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# Enzyme-assisted physicochemical enantioseparation processes: Part I. Production and characterization of a recombinant amino acid racemase

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ABSTRACT

The demand for enantiopure substances, e.g. for pharmaceutical applications or fine chemical production, continues to increase. This has led to the development of numerous stereoselective synthesis methods. Nevertheless a large number of chemical syntheses still result in racemic mixtures making a subsequent enantioseparation step necessary and thus are restricted to a maximum yield of 50%. Our work focuses on strategies to overcome this limitation by combining physicochemical separation processes with enzymatic racemization of the unwanted enantiomer in order to produce enantiopure amino acids. This paper deals with the production and characterization of a suitable amino acid racemase with broad substrate specificity (EC 5.1.1.10) from *Pseudomonas putida* which we cloned into *Escherichia coli*. Two enzyme lyophilizates of different purity were obtained from which the crude (*CL*) was sufficient for the racemization of methionine (Met) and the pure (*PL*) was used for asparagine (Asn). Racemization reactions of *D*-/*L*-Asn in $H_2O$ and *D*-/*L*-Met in 95 vol.% 100 mM $KP_i$-buffer, 5 vol.% methanol (MeOH) at different pH values and temperatures were characterized. The studied range of reaction parameters was chosen in dependency on planned enantioseparation processes. We found increasing $V_{max}$ values when temperature was risen stepwise from 20 to 40 °C for both systems and when pH was shifted from 6 to 8 for the Met system. The presented results provide the basis for engineering enzyme-assisted physicochemical enantioseparation processes.



## 1. Introduction

Chirality in nature and living systems is of great impact for a variety of active compounds interacting with them. The molecular building blocks of life, such as amino acids and sugars that form peptides, proteins and polysaccharides exist of chiral structures. Thus, optically pure enantiomers are of great interest for the fine chemical and pharmaceutical industry [1,2]. Despite an increasing number of stereoselective synthesis there are still a lot of reactions that lead to racemic mixtures of the desired homochiral products. The scientific and economic relevance of homochiral substances has led to the development of numerous separation techniques based for example on chromatography [3–5], crystallization [6,7] and stereoselective biotransformations [8,9] just to mention a few. A major drawback of these resolutions is the yield which is principally limited to a maximum of 50%. An attractive solution to overcome this limitation is the combination of a physicochemical separation process e.g. chromatography [10] or preferential crystallization (PC) [11] with an enzymatic process to reracemise the unwanted enantiomer [12,13]. In contrast to stereoselective biotransformations, such as the reduction of prochiral ketones to optically active alcohols [14,15] where only one enantiomer can be produced by a single biocatalyst, here *L*- and *D*-enantiomers can be obtained according to the chosen process route.

Since enantiopure amino acids (AA) are of great industrial relevance, e.g. *L*-Met for infusion solutions or food additives (ca. 600 t/year), AA are an interesting substance group for this approach.

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**Table 1**
Bacterial strains and plasmids used for cloning of AA racemase 5.1.1.10 and transformation into competent cell line.

| Strains/plasmids | Genotype/phenotype | Reference/source |
|---|---|---|
| *P. putida* KT2440 DSM6125 | | Nelson et al., 2002 [26] |
| *E. coli* DH5α | supE44 Δ(lacZYA-argF)U196 (ϕ80ΔlacZM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | Woodcock et al., 1989 [27] |
| *E. coli* BL21 (DE3) | $F^-$ ompT $hsdS_B(r_B^- m_B^-)$ gal dcm (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene1) | Studier and Moffat, 1986 [28] |
| pET22b | T7-expression vector for *E. coli*, PelB signal sequence, $Ap^r$ | Novagen |

