PRODUCTION OF ENANTIOMERICALLY PURE COMPOUNDS BY BIOCATALYSIS IN NON-AQUEOUS SOLVENT OR RESOLUTION BY SUPERCRITICAL CARBON DIOXIDE EXTRACTION.

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ABSTRACT

Two different methods for the production of enantiomers based on high pressure technologies are presented herein: the resolution of racemic mixture by partial complex formation followed by extraction and a biocatalytic synthesis based on an alcohol dehydrogenase.

INTRODUCTION

Since biological organisms are built out of chiral compounds, many compounds which are active toward it are chiral, as well. Enantiomers of an active molecule usually have different biological activities. There is an increasing demand for chiral molecules. In order to reduce side effect of a drug, only the active component should be present in the pharmaceutical formulation. In the case of pesticide, a pure chiral active product instead of a mixture could lower the environmental impact of the treatment. Legislation evolves in that direction. [1]

Reactions used by industry are not stereoselective in many cases, and the mixture of the enantiomeres should be resolved. This means that the enantiomers should separated from each others. The methods of resolution used mostly are [2]: running a supplementary reaction with a chiral reactant (diastereoisomers are separated according to their different physical properties) crystallisation with a chiral salt, or chromatography using a chiral column.

Those methods are generally expensive, possibly contaminant (for example when extra use of solvent for chromatography leads to further waste), and have a poor atom efficiency. That's why stereoselective reaction is a field of chemistry of the biggest interest. Two alternatives for the production of enantiomerically pure molecules based on high pressure technologies are presented herein: the resolution by complex formation followed by supercritical extraction and the use of an enzyme and its associated coenzyme.

MATERIALS AND METHODS

Perfil 250™ (expanded and milled perlite for use as a filtering aid with specific surface area of 2.9 m^2/g) was kindly given by Baumit Co. (Budapest, Hungary). (±)-*trans*-1,2-cyclohexanediol (*trans*-1,2-cyclohexanediol abbreviated as CHD) was provided by Sigma-Aldrich Corp., (Budapest, Hungary). Tartaric acid was kindly given by Richter Gedeon Co. Budapest, Hungary. CO_2 (99.5 w/w% pure) was supplied by Linde Ltd. (Budapest), Hungary. LBADH, as a lyophilised powder, NADPH, NADP were provided by Jülich Fine Chemical, Germany. Heptane, acetone, isopropanol, pH = 7 phosphate buffer solution, $CuSO_4.5H_2O$, Folin-Ciocalteau's phenol reagent and potassium-sodium-tartrate tetrahydrate were provided by Merck. $MgCl_2.6H_2O$, Sodium hydroxide, orange silica gel, titrant solution, "composite 5" solution and solvent, Hydranal® for Karl Fisher titration were provided by Riedel-deHaën. Acetophenone, $Na_2CO_3.10H_2O$ and *R*- and *S*-1-phenylethanol racemic

mixture were provided by Fluka. Decane was provided by Aldrich. Sucrose, *R*- and *S*-1-phenylethanol standards, glass beads (425-600 µm) were provided by Sigma.

The extractions were performed with the supercritical extraction plant described in [3].

The reactions at non atmospheric pressure and the phase equilibrium measurement were performed in a "high pressure view cell". This apparatus consists of a large piston from 60 to 30 cm^3, equipped with a mechanical stirrer, heater and thermocouple. Sampling without depressurisation is made possible by an HPLC injection valve. As it is closed by sapphire windows at its two extremities, it can be seen through. The back sapphire window is mobile. This allows the operator to maintain a constant pressure by changing its size. Pressurized gas is supplied by a pump controlled by air pressure.

Gas chromatography. In the case of the analysis of *R*-1-phenylethanol, the Gas chromatograph was HP 5890 with the integrator HP 3392A. The column was: BetadexTM 120 30 m ×0.25 mm 0.25 µm film provided by Supelco and made of β-cyclodextrin. In the case of the analysis of (±)-CHD, the gas chromatograph was Agilent 4890D with the column Hydrodex-β-6-TBDM (25 m x 0.25 mm x 0.25 µm film, Macherey & Nagel, No.: 21519/11).

