Racemization of mandelic acid enantiomers in batch and fixed-bed reactors using immobilized mandelate racemase.

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Abbreviations: MR, mandelate racemase, ee%, enantiomeric excess

Abstract

Production of optically pure products can be based on simple unselective synthesis of racemic mixtures combined with a subsequent separation of the enantiomers. However, this approach suffers from a 50% yield limitation which can be overcome by racemization of the undesired enantiomer and recycling. Application of biocatalyst for the racemization steps offers an attractive option for high-yield manufacturing of commercially valuable compounds. Our work focuses on exploiting the potential of racemization with immobilized mandelate racemase. Immobilization of crude mandelate racemase via covalent attachment was optimized for two supports: Eupergit® CM and CNBr-activated Sepharose 4 Fast Flow. To allow coupling of enzymatic reaction with enantioselective chromatography, a mobile phase composition compatible with both processes was used in enzymatic reactor. Kinetic
parameters obtained analyzing experiments carried out in a batch reactor could be successfully used to predict fixed-bed reactor performance. The applicability of the immobilized enzyme and the determined kinetic parameters were validated in transient experiments recording responses to pulse injections of R-mandelic acid.

1. Introduction

Enantiomers of a given substance are characterized with indistinguishable physico-chemical properties but can exhibit largely different physiological responses in living organisms. For this reason the production of optically pure products plays a key role in pharmaceutical and fine chemical industries. Pure enantiomers can be obtained by stereoselective synthesis, synthesis from chiral pool or synthesis of 50/50 racemic mixture and subsequent separation. The last strategy is used in many industrial production processes. This classical approach suffers from a 50% yield limitation when undesired enantiomer is discarded. A powerful concept to access pure enantiomers and to achieve high yields is the application of dynamic kinetic resolution (DKR) using an enzyme for selective conversion of one of the enantiomers combined with a racemization catalyst. In this filed in particular metalorganic chemical catalyst have been proven to be very attractive [1,2]. Apart from kinetic resolution concepts, there is a second way of overcoming yield restriction. Incorporation of a racemization step applied after enantioseparation and recycling the side stream can be very attractive. Repeatedly carried out joint separation and racemization can significantly enhance yields. Theoretically up to 100% yield can be reached in this way, increasing efficiency and improving process-economy.

Several options of processes integrating enzymatic reactions with continuous chromatography were presented in a review by Bechtold et al. [3]. A systematic conceptual design including racemization was proposed for planning of integrated processes for the production of pure enantiomers by Kaspereit et al. [4]. Described process configurations were based on enantioselective chromatography, racemization and crystallization. A concept of combined enantioseparation and racemization with amino acid racemase was investigated recently [5–7]. Potential of racemization coupling with chromatography was shown for methionine whereas the conglomerate forming asparagine was separated by preferential crystallization. In this paper we will focus on coupling of enzymatic racemization with enantioselective chromatography.
Catalysis of racemization by means of an enzyme is an attractive option mostly due to mild reaction conditions more compatible with separation step. Enzymatic racemization is however still underutilized in industrial processes. The most widely used racemization techniques – thermal racemization, base and acid-catalyzed racemization – often require harsh reaction conditions. Importantly the use of biocatalyst prevents occurrence of side reactions and product decomposition. An extensive review on racemization techniques used in industry was published by Ebbers et.al. [8]. Use of enantioselective chromatography as a separation step has a great potential due to the high number of available stationary phases ready for use with various enantiomers.

Minimization of enzyme costs and process integration should be considered when use of biocatalyst is planned. Coupling of racemization and enantioselective chromatography requires high enzyme stability in chromatography compatible mobile phase and convenient re-use of the biocatalyst. Depending on the stability of the free enzyme and the specificity of the chromatographic step it can be a challenging task and often requires immobilization of the biocatalyst [9–13]. Immobilization influences stability of the enzyme in working conditions by several mechanisms. The enzyme structure can be fixed with attachment via several points on its surface. Such restriction of structure movement increases resistance to denaturation and unfavorable conformational changes [11,14]. In case of immobilization of crude enzyme preparations the spacial separation in the pore prevents enzyme deactivation by aggregation or proteolysis. Therefore in some cases a need for expensive enzyme purification can be eliminated. Most importantly immobilization enables easy enzyme retention in the reactor, enzyme re-use and facilitates product downstream processing preventing contamination by the catalyst. The immobilized enzyme can be packed into a reactor and can easily be integrated with the chromatographic step [3].

