THE PRODUCTION OF FLAVOR ESTERS IN SUPERCRITICAL CARBON DIOXIDE.

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The ability of Novozyme 435 ® to catalyse the synthesis of flavor esters was investigated in supercritical carbon dioxide. Acetic anhydride was the acyl donor. Propanol, butanol, pentanol, octanol as well as isobutyl, isoamyl, isohexyl, and benzyl alcohols were used as substrates in equimolar ratio to the acylating agent. All the esters were synthesized in good yield (>80%) in short reaction time (<2 hour). As the carbon number of the alcohol increased, so did the esterification degree. On the contrary, the branching was found to decrease the product yield. These results could be referred to differences in enzyme specificity or steric hindrance phenomena. The same dependence on alcohol chain structure was found in n-hexane. In general terms, reaction rate and esterification extent were similar in both solvents. Consequently, supercritical CO_2 can be considered a good alternative to conventional organic solvents for the synthesis of food and health products where solvent residues are forcefully restricted.

INTRODUCTION

Short chain esters are flavor products of commercial importance. Traditionally, they have been produced by chemical synthesis since extraction from plant materials or the production by fermentation is too expensive for commercial exploitation. With increasing orientation toward ´natural` production, employment of enzymes has been gaining importance. Thus, lipases have been successfully employed in flavor and fragrances synthesis in nearly anhydrous organic solvents [1,2]. However, the use of toxic organic solvents for food and health products is progressively being restricted.

Few attempts have been made to use Supercritical Fluids (SCFs) as alternative solvents [3,4]. These solvents offer similar functions as organic solvents such as enhanced solubility of hydrophobic substrates, elimination of side reactions caused by water, and protection from microbial contamination. Moreover, it is possible to control the reaction environment and so, activity of enzymes, by solely pressure and temperature variations [3]. An additional benefit of these solvents lies in the easy coupling of reaction and separation. By operating a cascade of depressurisations (with a possible change in temperature) product fractionation may be achieved [5]. Since SCFs are usually gases at room conditions, the solvent can be easily removed without leaving any residue in the final product. For enzymatic reactions, supercritical carbon dioxide (SC-CO_2) has been the most studied one. Apart from being non-toxic, easily available and cheap, several enzymes including lipases exhibit stable activity in it [6].

The purpose of this work was to synthesize several esters by esterification of acetic anhydride with different alcohols in SC-CO_2. The alcohols were of chain length up to eight carbons, and different structure: aliphatic, normal and branched, and aromatic. Immobilized lipase from *Candida antarctica* (Novozyme 435®) was used as enzyme. The specific objectives were: a) to investigate the effect of alcohol chain on esterification extent, and b) to compare the reaction rate in SC-CO_2 versus n-hexane.

MATERIALS AND METHODS

Materials

Pressurized liquid carbon dioxide at about 5 MPa, >99,998% purity, was supplied by Air Liquid. *Candida antarctica* lipase B *(*Novozyme 435®), immobilized on a macroporous acrylic resin with a water content of 1-2% w/w was kindly provided by Novo Nordisk, Denmark. All reactants and products were provided by Aldrich of analytical grade. Acetonitrile (HPLC grade, Scharlau) was used in the High Performance Liquid Chromatography (HPLC) analytical procedure.

Experimental apparatus.

A 50 ml-batch reactor equipped with agitation, temperature and pressure reading devices, inlet and outlet connections and a rupture disk set at 25 MPa was used to conduct the tests. Temperature was controlled by a heating jacket. The CO_2 feeding line consisted of a CO_2 storage tank, a cooler, and a membrane pump.

Procedures.

The reactants and the enzyme were initially loaded to the reactor. After closure, the CO_2 was pumped up to the desired pressure and temperature was established. Agitation was fixed at 500 r.p.m. Samples were withdrawn at various intervals to determine product concentration. All tests were carried out in equimolar concentration of 0.8 M with an enzyme concentration of 3.3% (w/w). This concentration was calculated on the basis of the weight of the immobilized enzyme (e.g. enzyme + support) and not of actual weight of enzyme protein. Same procedure was used for n-hexane tests, except that operation was carried out at atmospheric pressure.

Analytical methods.

