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One pot kinetic resolution and product separation with corn germ oil and supercritical carbon dioxide

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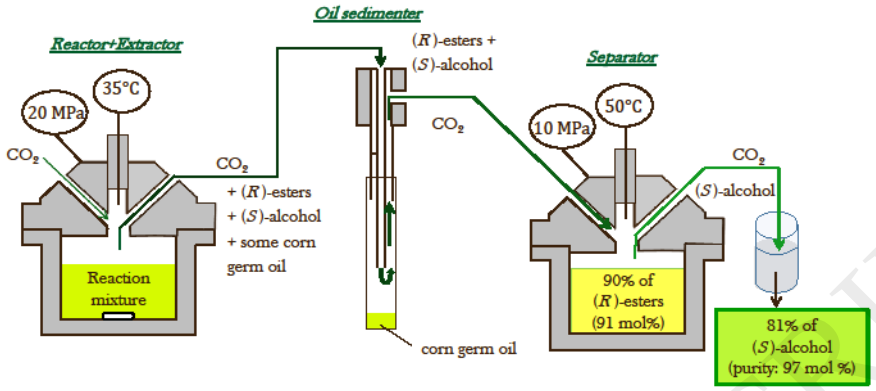
GRAPHICAL ABSTRACT



HIGHLIGHTS

- Lipase catalyzed kinetic resolution with corn germ oil as natural esterifier
- Excellent enantioselectivities and high yields at neat and biphasic conditions
- Outstanding separation of products by sequential pressure drops
- Design supported by phase equilibrium measurements

Abstract

Enantioseparation of 1-phenylethanol with corn germ oil, obtained by supercritical carbon dioxide, is efficiently catalyzed by Novozyme 435. The reaction has high enantioselectivity (E>1000) and the equilibrium conversion is approx. 90% at 50 °C both at ambient pressure and at 20 MPa. The esters and the remaining alcohol can be separated by supercritical carbon dioxide extraction and sequential pressure drop using a pressurized and an atmospheric

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separator. The determination of suitable pressure and temperature conditions were supported by phase equilibria measurements and distribution ratio determination.

Keywords: lipase, selective separation, fatty acid esters, enzyme, gas chromatography, process development

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Introduction

Enantiomerically enriched or preferably enantiopure products have a broad commercial potential due to the different bioactivity and bioavailability of the enantiomers. The global chiral market is estimated to reach $7.6 Billion with 41% share of European companies. [1] Enantiopure secondary alcohols are used as chiral building blocks and synthetic intermediates in asymmetric syntheses, pharmaceutical-, agrochemical- and fine chemical industries. Biological approaches as biocatalysis and bioconversion are advantageous due to their high efficiency, mild reaction conditions, stereospecificity and low environmental impact. Kinetic resolution of racemic secondary alcohols is an efficient method to obtain optically active alcohols. Lipases possess wide substrate specificity, moreover they do not require cofactors and their activity is also maintained in non-conventional solvents like supercritical fluids and ionic liquids. [2] As ester donors natural oils might also be applied instead of synthetic compounds. [3] The special advantages of using supercritical carbon dioxide as a solvent are its beneficial properties in product separation. [4]

The enzyme catalysed kinetic resolution of 1-phenylethanol is a widely studied model reaction. These reactions have been studied in organic solvents [5,6], in supercritical carbon dioxide ($scCO_2$) [4,7], in ionic liquids [8,9], an in a $scCO_2$/ionic liquid biphasic system [10]. Publications are available on the optimisation of the operation parameters of the resolution [7,10,11], kinetics and reactor modelling can also be found in the literature [12], and a suitable continuous equipment was also designed for this kinetic resolution [13]. In the studies typically vinyl acetate was applied as an esterification reagent, but in some cases good enantioselecitivties and conversions have also been achieved with other vinyl esters. [14] The carbon chain length of the ester moiety strongly affects the reaction rate. Vinyl butyrate was selected as optimal esterification agent regarding the reaction rate maximisation under atmospheric conditions. [4,10] Although vinyl esters are advantageous, because the

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equilibrium is shifted towards the formation the products by the immediate oxo-enol tautomerisation of the vinyl alcohol to acetaldehyde [15], vinyl acetate is highly volatile resulting in obvious environmental effects and losses and downstream processing [4,16] might also be problematic. Due to the wide acceptable substrate range of the popular lipases, as *Candida antarctica* lipase B (CALB), besides simple vinyl esters modified vinyl esters [17] and also glycerine based reagents [18] are applicable without a relevant loss of the selectivity or the activity.

