmitate production is shown in Figure 2.

The best results for the synthesis of fructose palmitate were obtained in EMK (72%) after 24 hours. A conversion of 67% was achieved in acetone, in 2–methyl 2–butanol (51%) and in *t*–butanol (43%). The obtained results are in agreement with published results for glucose palmitate production catalyzed by the same lipase [16].

Rapid solidification of the samples occurred when the syntheses were performed in EMK and acetone which made the analyses difficult. The use of *t*–butanol is restricted in food production [1], therefore all further reactions were performed in 2–methyl 2–butanol. The selected organic solvent is non–toxic and a suitable food solvent [5] in which lipases have proved to have good stability [18].

Particle size distribution of lipase from *Candida antarctica* B

Because some of the organic solvents are deleterious to lipases, lipase Novozym 435 from *Candida antarctica* B was incubated in 2–methyl 2–butanol for 24 hours at 60°C. The particle size distribution of the untreated lipase and lipase which was previously treated in the chosen solvent was determined in order to determine the stability of the lipase in the selected medium.

Although high yields of fructose palmitate can be obtained by enzymatic synthesis in selected organic solvents, the method is difficult to scale up. The removal of water from the reaction mixture by the addition of large concentration of molecular sieves increases the production costs. The synthesis of fructose palmitate in 2–methyl 2–butanol without molecular sieve at 60°C leads to poor conversion yields of 14% after 72 hours of reaction (data not shown). Therefore, the synthesis was also performed in SC CO_2 where the reaction can be performed in high yields without addition of molecular sieve [12]. For that reason, lipase was also incubated in SC CO_2 at 60°C and 10 MPa for 24 hours. Figure 3 shows the change of the mean particle size by treatment with carbon dioxide or 2–methyl 2–butanol.

The mean particle size of lipase treated in 2–methyl 2–butanol was smaller (408 μm) compared to the untreated lipase (464 μm). The reason for the decrease in the mean particle size of lipase could be that 2–methyl 2–butanol is deleterious to immobilized *Candida antarctica* lipase, resulting in the partial dissolution of its carrier. The mean particle size of lipase treated in SC CO_2 was larger (508 μm). This phenomenon could be explained by swelling of the immobilized lipase.

The change of lipase morphology by treatment with carbon dioxide or 2–methyl 2–butanol was analysed by SEM.

The images of untreated lipase and lipase treated with carbon dioxide and 2–methyl 2–butanol are shown in Figure 4.

The SEM images of untreated and treated lipase showed that the morphology of the immobilized lipase was not affected by 2–methyl 2–butanol or SC CO_2 within 24 hours.

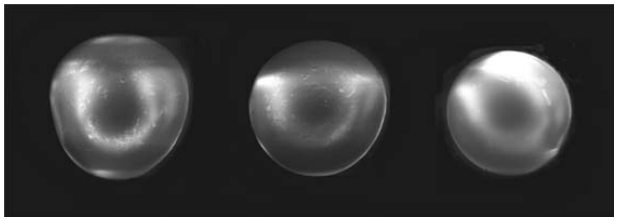

Figure 4. SEM of untreated (left) and treated lipase (middle: treated with carbon dioxide, right: treated with 2–methyl 2–butanol)

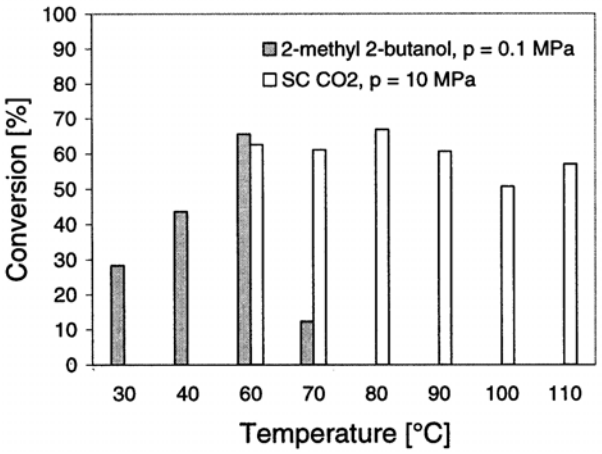

Figure 5. Influence of temperature on the fructose palmitate synthesis.