In this paper we investigated the potential of mandelate racemase (MR) [EC 5.1.2.2] as a biocatalyst for racemization of mandelic acid and some of its derivatives. The main objective of the study was the development of stable immobilized enzyme with high activity in conditions compatible with enantioselective chromatography. For this reason MR immobilization was optimized for two commercially available carriers: Eupergit® CM and CNBr-activated Sepharose 4 Fast Flow. Subsequently, we characterized one of the prepared biocatalysts (MR immobilized on Eupergit® CM) and studied its performance in batch and fixed bed reactors. A mathematical model was used to quantify the initial rates in batch runs in order to parametrize a standard kinetic model, which was then validated comparing predicted and measured fixed-bed reactor outlet concentrations under steady state conditions.
A final validation was performed by recording and interpreting responses to pulses of R-mandelic acid injected into the same fixed-bed reactor. This scenario simulates internal profiles exploited in enantioselective chromatography.

2. Materials and methods

2.1 Materials

The pET-15b plasmid with mandelate racemase gene was kindly donated by Prof. Stephen L. Bearne, Dalhousie University, Halifax, Canada [15]. Supports used for enzyme immobilization were Eupergit® CM (Sigma-Aldrich, St. Luis, MO, USA) and CNBr-activated Sepharose 4 Fast Flow (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Protease inhibitor cocktail set VII from Calbiochem was purchased from Merck, Darmstadt, Germany and Rotilabo PES filters with 0.2 µm pores from Carl Roth, Karlsruhe, Germany.

Two Chirobiotic T columns were used (Sigma Supelco, St. Louis, MO, USA). One (column C1) was applied in concentration analysis (5 µm, L=150 mm, d=4.6 mm) and another one (column C2) was connected with the outlet of the fixed-bed (16 µm columns, L=150 mm, d=10 mm). The mobile phase is discussed under 2.3 and 2.6.

Substrates used for enzymatic reactions were R-mandelic acid and R-3-chloromandelic acid purchased from Sigma Aldrich Chemie (Sigma-Aldrich, St. Luis, MO, USA), S-4-methoxymandelic acid from Beta Pharma Scientific (Branford, CT, USA) and S-2-chloromandelic acid from AK Scientific (Union City, CA, USA).

2.2 Enzyme production

E. coli BL21(DE3) cells were transformed by heat shock with the pET-15b plasmid encoding mandelate racemase with N-terminal StrepII-tag [15]. Cells were initially grown in minimal medium with addition of 0.5% glucose, 5 µg/ml thiamine and 100 g/ml ampicillin at 37°C. After induction of mandelate racemase expression with 200 µM isopropyl-b-D-thiogalactopyranoside (IPTG) at OD_{420}=0.5 the overproduction was performed at 20°C until OD_{420}=1.3. Cells were harvested by centrifugation (5000 g, 15 min, 4°C). The expression conditions have shown to decrease formation of inclusion bodies and increase the amount of active mandelate racemase in extracts.

The cell pellet from each 0.5 l of culture was re-suspended in 5 ml of 150 mM Tris-HCl pH 8.0 or coupling buffer used for immobilization (section 2.4) with addition of 60 µl of protease inhibitor cocktail set VII (Calbiochem). The soluble proteins fraction was extracted
by sonication with 7x30s bursts with 5 minutes cooling intervals at 4°C. Obtained extracts had activities of approximately 180 U/mg ± 20 U/mg of protein. Typically about 50 g of protein per each liter of expression culture was obtained. The extracts, further referred to as crude MR preparations, were stored at 4°C and used within 2 weeks during which enzymatic activity was proven to be stable.

2.3 Enzyme activity

Enzymatic activity measurements were performed in 50 mM HEPES buffer pH 7.5 with 3.3 mM MgCl₂ at 25°C. The reaction buffer is further referred to as solvent S1. The 10 g/l (65.7 mM) R-mandelic acid was used as the substrate if not stated otherwise. The activity values were expressed in U/mg of total protein or U/g of wet carrier (in case of immobilized enzyme). 1 U corresponds to the amount of enzyme required for conversion of 1 μmol of substrate in 1 minute.

**HPLC method.** The carrier with immobilized MR was conditioned in reaction buffer for 15 minutes before each experiment. Buffer was removed by suction drying on a glass frit filter. 15mg of wet carrier with immobilized MR was weighted into Eppendorf vials and 2 ml of substrate was added. The suspension was gently agitated for 1, 2, 5 and 8 minutes. After that time the reaction was terminated by filtration through Rotilabo PES filters with 0.2 μm pores and 1 minute heating at 90°C. Concentration of R-mandelic acid was determined on column C1 with 0.3M TEAAv buffer pH 4.02 with 20% methanol as mobile phase (Jandera et.al 2001). Measurements were performed on HPLC Agilent 1260 series (Agilent, Palo Alto, CA, USA) with detection at 254 nm. HPLC method was used for measurements of activity of immobilized MR during screening of coupling conditions and screening of immobilized enzyme substrate spectrum. All measurements were repeated twice.