RESULTS

1. Resolution by partial complex formation and SC CO_2 extraction.

The first method was the resolution by partial diastereomeric complex formation [4] followed by extraction with supercritical carbon dioxide. Supercritical carbon dioxide (SC CO_2, CO_2 above Tc = 31.1 °C and Pc = 73.8 bar) is renewable, inexpensive and safe (non toxic, non explosive, non flammable), the physical properties can be tuned by playing on pressure and temperature, its viscosity is intermediate between gas and liquid; this allows better mass transfer than using conventional solvent. The solvent is easily separated from the product by releasing the pressure. It is suitable for extraction due to its low viscosity and high diffusivity. Its "tunable" properties make it appropriate for separations, as well. This method was applied to the resolution of the racemic mixture (±)-CHD with (*R*,*R*)-tartaric acid as a resolution agent. The principle of this resolution is illustrated in Figure 1. A complex is preferentially formed between (*R*,*R*)-CHD and (*R*,*R*)-tartaric acid so that most (*S*,*S*)-CHD remained uncomplexed. Since the complex is less soluble in CO_2 the (*S*,*S*)-CHD was extracted by SC CO_2 at first. By releasing the pressure, it was easily separated from the solvent, SC CO_2, which can be recycled. (*R*,*R*)-CHD was subsequently raffinated, separated from tartaric acid. The results given in the 3 next paragraphs are based on experiments repeated 5 times.

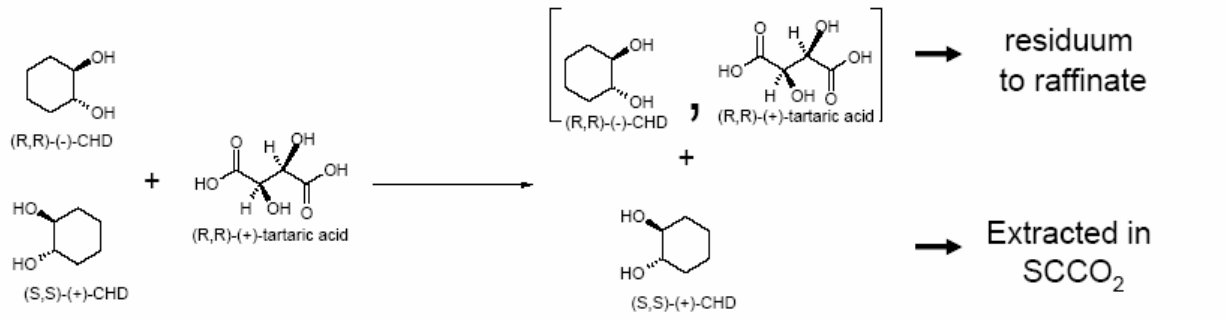

Figure 1: Principle of the resolution of (±)-*trans*-1,2-cyclohexanediol (*trans*-1,2-cyclohexanediol abbreviated as CHD) by complex formation followed by an extraction in CO_2.

Partial complexation of (±)-*trans*-1,2-cyclohexanediol.

1.000 g (±)-CHD (8.6 mmol) and 0.646 g (*R*,*R*)-tartaric acid (4.3 mmol) were separately dissolved in 15 ml of ethanol. These two solutions were mixed and a precipitate appeared. 1.5 g of expanded perlite was added to it. The ethanol was rotoevaporated at moderate temperature and the solid was let to dry out overnight at room temperature and atmospheric pressure thoroughly.