Certain research works studied the downstream processing after the kinetic resolution as well. Typically the product was extracted with and organic solvent from the reaction mixture (e.g. diethyl ether, methanol) or the volatility difference of the substrate and the product allowed a distillation based separation [3,16,18]. Another possibility is the separation by sequential pressure drop after a kinetic resolution performed in $scCO_2$. Paiva et al. used four separators in series after the column operating at 15 MPa and 45 °C. Temperatures of the separators 1-3 were 52.5 °C and their pressure ranged from 13 MPa to 7.5 MPa, temperature of the last separator was set to 50 °C. Their reaction used vinyl laurate as an esterification agent. After the four staged sequential separation both (*S*)-1-phenylethanol and (*R*)-1-phenylethyl-laurate achieved 95% purity. However the four staged separation system made it possible to recover only 1 %(w/w) of phenylethanol from the reaction mixture, about 99% of the initial feed remained in the liquid phase of the separators. [4]

Corn germ oil is widely used in the food industry.[19] Various food products contain corn germ oil like biscuits, ice creams, instant soups. Corn germ oil is thermally stable and is often used in cooking. Its hydrogenated form is an additive of margarines. In the cosmetic industries corn germ oil is a constituent of shampoos and balms for dry hair. The main components of corn germ oil are triglycerides, but as minor component free fatty acids, pigments, phytosterols, waxes etc. can also be detected. Corn germ oil might have as high as 57.8-62.0% linoleic acid

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content which is an essential fatty acid, while the content of saturated fatty acids is typically below 15%. [19] Corn germ oil might be produced from the corn germ by cold pressing, solvent extraction or supercritical fluid extraction. [20] Besides applications of corn germ oil in the food and cosmetic industries, it can also be a raw material of biodiesel production. Lipase catalysed transesterification of corn germ oil in a supercritical fluid was already studied in the late 1990s and the research is still running [21,22].

The aim of our work was to develop a kinetic resolution reaction and combined separation method with an increased efficiency compared to previous works to produce enantiopure secondary alcohols using renewable reagents only.

2. Materials and methods

2.1. Materials

1-Phenylethanol (PE), (≥98% purity), CALB immobilised on a macroporous resin (Novozyme-435), linoleic acid (≥99.9%), oleic acid (≥99.0%), palmitic acid (≥99.9%), benzyl alcohol (BA) (≥99.8%) and silicagel (100-200 mesh, medium pore size 30 Å) were purchased from Sigma-Aldrich Chemie GmbH. Solvents as *n*-hexane (>95%) and ethyl acetate (99.5%) were supplied by Merck KGaA. Carbon dioxide (>99.5%) used was from Linde Hungary Co. Corn germ oil was a supercritical carbon dioxide extract obtained at 45 MPa, 50°C in our laboratories.

2.2. Determination of solubilities and distribution coefficients

Solubility and distribution coefficient measurements were performed in a Pickel cell (New Ways of Analytics GmbH), which is a variable volume view cell unit with possibility to take samples from the upper and lower phases at constant pressure and temperature. Solubility was determined by standard cloud point measurement method. Distribution ratios were determined by sampling both phases at contant pressure and temperature followed by the quantitative gas chromatographic analysis of the samples (see 2.5).

2.3. High pressure enzyme catalysed reaction and product separation

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Kinetic resolutions were performed in a custom made 36 mL high pressure autoclave according to a previously published methodology. [23,24] For the high pressure experiments (*R*,*S*)-PE (0.033 g, 0.27 mmol) and CALB (0.020 g) were measured in the autoclave and corn germ oil was added (1550 µl, 1.30 g, 1,47 mmol in trioleate equivalent). The autoclave was heated to the desired temperature and pressurized with CO_2 to the desired pressure. The reaction mixture was stirred with a magnetic stirrer typically at 250 rpm. To follow the time course of the conversion samples were taken periodically from the $scCO_2$ phase.

The setup newly designed for the separation of the products and the remaining substrate is pictured in Fig. 1. Major elements are two autoclaves (36 mL each, one serves as a reactor/extractor, one as a high pressure separator), an oil sedimenter (10 mL) and an atmospheric separator.