Therefore we chose to use an already well known amino acid racemase with a broad specificity (EC 5.1.1.10) [16] as biocatalyst which was cloned into *E. coli* BL21 (DE3). We analyzed kinetic properties of two differently pure enzyme preparations with respect to racemization of the enantiomers of methionine (Met) and asparagine (Asn). Met, which is a racemic compound forming system and thus can be enantioseperated by PC [7], has been chosen as possible substrate for the application in an integrated enzymatic racemization process with chromatographic enantioseparation; Asn has been chosen because it is a suitable substance for enantioseparation by PC. Bechtold and coworkers [17] recently presented a detailed characterization of an amino acid racemase from *Pseudomonas putida* DSM 3263 (which is identical to the one used in our study) in which they concentrated on the racemization of *D*- and *L*-Met in organic-aqueous solutions. Our work is focusing on two different reaction systems: (1) racemization of *D*- and *L*-Met in 95 vol.% 100 mM $KP_i$-buffer (pH 7.0), 5 vol.% MeOH, which has been chosen suitable for the combination with chromatographic enantioseparation of methionine. (2) Racemization of *D*- and *L*-Asn in $H_2O$ which can be separated by PC. PC can be an interesting and highly efficient method for the optical resolution of conglomerate forming substances. In contrast to racemic compounds where both enantiomers coexist in one unit cell, a conglomerate is defined as a mechanical mixture of crystals of both enantiomers [18]. Nevertheless, only a few natural amino acids do belong to the group of conglomerates such as threonine (Thr) and asparagine [19]. PC of Thr in aqueous solutions has already been studied in detail by Elsner et al. [11] and others [20,21]. To the best of our knowledge so far there is neither published data about preferential crystallization nor about racemization of Asn.

## 2. Experimental

### 2.1. Materials

NaCl and $(NH_4)_2SO_4$ were obtained from KMF Laborchemie Handels GmbH (Lohmar, Germany), yeast extract and $NaH_2PO_4$ were purchased from Merck (Darmstadt, Germany). Isopropyl-β-D-thiogalactopyranosid (IPTG) and carbenicillin disodium salt were obtained from Carl Roth GmbH (Karlsruhe, Germany), pyridoxal-5′-phosphate (PLP) was obtained from Serva Feinbiochemica (Heidelberg, Germany). Diethylaminoethyl (DEAE) sepharose fast flow was purchased from Amersham Biosciences (UK). For SDS-polyacrylamid gel electrophoresis the NuPAGE test kit from Invitrogen (Carlsbad, CA, USA) and the Precision Plus Protein™ Standard Dual Color from Bio-Rad (Hercules, CA, USA) were used. Ultrapure water (Milli-Q by Millipore, Schwalbach, Germany) has been used exclusively during all procedures. All other chemicals were either purchased from Sigma–Aldrich or Fluka (Munich, Germany) and were of the highest available purity.

### 2.2. Cloning and transformation

*Bacterial strains and plasmids*. The strains and plasmids used are listed in Table 1. *P. putida* KT2440 and *E. coli* DH5α were used for cloning experiments and *E. coli* BL21 (DE3) as a heterologous expression host for plasmid-encoded racemase (orf PP3722).

*Media and growth conditions*. LB medium was routinely used to cultivate *E. coli* strains. Precultures for all experiments were prepared overnight in 5 ml LB medium in glass tubes at 37 °C. Plasmid-carrying *E. coli* cells were selected with 100 μg/mL ampicillin.

*DNA manipulation and PCR*. DNA fragments were amplified by PCR standard methods. DNA modifying enzymes (Fermentas, St. Leon-Rot, Germany) were used according to manufacturer's instructions. Plasmid DNA was prepared using the "HiSpeed Plasmid Midi" kit (Qiagen, Hilden, Germany).

*PCR amplification, cloning and expression of racemase*. Genomic DNA was isolated from *P. putida* KT2440 by using the DNeasyTissue-Kit (Qiagen). The gene was amplified by PCR. The PCR conditions used with Primers aaRaz NdeI up 5′-CAA *CAT ATG* CCC TTT CGC CGT ACC-3′ and aaRaz XhoI down 5′-TTT *CTC GAG* TCA *GTC GAC* GAG TAT CTT-3′ were as follows: initial denaturation 5 min at 98 °C, 35 cycles of 98 °C for 45 s, 56 °C for 45 s, 72 °C for 60 s and a final elongation 10 min at 72 °C.

TripleMaster polymerase used in PCR was purchased from Eppendorf (Hamburg, Germany). PCR was performed according to the manufacturer's recommendations. The size of the PCR product was checked on a 1% agarose gel and the product was excised from the gel and purified with a "Perfect Gel-Cleanup-Kit" (Eppendorf) used according to the manufacturer's protocol. The fragment was digested with NdeI/XhoI and ligated in pET22b which was hydrolyzed by the same restriction enzymes. *E. coli* DH5α was transformed with the resulting ligation.