**Polarimetric method** for measurements of activity of free enzyme. 40 μl of extract was added to 4 ml of substrate and immediately injected into 2 ml cell of MCP 500 Modular Circular Polarimeter (Anton Paar, Graz, Austria). Optical rotation was measured at 435 nm and 25°C. The response between 0.5 and 10 minutes was used for calculation of initial reaction rate. Measurements were repeated twice. Specific activities of free enzyme were given in U/mg of total protein. Protein concentrations were measured with Bradford methods with bovine serum albumin as a standard of concentration [16].

2.4 Enzyme immobilization

*CNBr-activated Sepharose 4 Fast Flow:*
A standard immobilization procedure provided by the producer was adopted. After pre-activation with 1 mM NaCl the 100 mM NaHCO$_3$ solution pH 8.3 was used as coupling buffer. 0.85 ml of crude enzyme preparation with total protein concentration of 2.5 – 10 mg/ml was added to 0.5 g of wet adsorbent. Coupling was performed at 4°C for 18 and 24h. After that time the adsorbent was washed on a glass frit and left for 2h in 50 mM glycine solution to block uncoupled active groups. After alternating washes with 50 mM Tris with 1 M NaCl pH 8.0 and 50 mM glycine with 1 M NaCl pH 3.5 buffers, the gel with immobilized mandelate racemase was stored in 150 mM Tris-HCl pH 8.0 with 20% ethanol at 4°C. Influence of following immobilization conditions was investigated:

a) Coupling time (18h and 24h)

b) Enzyme load, expressed as ratio of mg of protein to mg of wet carrier used for immobilization. The range 1:59 – 1:235 was tested.

_Eupergit® CM:_

A direct binding procedure via oxirane groups was adapted [17]. Immobilization was performed in HEPES buffers with various concentration and pH values. 4 ml of crude enzyme preparation in corresponding coupling buffer (1 mg/ml protein concentration) was added to 80-320 mg of dry adsorbent. The coupling was performed at room temperature for 24h and 72h. Next support with immobilized enzyme was washed thoroughly with corresponding coupling buffer and with 150 mM HEPES buffer. The uncoupled binding sides were blocked with 50 mM glycine for 45 minutes. Immobilized enzyme was stored in 150 mM Tris HCl pH 8.0 with 0.05% sodium azide at 4°C.

Influence of following immobilization conditions was investigated:

a) Coupling time (24-72h)

b) Coupling buffer concentration (0.35-1M HEPES)

c) Coupling buffer pH (6.9-8.2)

d) Enzyme load expressed as ratio of mg of protein to mg of dry carrier used for immobilization. The range 1:20 – 1:80 was tested.

Recovery of enzymatic activity was expressed as a percentage of total activity of immobilized enzyme compared to total activity of extract used for the immobilization on a given amount of carrier: A=100%(B/C), where A=recovery of enzymatic activity, B=total immobilized activity and C=total activity of extract used for immobilization.

### 2.5 Reaction in batch reactor
Immobilized MR was conditioned in reaction buffer (solvent S1 – 50 mM HEPES buffer pH 7.5 with 3.3 mM MgCl₂ or solvent S2 – 20 mM HEPES 3.3 mM MgCl₂ pH 6.8 with 20% methanol) for 15 minutes before each experiment in a stirred tank. Next, buffer was removed by suction drying on glass frit filter. 0.6 g of wet carrier particles was added to 60 ml of substrate (dosage D=10g/l, eq. 2) in reactor with stirrer rotation of 350 rpm. The solution was recycled via a peristaltic pump through 2 ml cell of MCP 500 Modular Circular Polarimeter. The optical rotation was measured at 435 nm at 25 °C. The method was used to obtain kinetic parameters of racemization reaction catalyzed by immobilized enzyme. The data from first 10 minutes of the reaction were fitted to linear equation. All measurements were repeated twice.

2.6 Performance in fixed-bed reactor

MR immobilized on Eupergit® CM was packed in Tricorn 5/20 column (GE Healthcare Life Sciences, Uppsala, Sweden) with a slurry method obtaining bed height of 2.6 cm. 0.356 g of immobilized enzyme was packed with a dosage D=1512 g/l (eq. 2). The fixed-bed reactor was connected to the HPLC system (Agilent Technologies 1260). The concentrations at the reactor outlet were measured in two ways. In case of constant injection of the substrate, samples were collected at reactor outlet and analyzed with MCP 500 Modular Circular Polarimeter. Alternatively 100 µl pulse injections of substrate were done and reaction products were separated by column C2 connected directly with the outlet of the enzymatic racemization reactor. The signal was recorded with a diode array detector at 254 nm. The system was operated in solvent S2 as mobile phase with flow rates in the range of 0.2 to 3 ml/min. The quantities of R and S-mandelic acid separated by chromatographic column were calculated using Peak Analyzer tool of Origin Pro 8.6 software. All measurements were repeated twice.