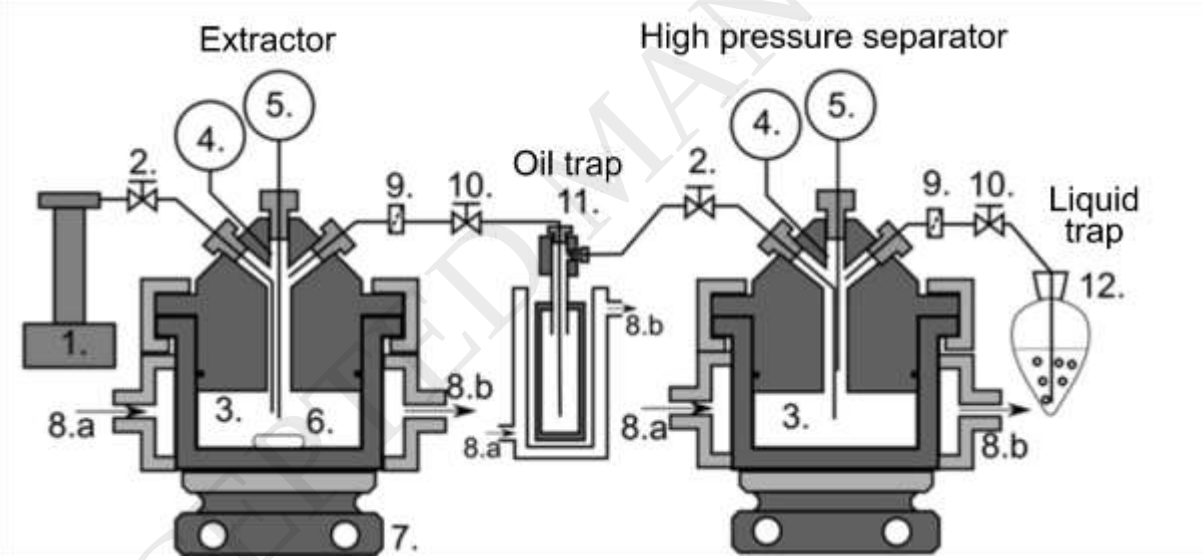

Fig 1. Reactor with a high pressure and an atmospheric separator. (1: ISCO 260D CO_2 pump, 2: inlet valve, 3: reactor or separator, 4: manometer and pressure transducer, 5: thermocouple, 6: mixer, 7: magnetic stirrer, 8a and 8b: heating in and outlets, 9: filter, 10: outlet vales, 11: oil sedimenter, 12: liquid trap as atmospheric separator)

2.4. Atmospheric reference experiments

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As control experiments the reactions were also performed atmospherically in vials. The reactants, the solvent if any and the immobilized enzyme were measured in and the vials were closed. For reactions in *n*-hexane: PE 0.033 g (0,27 mmol, 169 mmol/dm^3), corn germ oil 0.5 g (0.57 mmol in trioleate equivalent, 356 mmol/dm^3), 1 mL *n*-hexane and 0.033 g (0,27 mmol, 169 mmol/dm^3) and 20 mg Novozyme 435. For neat reactions: PE 0.033 g (0,27 mmol, 169 mmol/dm^3), corn germ oil 1.30 g (1.47 mmol in trioleate equivalent, 919 mmol/dm^3) and and 20 mg Novozyme 435. Reaction mixtures were tempered by a water bath and stirred by a magnetic mixer at 400 rpm. 5 µl samples were taken periodically to follow the reaction. Concentrations of the samples were determined by gas chromatography.

2.5. Analytical methods

A gas chromatographic method was developed for the quantification of the remaining alcohol and ester products and the determination of the enantiomeric excess values using an achiral and a chiral separation method parallelly.

The analysis of fatty acid esters was performed by a Thermo Finnigan Focus GC, Zebron ZB-WAX plus column (100% PEG stationary phase, 30 m x 0.25 mm x 0.25 µm), the injector and the flame ionization detector both at 260 °C, head pressure 220 kPa, the mobile phase was helium. A 1:100 split ratio was applied. The following temperature program was used: 110 °C for 1 minute, 3 °C/min to 130 °C then 20 °C/min to 195 °C kept for 2 minutes followed by a 0.5 °C/min ramp to 205 °C and a 5 °C/min ramp to 230 °C, which was kept for 6 minutes. Retention times of the major components of interest: 1-phelyethanol 6.8 min, phenylethyl-palmitate 31.2 min, phenylethyl-oleate 40.3, phenylethyl-linolate 41.7. Benzyl alcohol (BA) was added in predetermined concentration as the internal standard during the analysis to determine the concentration of PE. Taking into account the PE concentration and the response factors, which were ascertained from GC analysis performed earlier, the concentration of fatty acid esters could be obtained.