Alcohol and acetate concentration was determined by Gas Chromatography with a Varian Gas Cromatograph equipped with a hydrogen flame ionization detector and a TR-FFAP column (60-m length x 0.32-mm ID). Helium was used as a carrier gas at a flow rate of 29.5 ml/min. Column oven ranged between 75 and 100ºC depending on the compound. Injector and detector temperatures were 250ºC.

Benzyl acetate formation was followed by HPLC by gradient elution method using a mixture of water and acetonitrile (50:50). The chromatograph (Varian 9050) was equipped with a Water Spherisorb-C18 column (250 x 4.2 mm) and an ultraviolet detector KONIK KNK 500 working at a wavelength of 270 nm.

RESULTS AND DISCUSSION

In a previous work, the synthesis of isoamyl acetate by esterification with isoamyl alcohol was investigated. Several acyl donors and two different lipases were tested [7]. Acetic anhydride probed to be the most appropriate acyl donor and Novozyme 435 ® was the most efficient enzyme. Now, different acetates used as food aromas were synthesized. The general reaction scheme is:

acetic anhydride + alcohol Û acetate + acetic acid

The reported percentage esterification was calculated as acetate formation. Calculations based on alcohol conversion were found to be in good agreement. Average uncertainty in reported data was ± 8%. Esterification extent was compared with n-hexane results. The most significant findings are summarised below.

Effect of alcohol chain length

Seven primary alcohols were tested: four with linear chains (propanol, butanol, pentanol, octanol), three with a branch chain (isobutyl, isoamyl and isohexyl alcohols) and one with an aromatic ring (benzyl alcohol). The effect of alcohol side chain is shown in Figures 1 and 2. When comparing linear alcohols, as the alcohol chain increased, so did de reaction rate and percentage of esterification. It seems that the enzyme presented better affinity for the longest alcohols. The differences were small though. Similar behavior was also observed by Gatfield with soluble *Mucor miehi,* who reported an increase in yield with the alcohol chain length [8]. Conversely, with immobilized *Mucor* sp., *Aspergillus* sp. and *Rhizopus* lipases, yields decreased by increasing the number of carbon atoms in the alcohols, based on organic solvents assays [9,10].

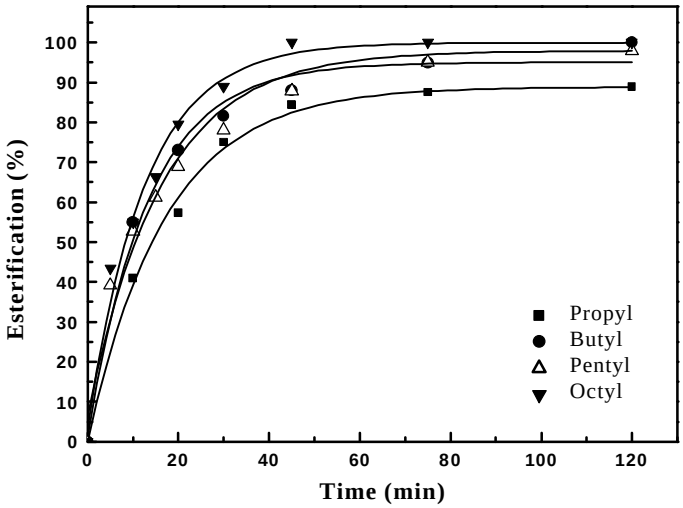

Figure 1: Effect of linear alcohol chain length on percentage of esterification. (solid line:exponential fitting).

The impact of branching was more significant. Accordingly, the presence of a methyl group in the chain was found to decrease the reaction rate; e.g. the isobutyl acetate formation was slower than that of butyl acetate (Figure 3). Additionally, the closer the methyl group was to the hydroxyl group, the lower the alcohol conversion. Finally, the aromatic ring, which was the bulkiest group, caused the lowest esterification rate.