3. Results and discussion

3.1 Mandelate racemase immobilization on CNBr-activated Sepharose 4 Fast Flow

Immobilization of MR crude preparation was tested for two materials known to provide multipoint covalent attachment of proteins: CNBr-activated Sepharose 4 Fast Flow and Eupergit® CM. Due to different properties and coupling mechanisms of these adsorbents the final effects of immobilization can differ. Various concentration and accessibility of immobilized enzyme molecules can be obtained for the two materials. A careful optimization of binding conditions was performed to find the appropriate immobilization strategy for further investigation.
CNBr-activated Sepharose 4 Fast Flow is based on Sepharose 4 Fast Flow carrier with physical stability improved by crosslinking of the agarose matrix. It can withstand pressure drops higher than conventional agarose supports therefore it is useful for industrial purposes. During the coupling reaction cyanate ester and imidocarbonate active groups of CNBr-activated Sepharose 4 Fast Flow react with primary amines on the protein surface. The immobilization time did not affect the final activity significantly which suggests that 18 hours of coupling was sufficient. Highest activity values of the immobilized MR (250 U/g of carrier) was obtained for enzyme load 1:118 and 1:59. Further increase of ratio of used support leads to decreased immobilized enzyme activity possibly due to incomplete saturation of stationary phase. The highest recovery of enzymatic activity (35%) was obtained at the highest enzyme load of 1:235.

3.2 Mandelate racemase immobilization on Eupergit® CM

Eupergit® CM is an epoxy activated porous acrylic support with high ligand density of 300 µmol/g of dry support promoting multipoint attachment. Binding on the epoxy supports is a two stage process. The initial adsorption is driven by hydrophobic interaction. Only after hydrophobic adsorption the epoxy groups can react with amino, thiol, aromatic hydroxile, carboxy and imidazoline groups on the protein surface. The type of protein groups available for reaction depends on coupling pH. For this reason orientation of protein and number of covalent bounds differ depending on the coupling conditions [18]. Immobilization of crude MR on Eupergit® CM was optimized first with respect to coupling time and pH (in 0.35M HEPES buffer). In the next step influence of buffer concentration and enzyme load was investigated.

As can be seen in fig. 1a improved coupling was achieved at pH 8.2. Amino, tiol and phenol groups of amino acids have a higher degree of immobilization on epoxy carriers at basic pH values [18]. Based on our results, these groups are primarily responsible for binding mandelate racemase. At neutral and slightly acidic conditions the protein acidic groups are primarily involved in coupling [19,20].

Immobilization time had a significant influence on the binding efficiency, especially in the case of coupling at pH 8.2. The activities of the immobilized enzyme increased over 4 times between 24h and 72h of immobilization. This is consistent with the fact that during immobilization on epoxy activated supports the second step of coupling, namely the covalent binding, is very slow. The formation of multiple bounds is thought to require prolonged periods of time for precise alignment of protein residues against the adsorbent epoxy groups.
and for coupling itself [21]. Based on the results, pH 8.2 and a coupling time of 72h were chosen for further investigation of impact of buffer ionic strength on coupling process.

Increased ionic strength of coupling buffer is required to promote the hydrophobic interaction [17,20]. Three concentrations of HEPES buffer pH 8.2 were investigated: 0.35 M, 0.7 M and 1 M. Increase of ionic strength should promote the immobilization process, as long as its value is not high enough to affect the structure and solubility of target enzyme. Mandelate racemase proved to be stable in all tested buffer concentrations and the highest values of enzymatic activity and recovery of enzymatic activity were obtained in 1 M HEPES buffer concentration (Fig 1b).

The influence of protein load between 1:20 and 1:80 was studied as well. The highest enzymatic activity values, up to 370 U/g, are obtained for the lowest enzyme load (fig. 1b). The highest recovery of mandelate racemase activity was however obtained for enzyme load of 1:60. The decreased recovery of enzymatic activity values for enzyme load of 1:20 and 1:40 may be a consequence of steric effects during coupling and/or hindrance of active sides of immobilized mandelate racemase due to high density of immobilized proteins. Based on the results optimal conditions of coupling providing highest recovery of enzymatic activity and high enzymatic activity were chosen.