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Enantiomeric excess values of the ester products were determined by chiral gas chromatography after the isolation of the esters followed by a methanolyzis. Components of the reaction mixture were isolated by column chromatography (silicagel 100-200 mesh, 30 Å avarage pore size, height of the column 30 cm, diameter of the column 1 cm, eluent 95:5 *n*-hexane : ethyl acetate). The isolated remaining alcohol was directly analyzed while the esters were dissolved in methanol and $NaHCO_3$ catalyst was added. During an overnight stirring the methanolyzis was completed and the methyl esters of the fatty acids and 1-phenylethanol were obtained. Enantiomeric excess of the latter is equal to the enantiomeric excess of the ester products and was determined in each case by chiral gas chromatography according to the following method: Thermo Finnigan Trace GC, AGILENT J&W HP-CHIRAL-20B column (stationary phase β-cyclodextrin (35% phenyl)-methylpolysiloxane, 30 m x 0.25 mm x 0.25 µm), temperatures of the flame ionized detector and of the injector were 250 °C, head pressure 175 kPa, as a mobile phase helium was applied. Temperature program: 110 °C for 1 min, a 5 °C/min ramp to 145 °C kept for 1 minute. (*R*)-PE 6.75 min, (*S*)-PE 6.9 min.

2.6. General calculation methods

Equation to calculate enantiomeric excess is given in eq. 1.

$$ee = \frac{c^* - c}{c^* + c} \qquad (1)$$

where c^* is the concentration or peak area of the major enantiomer, while c is the concentration of peak area of the minor enantiomer.

Enzyme catalyzed reactions are typically evaluated by enantioselectivity, which is in principle defined as eq. 2, but in practice it is typically calculated by eq. 3. from conversion and enantiomeric excess values:

$$E_{R,S} = \frac{\left({}^{k_{cat}}/_{K_m}\right)_R}{\left({}^{k_{cat}}/_{K_m}\right)_S} \qquad (2)$$

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where k_{cat} is the turnover number K_m is the Michaelis constant, indices *R* and *S* refer to the relevant enantiomer.

$$E = \frac{ln\left[\frac{1-ee_S}{1+(ee_S/ee_P)}\right]}{ln\left[\frac{1+ee_S}{1+(ee_S/ee_P)}\right]} = \frac{ln[(1-X)(1-ee_S)]}{ln[(1-X)(1+ee_S)]} \qquad (3)$$

where ee_S is the enantiomeric excess of the substrate, ee_P is the avarage enantiomeric excess of the products, while *X* is the conversion.

The conversion was directly calculated from the peak areas of the PE enantiomers in the reaction mixture assuming full selectivity of the reaction and this estimation gave the conversion with a 1% uncertainty according to the control measurements and was much simpler to calculate routinely. Yields of the esters (*Y*) were calculated from peak areas of the achiral chromatograms taking into account the response factors.

3. Results and discussion

The general scheme of the reaction is pictured in Scheme 1. As corn germ oil contains various fatty acids forming triglycerides,R_1 R_2 and R_3 might refer to different or similar hydrocarbon chains. Our own measurements resulted in a fatty acid distribution presented in Table 1.

Table 1. Fatty acid distribution of corn germ oil used in this study.

Fatty acid	Mol% of total fatty acid
$C_{16:0}$ (Palmitic acid)	10.8
$C_{16:1}$ (*cis*-7 Hexadecenoic acid)	0.1
$C_{18:0}$ (Stearic acid)	1.9
$C_{18:1}$ (Oleic acid)	29.6
$C_{18:2}$ (Linoleic acid)	55.6
$C_{18:3}$ (Gamma-Linolenic acid)	1.0
$C_{20:0}$ (Arachidonic acid)	0.4
$C_{20:1}$ (Paullinic acid)	0.3
$C_{22:0}$ (Behenic acid)	0.1