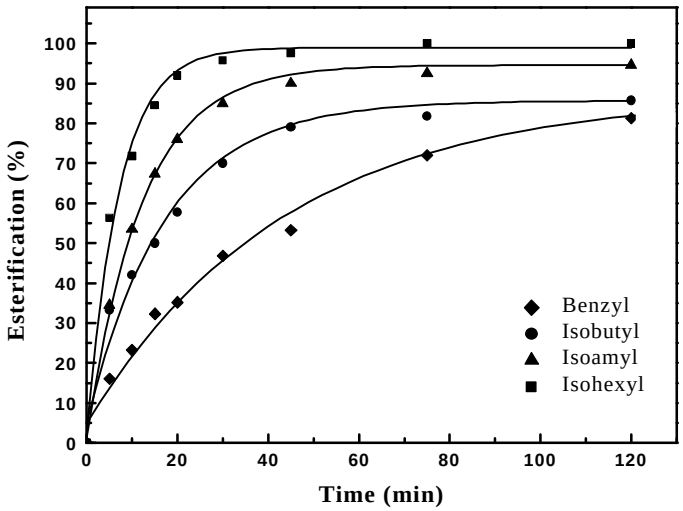

Figure 2: Effect of type of alcohol branch on reaction rate.

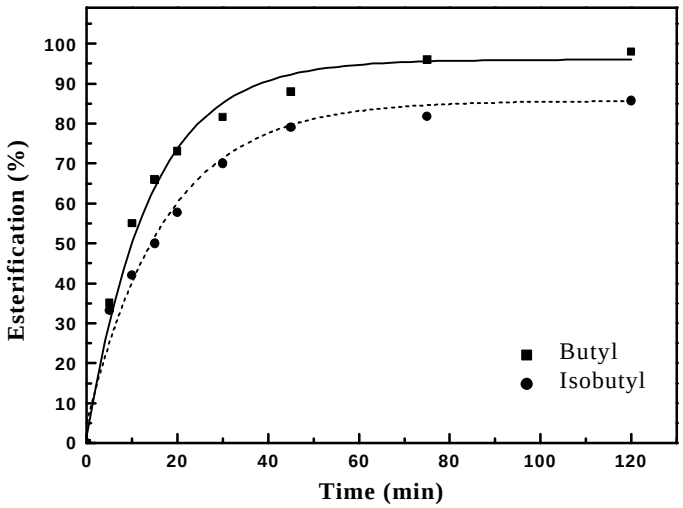

Figure 3: Effect of alcohol chain (linear or branched) on esterification extent.

On the other hand, Novozyme 435 ® was very efficient in catalyzing this type of reaction, achieving high conversion rates in short periods of time. For example, the isoamyl acetate production was 98% at 45 min, while it was necessary 72 hours to obtain 45% conversion using *Candida cylindrae* lipase in n-heptane [11]. The differences could not be solely explained by the solvent employed as will be demonstrated next.

Effect of solvent nature

Supercritical fluids exhibit lower viscosities and higher diffusivities to those of conventional liquid solvents; so they could be beneficial for reactions that are diffusionally limited. For example, Cernia et al., working on the esterification with secondary alcohols of acetic anhydride, found that reaction rates in SC-CO_2 were significantly higher than in organic solvents. The authors explained the differences in terms of enhanced mass transport properties and higher enzyme stability [12]. However, the advantages of using SC-CO_2 as reaction medium over organic solvents have not been always so clear. On the contrary, other authors reported a negative effect of SC-CO_2 on lipases activity [3,4]. This effect was attributed to the interactions between CO_2 and the enzyme. Kamat et al., proposed that CO_2 may form covalent complexes with free amino groups on the surface of the enzyme [13]. Other reason could be the partial drying of the enzyme due to the greater affinity of the SC-CO_2 for water. Nevertheless, when the enzyme was rehydrated [3] or tested in water-saturated SC-CO_2 [4], its catalytic activity was recovered.

To elucidate the impact of solvent nature in our system, parallel work was conducted in n-hexane. Tests were run using the same reactor, which means analogous shaking mode and agitation speed. As example, a linear alcohol (octyl) and a branched (isobutyl) alcohol formation in SC-CO_2 and n-hexane is compared in Figure 4. Also, the percentage of esterification achieved with all alcohols, after two hour-run, in both solvents, is shown in Table 1. As observed, the results were alike regarding both, the esterification percentage and the reaction rate. It seems that in our system, mass transfer effects were negligible, so no specific advantages of using SC-CO_2 were obtained.