Immobilization of MR on both tested adsorbents provided enzyme preparations with high enzymatic activity values. The highest activity values as well as the highest recovery of enzymatic activity were obtained for Eupergit® CM. Moreover mechanical and chemical properties of this carrier are attractive for use in industrial applications. Eupergit® CM carrier can be used at pH values ranging from 0 to 14 without swelling or shrinkage. The mechanical stability of Eupergit® CM assures a broad choice of reactor mode alternatives [17]. It can be used in packed-bed reactors characterized by superior reaction rates or stirred-tank reactors offering simplicity and low-pressure. For all the above reasons MR immobilized on Eupergit® CM was used for further investigation.

Crude mandelate racemase was immobilized on Eupergit® CM with enzyme load of 1:60 by 72h coupling in 1M HEPES buffer pH 8.2. 12 g Eupergit® CM with immobilized MR was prepared and used for further characterization.

Conversion of 0.6 g of R-mandelic acid in batch reactor experiments was carried out for three enzyme dosages D=5, 10 and 15 g/l (eq. 2). 90 % racemization was achieved after 58, 20 and 14 minutes respectively. Significant reaction rates were obtained in comparison to previously reported DEAE cellulose immobilized MR [22] where complete racemization of 0.5 g of R-mandelic acid with immobilized enzyme with dosage D=100 required 3 hours.
It should be mentioned here, that another attractive aspect for applying immobilized enzyme is the improved storage and reaction stability. MR immobilized on Eupergit® CM was stored at +4°C in 100 mM Tris HCl buffer pH 8.0 with 0.05% sodium azide for 2 months. At the end of the storage period over 90% of initial activity remained. During this time activity of free enzyme stored at the same temperature decreased to 67% and activity of free enzyme stored frozen at -20°C remained unchanged. Reaction stability of the immobilized enzyme was satisfying as well. Enzyme remained stable over course of 5 days of constant measurements in fixed-bed reactor.

3.3 Substrate specificity of immobilized mandelate racemase

The wide substrate spectrum of MR makes it especially interesting for use in industrial biotransformations of non-natural substrates. The substrate tolerance of free wild type enzyme was summarized in a review by Felfer et al. [23]. Some of the mandelic acid derivatives are of interest due to ease of enantiomers separation. For instance the S-2-chloromandelic acid crystallizes as conglomerate forming system and can be therefore easily separated by preferential crystallization. The reaction rates of free and immobilized MR were measured with four different substrates (table 1). The first three substrates are known to be racemized by MR whereas the activity against S-2-chloromandelic acid is negligible [23,24]. Analogous results were obtained for free and immobilized MR in this study, showing no significant influence of immobilization for tested substrates. Further studies were performed exclusively for the mandelic acid system.

3.4 Rate of the enzymatic reaction in a batch reactor.

The reaction rate can be calculated based on following equation:

\[
r = \frac{1}{\nu_i n_i} \frac{d n_i}{d t} = \frac{1}{\nu_i m_E} \frac{d m}{d t} = \frac{1}{\nu_i D} \frac{d c_i}{d t}
\]

(1)

Where \( r \) is reaction rate, \( t \) is time, \( \nu_i \) is a stoichiometric coefficient (for racemization reaction \( \nu_i = -1 \)), \( m_E \) is the mass of the solid particles carrying the immobilized enzyme, \( V_L \) is volume of reaction liquid phase, \( n_i \) are moles of substrate \( i \), \( c_i \) is concentration of substrate \( i \), and \( D \) is the already mentioned enzyme dosage expressed as:

\[
D = \frac{m_E}{V_L}
\]

(2)
Racemization mechanism can be described by reversible form of the Michaelis-Menten kinetics which can be expressed with the following equation for conversion of R-mandelic acid [26,27]:

\[
r = \frac{r_{\text{max},R} C_R - r_{\text{max},S} C_S}{K_{M,R} + \frac{C_R}{K_{M,R}} + \frac{C_S}{K_{M,S}}} + 1
\]

(3)

Where the \(C\) are the mandelic acid enantiomer concentrations, \(r_{\text{max}}\) is maximum reaction rate, \(K_M\) is Michaelis-Menten constant and subscripts \(R\) and \(S\) indicate values for R- and S-mandelic acid respectively. In case of mandelate racemase it has been shown in previous studies that reaction rates in both directions are similar [28,29]. Therefore we can assume that \(K_{M,R} = K_{M,S} = K_M\) and \(r_{\text{max},R} = r_{\text{max},S} = r_{\text{max}}\), which provides:

\[
r = r_{\text{max}} \frac{(C_R - C_S)}{K_M + C_R + C_S}
\]

(4)

The initial rates \(r_0\) of only R-mandelic acid racemization (\(C_{S,0} = 0\)), which can be most easily analysed, are:

\[
r_0 = r_{\text{max}} \frac{C_{R,0}}{K_M + C_{R,0}}
\]

(5)

From a series of initial rates observed in conventional isothermal batch reactor experiments we estimated the two kinetic parameters \(r_{\text{max}}\) and \(K_M\), both for the free and the immobilized enzyme applied at the optimal conditions in Solvent 1 (section 2.3) for R-mandelic acid racemization (table 2, fig.2).