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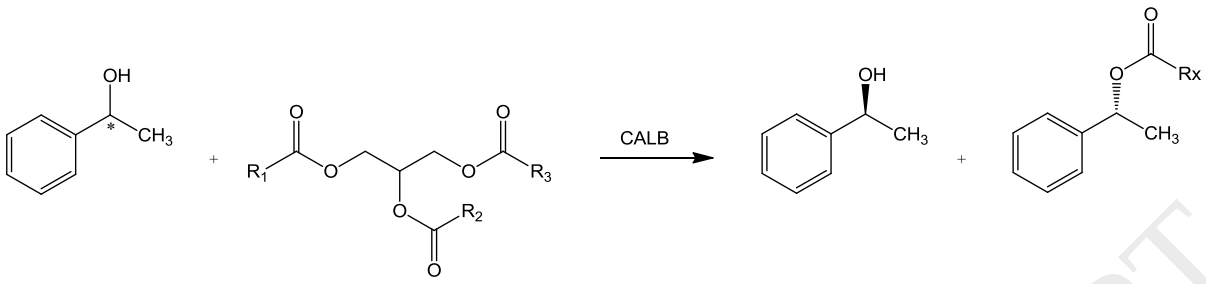
$C_{22:1}$ (Erucic and cetoleic acids) 0.2



Scheme 1. Reaction scheme of 1-phenylethanol with corn germ oil catalysed by *Candida antarctica* B enzyme preparation, resulting in selective ester formation (ee>99%).

The reaction has a high enantioselectivity both at atmospheric conditions and if done in a corn germ oil – supercritical carbon dioxide biphasic system. Concentration of remaining 1-phenylethanol as a function of reaction time is shown in Fig. 2. Conversions are not complete, but an equilibrium is reached after appr. 3-5 hours (final conversions are between 45.5-48.8% instead of 50%, see Table 2.). Although the most accepted mechanism theory for the of lipase catalysed reactions is the ping-pong bi-bi mechanism [25–27], if there is a large excess of one of the reactants, as it is in this case, first order kinetics might be also applied. [28] Enantioselectivities of all reactions were >1000 meaning highly enantioselective catalysis. Apparent reaction rate coefficients (k_{app} defined by eq. 4 assuming an apparent first order kinetics), equilibrium conversions (X_{eq}) obtained at neat conditions, in *n*-hexane solvent (homogenuous phase) or in supercritical carbon dioxide – corn germ oil biphasic systems at 10 and 20 MPa are summarized in Table 2..

$$X(t) = X_{eq} \cdot \left[1 - \exp\left(k_{app} \cdot t\right)\right] \qquad (4)$$

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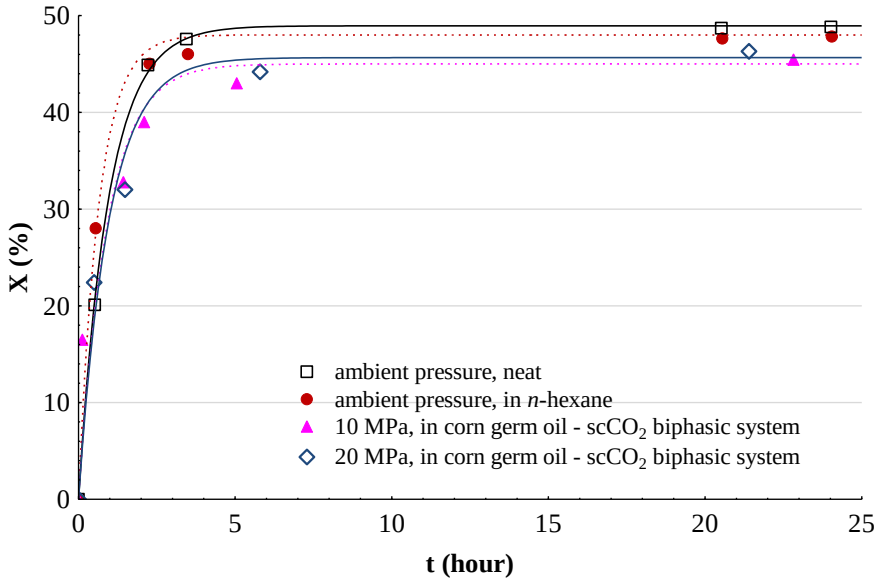

Fig. 2. Conversion as a function of reaction time, initial 1-phenylethanol concentration 160 mmol/dm^3 oil, 20 mg Novozyme 435, 40 °C.