Extraction of uncomplexed *trans*-1,2-cyclohexanediol, (*S,S*)-*trans*-1,2-cyclohexanediol.
The extraction was carried out at 150 bar and 48 °C with an average flow of CO_2 of 20 g/min and the total weight of CO_2 was 470 g. It was checked by a second extraction in the same condition but with 180 g of CO_2 that the "extractable" part of the CHD which mostly corresponds to the uncomplexed (*S*,*S*)-CHD had been extracted. Indeed only a small amount, inferior to 20 mg, was extracted in this second step. The average amount of CHD extracted during two consecutive extractions was 0.53 g and had an enantiomeric excess (*ee*) of about 60 %.

Raffination of the residuum, (R,R)-trans-1,2-cyclohexanediol.
The raffination is based on the acidic properties of tartaric acid: the tartaric acid treated with a basic aqueous solution. Water was then rotoevaporated and CHD was extracted by chloroform. The average quantity of product isolated after raffination was about 0.3 g and had an enantiomeric excess of 80 %.

The F-parameter or selectivity is used for the evaluation of a resolution and defined as [5]:

$$F = Yield_{Extraction} * ee_{extract} + yield_{raffination} * ee_{raffinate} \qquad \text{Equation 1}$$

The F-parameter takes value between 0 and 1 and the higher it is the better the resolution is. In this case, F was 0.6.

2. Enzyme-catalysed reaction in non-aqueous media.

Another method for the production of enantiomers is based on the chiral catalysts of life: enzymes. Moreover, they are also important catalysts for industrial purposes, especially food industry. The advantages of biocatalysis are the following [1, 6]: Enzyme are renewable, biodegradable and non toxic catalysts; they present good kinetic (high turnover) and high selectivity (important enantiomeric excess and conversion and no protection step needed). Moreover, they require only mild reaction conditions (low temperature, moderate pH and so on).

The "natural" solvent for biocatalysis is water. However the slight water solubility and/or stability of certain compounds in water limit the performance of the reaction. Moreover, an extraction of the medium by an organic solvent is needed to recover the product. This step causes extra costs and the use of contaminant, toxic solvents like hexane.

It was discovered in the mid-1980's that enzymes are surprisingly active in organic solvents. The advantages for the use of organic solvents in biocatalysis can be listed [7]: the solubility of reactants is increased, the non-enzymatic (spontaneous) and side reactions (products not stable in water.) eliminated. The stability of enzymes can be enhanced, as well as the stability of the coenzyme. In the case of coenzyme dependant reactions, permanent complexes of coenzyme and enzyme exist in organic solvent; hence no dissociation takes place, even at low affinity. But the following problems may be encountered: many enzymes are quickly deactivated in organic solvent, the reaction rate is sometimes low, solvents strongly inhibit certain enzymes, and aggregation of the enzyme is a limit to mass transfer. Immobilisation prevents this aggregation because enzyme and coenzyme are spread out over the area of the support.

Alcohol dehydrogenase (ADH) from *Lactobacillus brevis* (LBADH) [8] catalysed the conversion of acetophenone to R-phenylethanol, which was investigated as a model reaction. LBADH was chosen because of its availability, low price and promising application [9]. Indeed, LBADH presents a high enantiomeric excess for the synthesis of *R*-1-phenylethanol and other compounds. Actually, LBADH has a broad substrate range and therefore the synthesis could be applied or adapted to another product. It requires the coenzyme NADP/NADPH and catalyses the reaction which is at the first line in the Figure 2. NADPH is regenerated using a "sacrificial substrate": isopropanol is added to the system. It undergoes the reaction opposite to the substrate's (second line of Figure 2), which gives NADPH. In the last line of the Figure 2, the final yield is given where both NADP and LBADH are catalysts.