Recombinant plasmids were identified and further characterized by restriction enzyme digestion. The DNA sequence of the amplified racemase was verified by DNA sequencing (Sequiserve, Vaterstetten, Germany).

*E. coli* BL21 (DE3) was transformed with the expression plasmid. A single colony was inoculated to LB medium containing 0.4% (w/v) glucose and 100 μg/mL ampicillin and grown at 37 °C on a shaking incubator until an optical density at 580 nm of 0.5 was reached. IPTG was then added to a final concentration of 0.4 mM for induction of expression. The induced cells were incubated at 37 °C for 2 h and harvested by centrifugation at 14,000 rpm for 15 min. The expression was analyzed by SDS-PAGE.

### 2.3. Fermentation of recombinant E. coli BL21 (DE3)

*E. coli* BL21 (DE3) was grown in a modified LB medium containing 0.5% (w/w) trypton, 0.25% (w/w) yeast extract, 0.25% (w/w) NaCl, 0.025% (w/w) $MgSO_4$ heptahydrate, 0.013% (w/w) $NaH_2PO_4$, 0.5% (w/w) *D*(+)-glucose monohydrate and 50 μg/mL carbenicillin disodium salt. A 50 mL preculture was grown for 6 h at 37 °C and 200 rpm in a 250-mL shaking flask after inoculation with 1 mL of the cryoculture (stored at −80 °C). 5 times 200 mL of aforementioned LB medium in 1 L shaking flasks were inoculated with 10 mL of the preculture to yield a volume of about 1 L starting culture for large scale fermentation. The starting culture was grown over night (18 h) at 27 °C and 200 rpm until it reached an $OD_{600}$ of 4.7. A fed-batch fermentation was performed in a process-controlled stainless steel bioreactor (Chemup, Richard Stihler GmbH & Co KG, Lahr, Germany) with a working volume of 30 L (pH 7.0, 500 rpm, 37 °C).

**Table 2**
Specific activities and yields of crude lyophilizate, resuspended precipitate and lyophilizate of pooled active DEAE chromatography fractions. Specific activities were measured at 35 °C and an initial substrate concentration of 200 mM *L*-Asn in $H_2O$.

| Step | Mass/volume | Protein concentration | Specific activity [U/$g_{prot}$] | Total activity [U] | Yield |
|---|---|---|---|---|---|
| Crude lyophilizate | 10,000 mg | 77% | 381 | 2940 | 100% |
| $(NH_4)_2SO_4$-precipitation | 27.5 mL | 69.7 mg/mL | 560 | 1074 | 37% |
| DEAE chromatography and lyophilization | 175 mg | 68% | 3703 | 441 | 15% |

Therefore 20 L of LB medium (as described above but containing 2% (w/w) *D*(+)-glucose monohydrate and 0.02 vol.% antifoam in addition) were fed into the reactor and inoculated with 1 L starting culture. The expression of the recombinant amino acid racemase was induced with 100 μmol/L IPTG after 3 h when the fermentation broth reached an $OD_{600}$ of about 5.5. After induction *D*(+)-glucose monohydrate was fed to the fermentation broth (ca. 30 g/h) and the temperature was decreased to 27 °C. After 24 h 750 g cells (biowetmass, BWM) were harvested by centrifugation (20 min, 15,000*g*, 4 °C) and washed with 0.85% NaCl.

### 2.4. Purification of racemase

The overproduced racemase was released from the cytosol of *E. coli* BL21 (DE3) by ultrasonic cell-disruption in a continuous flow-cell sonifier (Branson W250, Danbury, CT, USA) from a 20% (w/w) cell suspension in 10 mM $KP_i$-buffer, pH 7.0 (containing 50 μM PLP and lysozyme from hen egg white) at 4 °C. Cell debris were removed by centrifugation (20 min, 20,000*g*, 4 °C), the supernatant was frozen portion wise at −20 °C, lyophilized for about 48 h and stored at 4 °C until it was used. A total mass of 93 g of crude lyophilizate (*CL*) could be obtained.