The comparison of kinetic parameters for free and immobilized enzyme in optimal conditions suggests an influence of additional effects upon immobilization. Value of \(K_M\) increased whereas \(r_{\text{max}}\) decreased nearly twice (\(r_{\text{max}}\) of 307.5 U/g of carrier is equal to 107.6 U/mg of protein for immobilized enzyme). Kinetic parameters can be altered after enzyme immobilization due to several effects. Mass transfer limitations may limit the apparent rate of reaction. Upon immobilization some of the enzyme molecules can be deactivated or some of the active sides can become inaccessible to the substrate leading to decrease of specific activity. Other steric effects influencing the association and dissociations steps of the reaction would affect apparent \(K_M\) value. Similar effect can be a consequence of small conformational changes of the enzyme upon immobilization or changes of the nano-environment such as charge density in proximity of the carrier surface. The change of the reaction buffer is another factor clearly affecting reaction kinetics (see below for details). The \(r_{\text{max}}\) and \(K_M\) obtained for
reaction catalyzed by immobilized racemase are therefore apparent values which lump the influence of several phenomena. The determination of exact causes of the changes of kinetic parameters was outside of the scope of this study. The knowledge of apparent kinetic parameters can be however used for prediction of reactor behavior.

Optimal coupling of chromatographic separation and enzymatic racemization requires use of identical mobile phase in both steps of the process [4]. A choice of specific solvent has to be done carefully since it will strongly influence the performance of both steps. On one hand in chromatography the mobile phase should provide high resolution and assure stability of stationary phase. On the other hand in racemization step the high reaction rates and biocatalyst stability should be assured. These two steps normally have different requirements for optimal mobile phase compared to standalone processes, therefore a compromise needs to be found.

In this work we used Chirobiotic T column for the separation of mandelic acid enantiomers [30]. It can be operated at pH between 3.8 and 6.8 and requires a use of an organic modifier. The separation of racemic mandelic acid on Chitobiotic T column was studied in three mobile phases: 20 mM HEPES 3.3 mM MgCl$_2$ pH 6.8 with addition of 10%, 15% and 20% of methanol. The highest resolution of enantioseparation was obtained for mobile phase with 20 % methanol. The enzymatic activity decreased compared to optimal reaction conditions (solvent S1) independent of the concentration of methanol. Therefore mobile phase with highest methanol concentration (solvent S2 - 20mM HEPES 3.3 mM MgCl$_2$ pH 6.8 20% methanol) was chosen as most compatible with both processes and used for further investigation.

Kinetics of immobilized MR reaction in solvent S2 is shown in fig.2c. Changes in mobile phase composition had significant influence on apparent $r_{max}$ – the value was nearly halved in comparison to optimal reaction conditions (table 2). The decrease of pH to suboptimal value has likely a significant influence on the reaction. The addition of methanol and decrease of buffer concentration can influence the enzyme performance as well. The activity of immobilized MR in solvent S2 is still significant and allows a rapid inter-conversion of mandelic acid enantiomers.

3.5 Evaluation of fixed-bed reactor performance

3.5.1 Steady state operation
The classical balance of a fixed-bed reactor is applied in our work as follows:

\[
\frac{\partial c_i}{\partial t} + u \frac{\partial c_i}{\partial z} = -D v_i \, r
\]  \hspace{1cm} (6)

where \( z \) is axial coordinate, \( u \) is superficial velocity, and \( r \) can be expressed with eq. 4. For a fixed-bed reactor operating under steady state the second term can be eliminated when the reaction time is substituted in a classical way by residence time \( \tau \) [31]. The equation is then fully analogous to eq. 1:

\[
\frac{dc_i}{d\tau} = -D v_i \, r
\]  \hspace{1cm} (7)

where residence time is

\[
\tau = \frac{V_L}{F} = \frac{A_L z}{A_L u}
\]  \hspace{1cm} (8)

where \( F \) is flow rate and \( A_L \) is cross section area.