Effect of temperature on the lipase activity in organic solvent and in SC CO_2

The synthesis of fructose palmitate in 2–methyl 2–butanol was performed at temperatures from 30 to 70 °C at atmospheric pressure and a rotational speed of 600 rpm. Esterification was also performed in SC CO_2 at temperatures from 60 to 110°C at 10 MPa and 600 rpm. The reactions were performed for 24 hours. The results are summarised in Figure 5.

The optimal temperature for fructose palmitate production in 2–methyl 2–butanol at atmospheric pressure was 60 °C which resulted in 65% conversion after 24 hours of reaction. Enzyme deactivation occurred at higher temperatures. Optimal temperature in SC CO_2 at 10 MPa was found to be 80 °C. A conversion of 67% was obtained after 24 hours of reaction, which was somewhat higher than obtained in organic solvent at 60°C. The published results regarding *Candida antarctica* lipase catalyzing the synthesis of glucose with lauric acid showed increased productivity with increasing temperature up to 70°C [12].

The highest conversion in SC CO_2 was obtained at higher temperature (80°C) compared to the reaction performed in 2–methyl 2–butanol at atmospheric pressure. The reason for enzyme deactivation at atmospheric pressure at temperatures higher than 60°C could be that the solvent used in the lipase–catalyzed synthesis is deleterious to *Candida antarctica* lipase at high temperatures, resulting in partial or complete deactivation. High conversion yields for fructose palmitate synthesis obtained in SC CO_2 at high temperatures could be due to the increased solubility of palmitic acid at high pressures [19] and the increased solubility of D–(–) fructose at high temperatures at a pressure of 10 MPa [20]. The Increased stability of the enzyme in SC CO_2 could be due to the rigidity of the immobilized lipase in SC CO_2 at high temperatures. When the synthesis of fructose palmitate was performed in 2–methyl 2–butanol at temperatures higher than 60°C, forces which stabilize the structure of the protein probably decreased in strength leading to the breakdown of its active conformation.

Effect of co–solvent on fructose palmitate production in SC CO_2

The addition of 2–methyl 2–butanol to SC CO_2 to enhance the solubility of fructose in non–polar SC CO_2 was investigated. The synthesis of fructose palmitate was performed at 60°C and 16 MPa varying the concentration of co–solvent between 20 and 50 mol%. The effect of cosolvent concentration on the conversion of palmitic acid after 24 hours of reaction is shown in Figure 6.

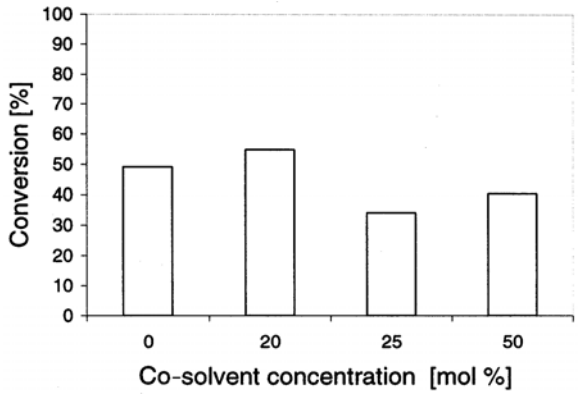

Figure 6. Influence of the co–solvent concentration on conversion of palmitic acid in SC CO_2, reaction conditions: 10 mmol fructose, 10 mmol palmitic acid, 10% lipase, 60°C, 16 MPa, 600 rpm, 24 h

The enhanced solvation power of SC CO_2 was observed when 20 mol % of 2–methyl 2–butanol were used as a cosolvent which resulted in 55% conversion of palmitic acid. With further increase of the co–solvent concentration, a decrease in the final conversion was observed. Increasing the concentration of co–solvent, the critical point of CO_2 and the co–solvent mixture changes and the supercritical phase is changed into a high–pressured liquid phase which results in a decrease of the mass transfer rate [15].