For further purification 10 g of *CL* were dissolved in 1000 mL of 10 mM $KP_i$, pH 7.0, and 176.0 g of $(NH_4)_2SO_4$ (30% saturation) were added to the solution. After 30 min the precipitate was removed by centrifugation (20 min, 10,000*g*, 4 °C) and additional 163.3 g of $(NH_4)_2SO_4$ (55% saturation) were added to the supernatant (1008 mL). The now forming precipitate was again removed by centrifugation; the pellet was resuspended in 100 mL of 10 mM $KP_i$, pH 7.0, and filtrated (pore size 0.8 μm). 111 mL of filtrate were washed with two volumes of $H_2O$ in an Amicon-cell (membrane: Pall Omega 10 kDa, A: 41.8 $cm^2$) and afterwards concentrated to 30 mL (all steps were done in an ice bath for cooling).

The concentrate was then applied with a flow of 5 mL/min to a DEAE-sepharose column (5 × 25 cm, operated by ÄKTApurifier from Amersham Biosciences, Uppsala, Sweden) which had been equilibrated with 5 column volumes of 10 mM KPi, pH 7.0. After the column had been washed (10 mL/min) with 2 volumes of the same buffer but containing 50 mM NaCl, the enzyme was eluted with the buffer supplemented with 100 mM NaCl. The active fractions, which have a specific absorption maximum at 420 nm, emerged at the beginning of elution and were pooled. The pooled fractions were washed with 3 × 90 mL of 10 mM $KP_i$, pH 7.0, in an Amicon-cell (membrane: Pall Omega 10 kDa, A: 41.8 $cm^2$) and concentrated to 10 mL. The enzyme solution was then frozen in liquid nitrogen and lyophilized. 175 mg of pure lyophilizate (*PL*) (light yellow powder) were obtained. To prevent a loss of the cofactor PLP during purification all buffers contained 20 μM of PLP.

### 2.5. Analytics

Enzyme assays were done at 650 rpm in 1.5 mL Eppendorf reaction vials which were placed in an Eppendorf Thermomixer. Calibrations were performed under assay conditions in the range of estimated analyte concentrations.

*Methionine*. Activities for racemization of Met solutions were measured by following the enantiomeric composition of the reaction medium via HPLC (Crownpak CR(+) column (0.4 cm × 15 cm), Daicel Chem. Ind.; $HClO_4$, pH 2; $T$ = 25 °C; 0.6 mL/min, $V_{inj}$ = 5 μL; $\lambda_{abs}$ = 200 nm). For kinetic studies activities of *CL* were measured as follows: 0.1 mg/mL of *CL* were added to 95 vol.% 100 mM $KP_i$-buffer, 5 vol.% MeOH, containing *L*- and *D*-Met ranging from about 7 to 200 mM. Initial reaction rates were determined by taking samples at three points of time. Therefore 100 mg reaction solution were diluted with 900 mg of $HClO_4$, pH 1.0, to stop the reaction and analyzed by HPLC to determine their enantiomeric composition. The slope of product formation, which was calculated by linear regression using Microsoft Excel, represents the initial reaction rates for the particular substrate concentration. (All samples were taken in duplicate, results represent mean values.)

*Asparagine*. Kinetic studies of *PL* were performed in aqueous *L*- and *D*-Asn solutions containing approximately between 20 and 250 mM Asn (depending on solubility at certain temperature) with concentrations of 0.1 mg *PL* per mL. Activity assays were done as described above but analyzed under different HPLC conditions ($HClO_4$, pH 1; $T$ = 0 °C; 0.4 mL/min).

A control showed that neither methionine nor asparagine racemize spontaneously after dilution with $HClO_4$, pH 1 (done by HPLC analysis after 1 h incubation time).

*Protein determination*. Protein concentrations of both enzyme preparations were determined at 30 °C by a standard Bradford assay [22] using a Shimadzu UV-1601 photometer.

*SDS-PAGE*. Gel electrophoresis was performed according to the manufacturer's instructions using the NuPAGE test kit by Invitrogen. A total protein loading of about 1 μg for *CL* and about 0.1 μg for *PL* each were applied to a gel pocket. A protein standard solution was applied to a nearby pocket and electrophoresis was performed at 200 V for 45 min. After developing and incubating the gel, it was scanned with a desktop scanner.