For the two enantiomers considered here, the following total mass balance can be exploited to express the concentration of one enantiomer by the concentration of the other:

\[
C_{T,0} = C_{R,0} + C_{S,0} = C_R(\tau) + C_S(\tau)
\]  \hspace{1cm} (9a)

where \( C_{T,0} \) is a total inlet mandelic acid concentration. We can introduce eq. 4 in consideration for batch reactor balance eq. 7 substituting \( C_S(\tau) \) with:

\[
C_S(\tau) = C_{T,0} - C_R(\tau)
\]  \hspace{1cm} (9b)

Obtaining following equation for batch reactor:

\[
\frac{dc_i}{d\tau} = -D \, r_{\text{max}} \frac{C_{T,0} - 2C_R(\tau)}{K_M + C_{T,0}}
\]  \hspace{1cm} (10)

Integrating eq. 10 we obtain:

\[
\int_{C_{R,0}}^{C_R(\tau(x))} \frac{K_M + C_{T,0}}{2C_{R}(\tau) - C_{T,0}} - dc_i = D \, r_{\text{max}} \int_{\tau=0}^{\tau(x)} d\tau
\]  \hspace{1cm} (11)

which leads to:

\[
C_R(\tau(x)) = \frac{1}{2}C_{T,0} + \frac{1}{2}(C_{R,0} - C_{S,0}) e^{-2\tau D \frac{r_{\text{max}}}{K_M + C_{T,0}}}
\]  \hspace{1cm} (12)
Fixed-bed reactor was operated first under steady state conditions with constant injection of 20g/l mandelic acid in solvent S2 with 100%, 60% and 40% R-enantiomer excess. Three flow rates were used to obtain various $\tau$ values. For each set of conditions 3 samples were collected during 30 column volumes. $C_R$ of each collected sample was obtained via polarimetric measurement to confirm that steady state in the reactor was reached. The experimental values were compared with prediction obtained from eq. 12 as shown in fig.3. For this the $K_M$ and $r_{max}$ form batch reactor experiments were used together with the dosage of enzyme packed in the fixed-bed reactor. The obtained match between experimental results and simulation of outlet concentrations of R-mandelic acid are satisfying. The results confirm, that the kinetic parameters obtained from analyzing batch runs with more reduced enzyme dosage are useful for prediction of the performance of a fixed-bed containing the enzyme in a more concentrated form enzyme and, thus, for optimizing operating conditions as e.g. flow rate or reactor size to achieve a desired conversion.

3.5.2 Transient operation

To validate compatibility of enzymatic reaction with enantioselective chromatography the chromatographic column C2 was connected directly after the outlet of fixed-bed reactor. R-mandelic acid in solvent S2 was injected in the racemization reactor and the mixture obtained in the reaction was separated on-line on C2 column. The influence of flow rate, R-mandelic acid concentration and injection value was investigated (fig.4). The complete racemization was obtained for the lowest flow rate of 0.2 ml/min as the highest residence time in the reactor was achieved. At higher flow rates the enantiomeric excess ($ee\%$) increased with increase of substrate concentration and decrease of $\tau$. At the highest load of mandelic acid and the lowest $\tau$ significant racemization was still obtained with outlet $ee\%$ of 56%. A chromatogram illustrating this result is shown on fig. 5a in comparison to complete racemization at lowest R-mandelic acid load and highest residence time (fig. 5c). It can be seen that with increase of the substrate load the peak separation becomes incomplete. A proper design of cut times could however assure a separation of the enantiomers and integrated processes with productivity increased by short batch times. The repeatability of racemization and separation for 4 mg injections and lowest $\tau$ proves high stability of the set-up even at overload conditions (fig. 5b).
The decrease of substrate conversion with decrease of \( \tau \) is consistent with results obtained in steady stare experiments. Complete racemization was obtained for all tested inlet concentrations when residence times in enzymatic reactor exceeded 1 minute. In the transient pulse experiments it was found that residence times below 0.3 minutes led to incomplete racemization which is in agreement with the steady state results discussed above (fig. 3). The more detailed quantification of pulse injections in fixed bed reactor would require solution of eq. 6 which is outside the scope of this study.

4 Concluding remarks

Crude mandelate racemase was immobilized on Eupergit\textsuperscript{®} CM and CNBr-activated Sepharose 4 Fast Flow. The highest enzymatic activity and recovery of activity were obtained on the former carrier. Various immobilization parameters were studied and optimal coupling conditions were found to be 72h coupling in 1 M HEPES buffer pH 8.2 with enzyme load of 1:60. The Eupergit\textsuperscript{®} CM-immobilized MR was characterized with respect to reaction kinetics, substrate specificity and stability. High operational stability allowed repetitive use in stirred-tank and fixed-bed reactors without loss of activity.