CONCLUSION

The lipase–catalyzed synthesis of fructose palmitate was performed in different organic solvents at atmospheric pressure, finding 2–methyl 2–butanol the most suitable one. Esterification was also performed in SC CO_2 resulting in conversion yields slightly higher than in 2–methyl 2–butanol after 24 hours of reaction. The yield was further increased by adding a small concentration of co–solvent to SC CO_2 at 16 MPa. The advantages of carbon dioxide as a new solvent for the enzymatic synthesis of sugar fatty acid esters make it a promising alternative to conventional organic solvents.

REFERENCES

[1] Y. Yan, U.T. Bornscheuer, R.D. Schmid, Lipase–catalyzed solid–phase synthesis of sugar fatty acid esters, Enzyme Microb. Technol. **25** (1999) 729–285.

[2] T. Watanabe, S. Katayama, M. Matsubara, Y. Honda, M. Kuwahara, Antibacterial carbohydrate monoesters suppressing cell growth of *Streptococcus mutans* in the presence of sucrose, Curr. Microbiol. **41** (2000) 210–213.

[3] A. Kato, K. Arima, Inhibitory effect of sucrose ester of lauric acid on the growth of *Escherichia coli*, Biochem. bioph. res. co. **42** (1971) 596–601.

[4] A.M. Klibanov, Asymetric transformations catalyzed by enzymes in organic solvents, Acc. Chem. Res. **23** (1990) 114–121.

[5] N. Khaled, D. Montet, M. Farines, M. Pina, J. Graille, Synthesis of sugar mono–esters by biocatalysis, Oleagineux **47** (1992) 181–190.

[6] A. Marty, W. Chulalaksananukul, R.M. Willemot, J.–S. Condoret, Kinetics of lipase–catalyzed esterification in supercritical CO_2, Biotechnol. Bioeng. **39** (1992) 273–280.

[7] A. Marty, D. Combes, J.–S. Condoret, Continuous reaction–separation process for enzymatic esterification in supercritical carbon dioxide, Biotechnol. Bioeng. **43** (1994) 497–504.

[8] M.D. Romero, L. Calvo, C. Alba, M. Habulin, M. Primožič, Ž. Knez, Enzymatic synthesis of isoamyl acetate with immobilized *Candida antarctica* lipase in supercritical carbon dioxide, J. Supercrit. Fluid **33** (2005) 77–84.

[9] S. Srivastava, G. Madras, J. Modak, Esterification of myristic acid in supercritical carbon dioxide, J. Supercrit. Fluid **27** (2003) 55–64.

[10] H. Vija, A. Telling, V. Tougu, Lipase–catalyzed esterification in supercritical carbon dioxide and in hexane, Bioorg. Med. Chem. Lett. **7** (1997) 259–262.

[11] F. Chamouleau, D. Coulon, M. Girardin, M. Ghoul, Influence of water activity and water content on sugar esters lipase–catalyzed synthesis in organic media, J. Mol. Catal. B **11** (2001) 949–954.

[12] C. Tsitsimpikou, H. Stamatis, V. Sereti, H. Daflos, F.N. Kolisis, Acylation of glucose catalyzed by lipases in supercritical carbon dioxide. J. Chem. Technol. Biotechnol. **71** (1998) 309–314.

[13] T.W. Randolph, D.S. Clark, H.W. Blanch, J.M. Prausnitz, Enzymatic oxidation of cholesterol aggregates in supercritical carbon dioxide, Science **239** (1988) 387–390.

[14] E. Castillo, A. Marty, D. Combes, J.S. Condoret, Polar substrates for enzymatic reactions in supercritical CO_2: how to overcome the solubility limitations, Biotecnol. Lett. **16** (1994) 169–174.