## 3. Results and discussion

### 3.1. Enzyme purification

The recombinant amino acid racemase was purified from *E. coli* BL21 (DE3) in two independent steps leading to two differently pure enzyme preparations. Lyophilization of the cell-free protein extract (from 750 g BWM) gave 93 g of crude lyophilizate (*CL*) with a protein concentration of 77 wt%. While *CL* showed no other reactions on Met than racemization (constant mass balance over several days determined by HPLC) it exhibited a so far unidentified side reaction on Asn that lead to a degradation of the *L*-enantiomer. Therefore 10 g of *CL* were further purified as described resulting in 175 mg of pure lyophilizate (*PL*) with a protein concentration of 68 wt%. Table 2 summarizes specific activities and yields of the purification steps. The purified enzyme preparation did not show the aforementioned side reaction on *L*-Asn anymore. Fig. 1 shows the SDS-PAGE protein band patterns of both preparations. While *CL* is a mixture of all proteins present in the cytoplasm of *E. coli* BL21 (DE3), the band pattern for *PL* shows only one sharp band below 50 kDa which

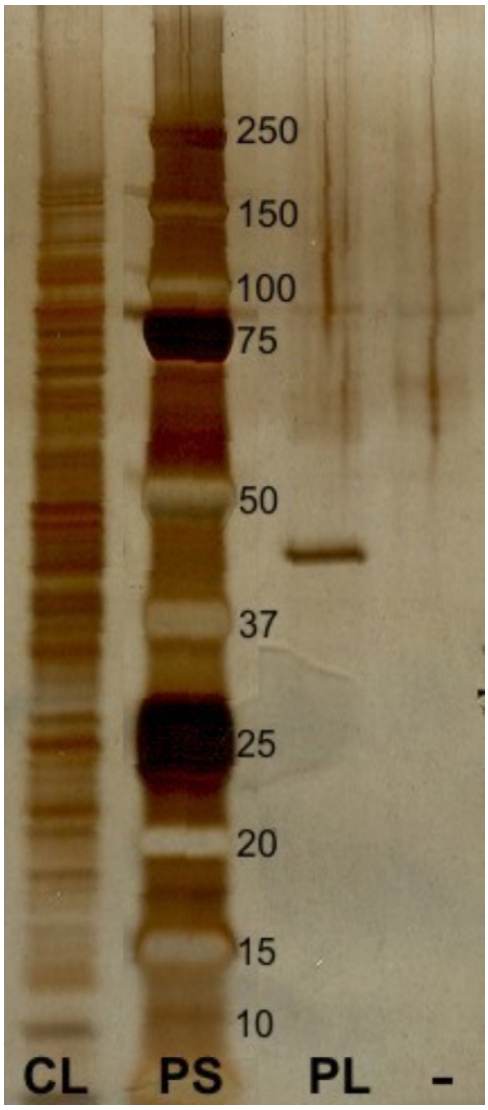


**Fig. 1.** SDS-PAGE of crude lyophilizate (*CL*), pure lyophilizate (*PL*) and protein standard (*PS*, molecular weights are given in kDa). The protein loading was about 1 μg for *CL* and 0.1 μg for *PL*. *PL* exhibits only one sharp band below 50 kDa, while *CL* shows a composition of all intracellular proteins. The right lane (-) represents an empty lane without any protein loading.

matches the MW of the cloned amino acid racemase (homodimer of 2× ca. 45 kDa).

### 3.2. Enzyme kinetics

The amino acid racemase which has been used for this work follows a reversible three-step Michaelis–Menten mechanism and uses PLP as cofactor. While no substrate is present, PLP is bound to a lysine residue in the active site via an internal Schiff-base. When a substrate molecule enters the active site, PLP is transferred from Lys to the α-amino group of the substrate to form an external Schiff-base. Racemization proceeds then via abstraction of the α-hydrogen atom to form a quinoid-type carbanion intermediate and subsequent reprotonation on the opposite or the original side. A more detailed description of the catalytic mechanism can be found elsewhere [12,23,24].