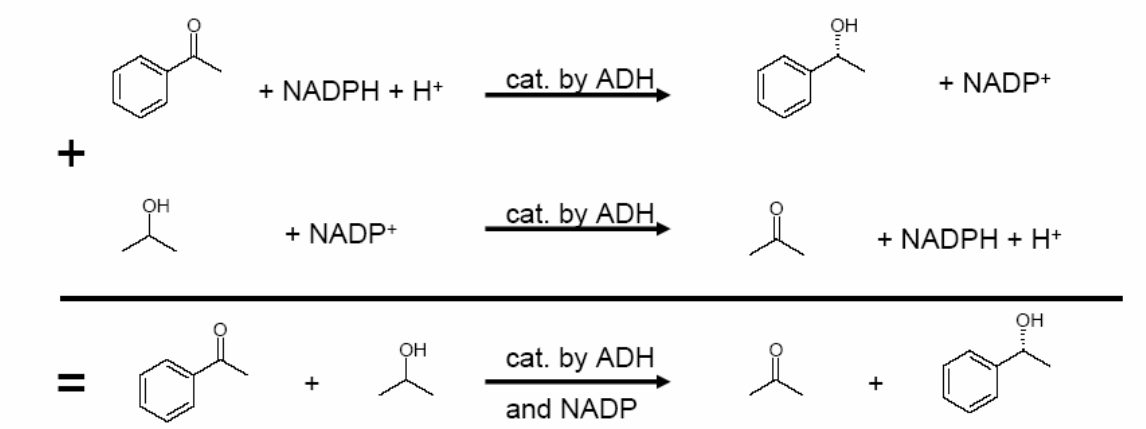

Figure 2: Reactions catalysed by LBADH, conversion of acetophenone and regeneration of NADPH by addition of isopropanol.

The three reaction media investigated are: single-phased aqueous system (enzyme and coenzyme solubilised in water, not presented herein), two-phased system water/propane (enzyme and coenzyme solubilised in the aqueous phase) and a single-phased system in propane (enzyme and coenzyme coimmobilised).

Reaction with immobilised enzyme in propane.

The method for co-immobilising the enzyme and the coenzyme on glass beads was adapted from [10]. Enzyme (25 mg) and coenzyme (11 mg) were diluted in the buffer in a beaker. The solution was stirred for 10 minutes at 4 °C. Then the support was added (5 g glass beads (425 µm-600 µm)). This mixture was stirred for 1 hour at 4 °C. Then the beaker was placed into a dessicator at the pressure of about 0.5 bar for 2 hours and, then, at the pressure of about 0.1 bar until it dried.

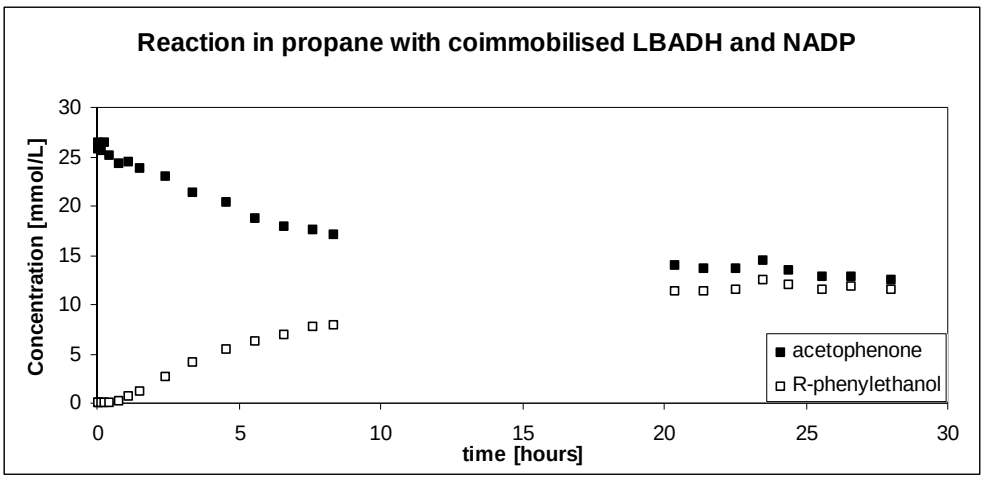

Figure 3: Concentration of acetophenone and R-phenylethanol in the propane phase according to time. Reaction of 1.5 mmol of acetophenone and 25 mmol of isopropanol with 11 mg NADP and 25 mg LBADH in propane at 30 °C and 30 bar.