It was further shown that racemization by immobilized mandelate racemase and enantioselective chromatography by Chirobiotic T column can be coupled using as a common mobile phase 20 mM HEPES 3.3 mM MgCl\textsubscript{2} pH with 20% methanol. Reactor performance under steady state conditions was successfully predicted by a mathematical model incorporating kinetic parameters obtained in batch reactor experiments incorporating the right enzyme dosage. Performance of the fixed bed reactor coupled with chromatographic column was also validated analyzing racemization and separation of mandelic acid enantiomers as a response to pulse injections.

This results of this work and the applied approach will be used for further design and optimization of joint reaction and separation steps.

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References:


Tables:

Table 1. Substrate tolerance of free and immobilized mandelate racemase (different mandelic acid derivatives).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity of free crude MR %</th>
<th>Relative activity of free MR from literature data % [23]</th>
<th>Relative activity of MR immobilized on Eupergit® CM %</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-mandelic acid (reference)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>R-3-chloromandelic acid</td>
<td>69.8</td>
<td>61</td>
<td>64.4</td>
</tr>
<tr>
<td>S-4-methoxymandelic acid</td>
<td>23.3</td>
<td>17</td>
<td>22.3</td>
</tr>
<tr>
<td>S-2-chloromandelic acid</td>
<td>1.2</td>
<td>≤1</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Table 2. Kinetic parameters of racemization of mandelic acid obtained by eq. 5.

<table>
<thead>
<tr>
<th></th>
<th>$r_{max}$</th>
<th>$K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free crude MR, solvent 1</td>
<td>220.6 U/mg protein ± 2.5</td>
<td>3.87 mM ± 0.4</td>
</tr>
<tr>
<td>MR immobilized on Eupergit® CM, Solvent 1</td>
<td>307.5 U/g carrier ± 4.6</td>
<td>11.24 mM ±1.0</td>
</tr>
<tr>
<td>MR immobilized on Eupergit® CM, Solvent 2</td>
<td>169.5 U/g carrier ±5.1</td>
<td>8.59mM ±1.2</td>
</tr>
</tbody>
</table>

1 1 U = 1 µmol/min

2 kinetic parameters for immobilized enzyme are apparent values
Figures:

Fig. 1 Optimization of crude MR immobilization on Eupergit® CM in HEPES buffer. Influence of coupling conditions on enzyme activity and recovery of enzymatic activity at various enzyme loads. a) Influence of coupling time and pH in 0.35M HEPES buffer: 24h coupling at pH 6.9 - black; 72h coupling at pH 6.9 - white; 24h coupling at pH 8.2 – light gray; 72h coupling at pH 8.2 – dark gray. b) Influence of coupling buffer concentration at pH 8.2, 72h coupling time and various enzyme loads: 0.35 M buffer – black; 0.7M buffer – white; 1M buffer dark gray. To assure that enzymatic activity is measured on basis of linear region of the reaction, the data with minimal R2 of 0.95 of the slope were used for calculation. Measurements were repeated twice.

Fig. 2 Kinetics of R-mandelic acid racemization of a) free crude MR in solvent S1, b) immobilized MR in solvent S1, c) immobilized MR in solvent S2. Reaction rates were obtained by analysis of the first 10 minutes of reaction with minimal $R^2 \geq 0.99$. Measurements were repeated twice. (D=10 g/L, eq.2)

Fig. 3 Prediction of fixed-bed reactor R-mandelic acid outlet concentration at overall inlet mandelic acid concentration of 20 g/l and varying ee%. Solid lines show predictions obtained with eq. 12, dashed line represent racemate concentration and symbols represent experimental data from fixed bed reactor. Square – initial 100% R-mandelic acid ee%, circle – initial 60% R-mandelic acid ee%, triangle – initial 40% R-mandelic acid ee%. Measurements were repeated twice. (D=1513 g/L, eq. 2)

Fig. 4 Influence of residence time in racemization reactor on mandelic acid ee% during coupled racemization-chromatographic separation in solvent S2. Four different amounts of the substrate were injected on the fixed-bed reactor: 4 mg (diamonds), 2 mg (triangle), 1 mg (circle) and 0.5 mg (square). The described process can be used for production of pure S-mandelic acid from racemic mixture as shown on Fig. 5b. The connecting lines are made only for better visualization of trends. Measurements were repeated twice.

Fig. 5 Separation profile of mandelic acid enantiomers on Chirobiotic T column after racemization of R-mandelic acid on fixed-bed reactor. a) partial separation and racemization after injection of 4mg of R-mandelic acid with 3 ml/min flow rate ($\tau=0.075$ min, eq. 8) b) 10 injections of 4 mg R-mandelic acid with 3 ml/min flow rate ($\tau=0.075$ min). c) complete racemization and separation after injection of 0.5mg R-mandelic acid with 0.2 ml/min flow rate ($\tau=1.13$ min). S-mandelic acid is eluted first.