The racemization can be described by a reversible three-step mechanism where the conversion of the *L*- to the *D*-substrate

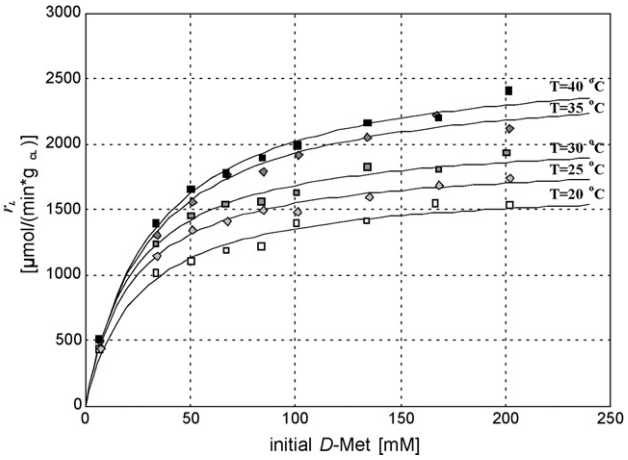


**Fig. 2.** Experimental data for *D*-methionine racemization with *CL* shown by symbols. Lines show data calculated by non-linear fitting for racemization of *D*-Met using $K_m$ and $V_{max}$ shown in Table 3 Solvent: 95 vol.% 100 mM $KP_i$-buffer (pH 7.0), 5 vol.% MeOH.

enzyme complex is pooled into one step:

$$E + L\text{-}AA \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E(L\text{-}AA) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E(D\text{-}AA) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + D\text{-}AA \qquad \text{(scheme 1)}$$

with

$$K_{m,L} = \frac{k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3}{k_1(k_{-2} + k_2 + k_3)}; \quad K_{m,D} = \frac{k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3}{k_{-3}(k_{-1} + k_{-2} + k_2)}$$

where *E* is the racemase, $k_i$ are the reaction rate constants for the single reactions and $K_{m,L/D}$ are the dissociation constants for the substrate/product binding to the enzyme for forward and backward reactions (under assumption that complex formation is not the rate limiting step).

From (scheme 1) the reaction rate can be derived as

$$r_L = C_E \cdot \frac{(V_{\max/D}/K_{m,D}) \cdot C_D - (V_{\max/L}/K_{m,L}) \cdot C_L}{1 + (C_L/K_{m,L}) + (C_D/K_{m,D})}, \quad r_D = -r_L, \qquad (1)$$

where $r_D$ and $r_L$ are the reaction rates for the formation of *D*-AA and *L*-AA respectively, $C_E$ is the enzyme concentration and $V_{max,L/D}$ are the maximum mass specific reaction rates (in $\mu mol\ min^{-1}\ g^{-1}_{enzyme\ preparation}$) for the racemization of either *L*- or *D*-AA. With approaching the equilibrium condition (racemic composition) the driving force of the reaction (numerator in Eq. (1)) becomes 0. The Haldane relationship (Eq. (2)) describes the relation between the equilibrium constant $K_{eq}$ of a reaction and the forward and backward kinetic constants of the catalyzed reaction. Under equilibrium conditions $K_{eq}$ must be 1:

$$K_{eq} = \frac{C_L}{C_D} = \frac{V_{\max,D}}{K_{m,D}} : \frac{V_{\max,L}}{K_{m,L}} = 1 \qquad (2)$$

#### 3.2.1. Methionine

$K_m$ and $V_{max}$ values were determined for the racemization of *D*- and *L*-Met solutions with *CL* in 95 vol.% 100 mM $KP_i$-buffer (pH 7.0), 5 vol.% MeOH at $T$ = 20–40 °C (with step of 5 °C) as well as at pH 6, 7 and 8 at 25 °C. Figs. 2–4 show the results of the kinetic studies for *D*- and *L*-Met racemization. Initial rates were measured as described above. In all cases less than 5% of the initial enantiomer concentration was isomerized to allow for initial rate conditions.