[15] J.–H. Heo, S.Y. Kim, K.–P. Yoo, Enzymatic preparation of a carbohydrate ester of medium–chain fatty acid in supercritical carbon dioxide, Biotecnol. Lett. **22** (2000) 995–998.

[16] L. Cao, A. Fischer, U.T. Bornscheuer, R.D. Schmid, Lipase–catalyzed solid phase synthesis of sugar fatty acid ester, Biocatal. Biotransform. **14** (1997) 269–283.

[17] M. Leitgeb, Ž. Knez, The influence of water on the synthesis of n–butyl oleate by immobilized Mucor miehei lipase, J. Am. Oil Chem. Soc. **67** (1990) 775–778.

[18] M.V. Flores, K. Naraghi, J.–M. Engasser, P.J. Halling, Influence of glucose solubility and dissolution rate on the kinetics of lipase catalyzed synthesis of glucose laurate in 2–methyl 2–butanol, Biotechnol. Bioeng. **78** (2002) 814–820.

[19] T. Bamberger, J.C. Erickson, C.L. Cooney, Measurement and model prediction of solubilities of pure fatty acids, pure triglycerides, and mixtures of triglycerides in supercritical carbon dioxide, J. Chem. Eng. Data **33** (1988) 327–333.

[20] J.–S. Yau, F.–N. Tsai, Solubilities of D–(–) fructose and D(+)–glucose in subcritical and supercritical carbon dioxide, J. Supercrit. Fluid **7** (1994) 129–133.

[21] International Standard ISO13320–1: Particle size analysis – Laser diffraction methods. Part 1: General principles, 1999.

IZVOD

SINTEZA ESTRA FRUKTOZE I MASNIH KISELINA KATALIZOVANE LIPAZAMA U PRISUSTVU NATKRITIČNOG UGLJEN DIOKSIDA

(Naučni rad)

Saša Šabeder, Maja Habulin, Željko Knez
Univerzitet u Mariboru, Fakultet za hemiju i hemijsko inženjerstvo, Laboratorija za separacione procese, Maribor, Slovenija

Estri šećera i masnih kiselina su biorazgradive površinski aktivne materije čija je primena značajna u proizvodnji hrane, kozmetičkih preparata i lekova. To su potencijalne substance koje se mogu iskoristiti umesto nekih drugih hemijski sintetizovanih površinski aktivnih materija koji se danas smatraju da su zagađivači životne sredine. Enzimska sinteza palmitata fruktoze katalizovane lipazom *Candida antarctica* B analizirana je u šaržnom reaktoru pod atmosferskim pritiskom u prisustvu različitih organskih rastvarača pri čemu je proučavan uticaj organskog rastvarača i temperature na brzinu esterifikacije, s obzirom da natkritični ugljen dioksid (SC CO_2) poseduje značajne prednosti u odnosu na klasične organske rastvarače jer se u složenim procesima može uvećati brzina hemijske reakcije kao i brzina prenosa mase u prisustvu SC CO_2. Nadalje ovaj ugušćeni fluid je netoksičan, nezapaljiv i može se nabaviti po niskoj ceni te je izabran kao medijum za reakciju esterifikacije fruktoze i palmitinske kiseline i dobijanja palmitata fruktoze. Uticaj temperature na brzinu esterifikacije u prisustvu imobilisane lipaze proučavan je na 10 MPa i dobijeni rezultati su upoređivani sa rezultatima esterifikacije pod atmosferskim pritiskom na istim temperaturama. Najveća konverzija od 67% je ostavrena nakon 24 h kada je reakcija esterifikacije realizovana na 80°C u prisustvu SC CO_2. Ispitivan je uticaj raspodele veličine čestica i morfologije netretirane i tretirane lipaze sa 2–metil 2–butanolom i SC CO_2 na brzinu procesa esterifikacije fruktoze i palmitinske kiseline.

Ključne reči: Fruktozni palmitat, Površinski aktivne materije, Lipaza, *Candida antarctica* lipaza, Esterifikacija, Organski rastvarači, Natkritični ugljen dioksid.