Table 2. Equilibrium conversion and reaction rate coefficients of the esterification reaction at 40 °C in various media.

Conditions	X_{eq} (%)	k_{app} (10^{-3}·min^{-1}·mg enzyme preparation^{-1})
neat	48.0	0.88
n-hexane	48.9	1.30
CO_2, 10 MPa	45.0	0.91
CO_2 20 MPa	45.6	0.86

The reaction seems to be the fastest in hexane, and slightly slower in carbon dioxide expanded oil and neat reaction. Apparent reaction rate coefficients in contact with CO_2 compared to those in *n*-hexane are not necessarily lower because of the possible lower activity of the enzyme preparation, but might be because of the distribution of the substrate between the oil and supercritical phases resulting in lower apparent activity. Distribution of PE and products between the oil and carbon dioxide phases is discussed in the following subchapter.

3.1. Determination of the solubility data

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The solubility of 1-phenylethanol in $scCO_2$ is already published [29,30] thus only control measurements were performed in the relevant concentration ranges (1.1-7.5 mg/g CO_2, 0.40-0.63 mmol/mol CO_2). 1-Phenylethanol was completely dissolved in CO_2 at these concentrations at 10-20 MPa pressure and 35-55 °C temperature ranges, as it was expected based on the published results. Solubility of the corn germ oil was tested at 96.3 mg/g CO_2 mass ratio (4.79 mmol in trioleate equivalent/mol CO_2, relevant to the reaction conditions) at 40-60 °C temperature and 10-20 MPa pressure ranges. Visible dissolution was not achieved, which is in accordance with the literature. Soares and coworkers studied the solubility of corn germ oil in $scCO_2$ in a temperature range from 40 to 80 °C and pressure between 20 MPa an 35 MPa. According to their data at 20 MPa and 40 °C they observed 3.5 g/kg solubility of the oil in the fluid phase [31]. Chen and coworkers measured the solubility of triolein in $scCO_2$, and at 10 MPa and 40°C they got 0.0004 molar fraction solubility [32]. Furthermore, solubility of CO_2 in the oil phase was determined at 10 MPa and 50 °C to be 0.313 g CO_2/g liquid phase. As in the reaction various esters are formed we decided to measure the apparent solubility of the ester mixture of the reaction by cloud point measurement. Results obtained at 1.45 mg/g CO_2 mass ratio are plotted in Fig. 3. The high solubility of PE in the whole pressure and temperature range and the limited solubility of the products at elevated temperatures and lower pressures (e.g. 50-60 °C and 10 MPa) suggested that a suitable reaction and separation coupling by sequential pressure drops might be possible. However, for a better design, distribution ratio (oil/CO_2) measurements were needed to maximize the separation factor of the PE and the products.

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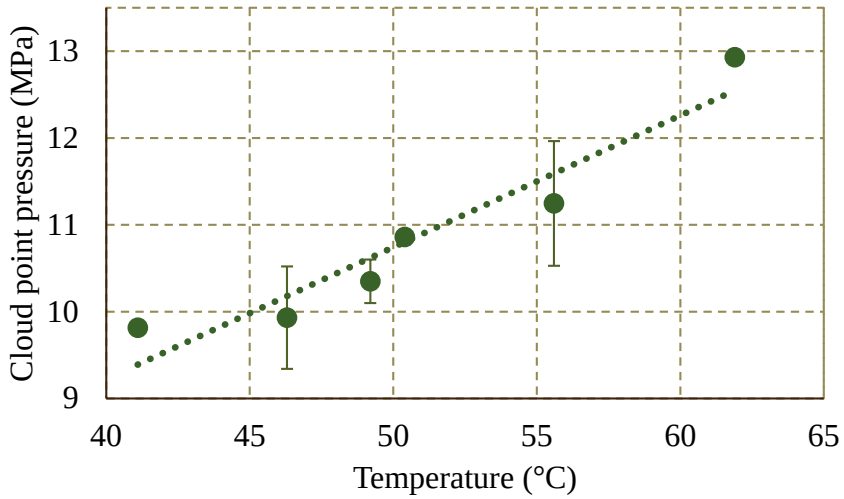

Fig 3. Cloud point pressures of the ester product mixture of the reaction at 1.45 mg/g CO_2 concentration.