The reaction (see Figure 3) was performed at 30 bar and 30 °C in propane inside the "high pressure view cell" with enzyme and coenzyme immobilised on glass beads. The water activity was set with Na_2SO_4 10/0 [11]. The initial rate was estimated to 1.4 mmol/min which corresponds to 0.056 µmol/(min.mg_{Enzyme}). The total turnover of this reaction related to the enzyme (tetramere) was

160 and related to the coenzyme 50. The yield was 45 %. The enantiomeric excess was superior to 99 %.

Reaction run in a biphasic system, water-liquid propane.

If the low solubility of substrate imposes a large reaction volume or if a substrate or product is instable in water so that it is important to reduce the time it spends in the aqueous phase, a biphasic system can be chosen instead of a non-aqueous medium for biocatalysis. Such a biphasic system is composed of an:

- Aqueous phase. It contains the catalysts (enzyme and coenzyme) and hydrophilic substrates and products. The reaction takes place in this phase.
- Organic water-immiscible phase. It stores the apolar/hydrophobic substrates and products and exchanges them with the other phase.

Reactions in a biphasic system water/liquid propane were run in the view cell. Three experiments were run in those conditions: the reactions were catalysed by 25 mg LBADH, 10 mg NADP, the substrate quantity was 7 g isopropanol and 0.18 g acetophenone. The temperature was set at 30 °C. The pressure, 30 bar, 100 bar, 200 bar, was the only varying parameter. It was noticed [12] that an increase in pressure enhances the catalytic properties of horse liver alcohol dehydrogenase (HLADH) by reducing substrate inhibition. The increase of pressure did not demonstrate a clear improvement of the kinetics. The total turnover was about 80 related to coenzyme and 300 related to enzyme.

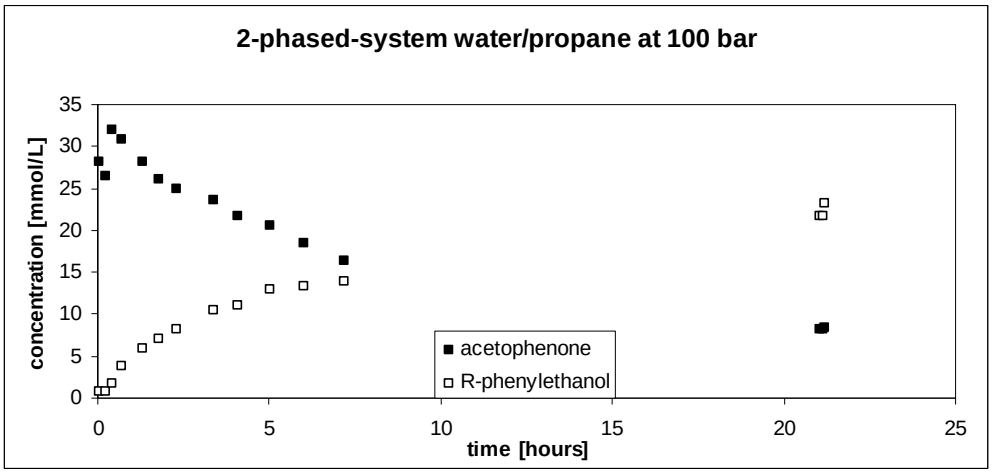

Figure 4: Concentration in the propane phase. Reaction of 1.55 mmol of acetophenone and 0.12 mol of isopropanol with 10 mL of buffered water and propane in the view cell at 30°C and 100 bar. Catalyst made of 10 mg NADP and 25 mg LBADH.

CONCLUSION

Two different methods enabling to produce enantiomerically pure compounds were investigated: a resolution method by partial complex formation in association with supercritical extraction and the synthesis using LBADH and NADP in non aqueous media. The F-parameter for the resolution was about 0.6. The product obtained by an enzyme-catalysed reaction was almost enantiomerically pure but the deactivation of the enzyme limited the conversion.

ACKOWLEDGMENT

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