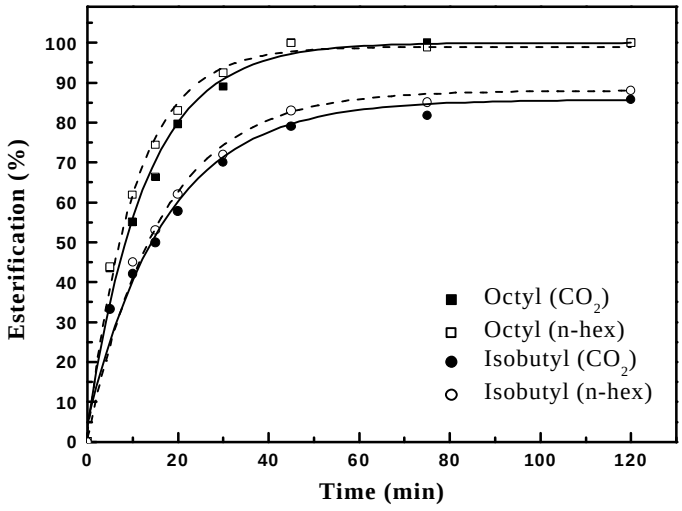

Figure 4: Effect of solvent nature on esterification rate.

CONCLUSIONS

Novozyme 435 ® was very efficient in catalysing the synthesis of flavor esters in SC-CO_2. The esterification dependence on linear alcohol chain length could be referred to differences in enzyme specificity, being more effective for longer alcohols. When branched alcohols were used, reaction rate and percentage of esterification decreased. The bulkier the

molecule was, the lower conversion was achieved. This fact could be explained by steric hindrance phenomena. Similar esterification extent and reaction rate were obtained in n-hexane and SC-CO_2. Consequently, SC-CO_2 can be considered a good alternative to conventional organic solvents for the synthesis of food and health products where solvent residues are forcefully restricted. Further advantages of using this solvent would be the facilitation of downstream and product separation.

Table 1: Esterification degree at two-hour run.

	Esterification (%)	
	n-Hexane	**SC-CO_2**
Propanol	89,9 ± 7,2	88,9 ± 7,1
Butanol	98,0 ± 7,8	99,0 ± 7,8
Pentanol	98,0 ± 7,8	97,9 ± 7,8
Octanol	100,0 ± 8,0	100 ± 8,0
Isobutyl	88,9 ± 7,2	85,7 ± 6,9
Isoamyl	89,9 ± 7,2	94,6 ± 7,6
Isohexyl	95,5 ± 7,6	100,0 ± 8,0
Benzyl	84,8 ± 6,8	81,3 ± 6,5

REFERENCES

[1] MANJÓN, A., IBORRA, J.L., AROCAS, A., Biotech. Lett., Vol. 3(5), **1991**, p.339.

[2] CHULALAKSANANUKUL, W., CONDORET, J.S., COMBES, D., Enz. Microb. Technol., Vol.14, **1992**, p.293.

[3] KNEZ, Ž., HABULIN, M., J. of Supercritical Fluids, Vol. 23, **2002**, p.29.

[4] CHULALAKSANANUKUL, W., CONDORET, J.S., COMBES, D., Enz. Microb. Technol., Vol.15, **1993**, p.691.

[5] MARTY, A., COMBES, D., CONDORET, J.S., Biotech. and Bioeng., Vol.43, **1994**, p.497.

[6] TANIGUCHI, M., KAMIHIRA, M., KOBAYASHI, T., Agr. Bio. Chem., Vol. 51 (2), **1987,** p.593.

[7] CALVO, L., ROMERO, M.D., ALBA, C., ORTIZ, F., GUTIERREZ, M., Chem. Eng. Transactions, Vol. 2, **2002**, p.181.

[8] GATFIELD, IL. In Biogeneration Aromas, Ed TH Parliament and R. Croteau, ACS Symposium series, Vol. 317, **1986**, p.310.

[9] LANGRAND, G., RONDOT, N., TRIANTAPHYLIDES, C, BARATTI, J., Biotech. Lett., Vol.12 (8), **1990**, p.581.

[10] ABBAS, H., COMEAU, L., Enz. Microb. Technol., in press.

[11] GILLIES, B., YAMAZAKI, H., ARMSTRONG, D.W., Biotech. Lett., Vol. 9 (10), **1987**, p.709.

[12] CERNIA, e., PALOCCI, C., SORO, S, Chem. Phys. Lipids, Vol. 93, **1998**, p.157.

[13] KAMAT, S., BECKMAN, E.J., RUSSELL, A.J. Enz. Microb. Technol., Vol.14, **1992**, p. 265.