Distribution ratios of selected phenylethyl esters and the 1-phenylethanol in the oil – carbon dioxide biphasic system was determined by sampling and analyzing the oil and fluid phases at 10 Mpa 50 °C and at 20 MPa 35 °C. The amount of the oil phase was 7.7 g at each experiments. Results are summarized in Table 3.

Table 3. Distribution ratio values of 1-phenylethanol and its esters between corn germ oil and supercritical carbon dioxide

Compound	Distribution ratio (in oil/in CO_2, g/g (mol/mol))	
	10 MPa, 50 °C	20 MPa, 35 °C
1-phenylethanol	41±2.7 (832±54.2)	5.6±0.6 (113±12.0)
1-phenylethyl palmitate	8160±510 (163942±10246)	16.4±2 (329±40.2)
1-phenylethyl oletate	12453±823 (250192±16535)	19.5±2.6 (392±52.2)
1-phenylethyl linolate	9572±625 (192310±12557)	18.5±1.4 (372±28.1)

Distribution ratio values indicate that separation factors of ester products and PE can be as high as 200-300 g/g at 10 MPa 50 °C while they are below 5 g/g at 20 MPa 35 °C. Thus a coextraction of PE and the products is possible at 20 MPa pressure and 35 °C temperature with CO_2 leaving the oil phase with the immobilized enzyme in the reactor. At 10 MPa and 50 °C

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precipitation of the products is possible, while PE will be still mostly in the fluid phase since the oil-to-carbon dioxide ratios are small.

Furthermore, these observations support that the immobilized enzyme catalyzed reaction is performed in a CO_2 enriched oil phase while PE is distributed between the two phases resulting in a slightly lower apparent reaction rate than at neat conditions (see 3).

3.2 Hybrid reaction and separation setup, proof of principle

Based on the results and hypothesis described in part 3.1 the construction was built as presented in Fig 1. in part 2.3. At first trials the oil sedimenter was missing, but its installation was necessary due to the issue that the high CO_2 flow in the reactor/extractor resulted in drop drifting. The oil sedimenter operates at the same conditions as the extractor and the oil phase trapped in it had similar product and alcohol concentrations as the oil phase remaining in the extractor.

73-78% of PE was collected in the atmospheric liquid trap, while 73-90% of the esters were collected in the high pressure separator operating at 10 MPa and 50 °C, depending on the exact flow patterns. At an optimized CO_2 flowrate of 0.75 mL/min at 20 MPa and 35 °C (extractor) and stirring rate of 150 rpm (in the extractor only), using altogether 120 g of CO_2 during the separation we obtained 90% of the esters in 91 mol% purity, and 81% of the remaining alcohol in 97 mol% purity.

4. Conclusion

The resolution of racemic 1-phenylethanol with corn germ oil in $scCO_2$ was successfully developed with ≥45% equilibrium conversion concerning the racemic alcohol, and ≥99.5% enantiomeric excess of the ester products. Corn germ oil forms a biphasic system with $scCO_2$ up to 20 MPa, therefore the ester products and the remaining alcohol after the resolution process could be extracted with $scCO_2$ at 20 MPa and 35 °C. The separation process of alcohol and esters was based on sequential pressure drop (10 MPa at 50 °C and atmospheric), the design

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was supported by phase equilibria studies. Owing to the specified extraction - separation system, the ester products and the remaining alcohol could be separated with a purity over 90% and with the mass balance error below 10%. Further aim is to design a semi-continuous system based on the presented results for the resolution of continuous racemic alcohol feed and direct separation of enantiopure alcohol and ester by $scCO_2$.

Acknowledgments

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Figure captions

Fig. 1. Reactor with a high pressure and an atmospheric separator. (1: ISCO 260D CO_2 pump, 2: inlet valve, 3: reactor or separator, 4: manometer and pressue transducer, 5: thermocouple, 6: mixer, 7: magnetic stirrer, 8a és 8b: heating in and outlets, 9: filter, 10: outlet vales, 11: oil sedimenter, 12: liquid trap as atmospheric separator).

Fig. 2. Conversion a function of reaction time, initial 1-phenylethanol concentration 160 mmol/dm^3 oil, 20 mg Novozyme 435, 40°C.

Fig. 3. Cloud point pressures of the ester product mixture of the reaction at 1.45 mg/g CO_2 concentration.

Scheme 1. Reaction scheme of 1-phenylethanol with corn germ oil catalysed by *Candida antarctica* B enzyme preparation, resulting in selective ester formation (ee>99%).

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