$V_{max}$ values increase for *D*- and *L*-Met racemization with rising temperature (about 1.5-fold for *L*-Met from 20 to 40 °C) and pH values (about 2.5-fold for *L*-Met from pH 6 to pH 8). Noticeable here is the switch of $V_{max}$ values for *D*- and *L*-Met at pH 8. While at pH 6 and 7 $V_{max(L\text{-}Met)}$ is slightly higher than $V_{max(D\text{-}Met)}$, at pH

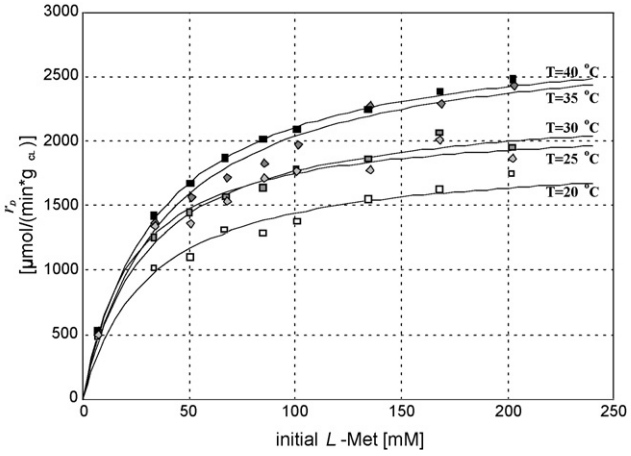


**Fig. 3.** Experimental data for *L*-methionine racemization with *CL* shown by symbols. Lines show data calculated by non-linear fitting for racemization of *L*-Met using $K_m$ and $V_{max}$ shown in Table 3 Solvent: 95 vol.% 100 mM $KP_i$-buffer (pH 7.0), 5 vol.% MeOH.

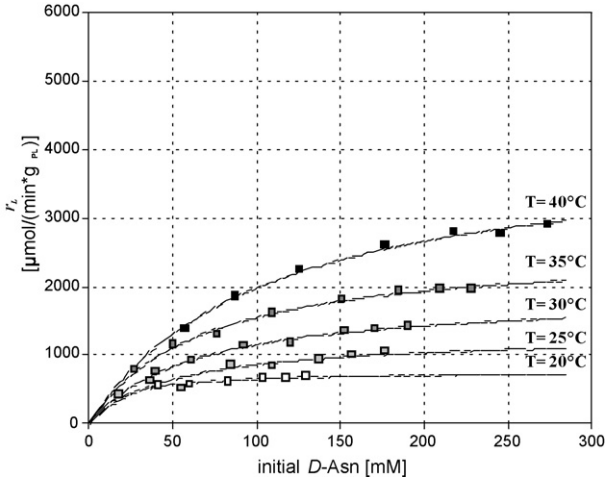


**Fig. 5.** Experimental data for *D*-asparagine racemization with *PL* shown by symbols. Lines show data calculated by non-linear fitting for racemization of *D*-Asn using $K_m$ and $V_{max}$ shown in Table 4. Solvent: 100% $H_2O$.

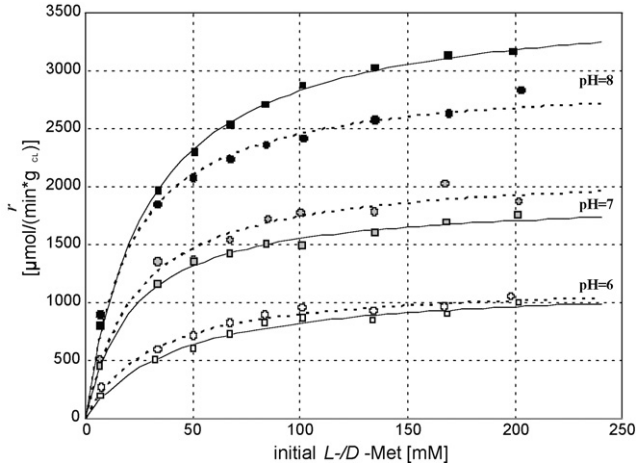


**Fig. 4.** Experimental data for *L*- (circles) and *D*-(squares) methionine racemization with *CL*. Lines show data calculated by non-linear fitting for racemization of *L*-Met (dotted) and *D*-Met (full) using $K_m$ and $V_{max}$ shown in Table 3. Solvent: 95 vol.% 100 mM $KP_i$-buffer, 5 vol.% MeOH, $T = 25\,^{\circ}C$.

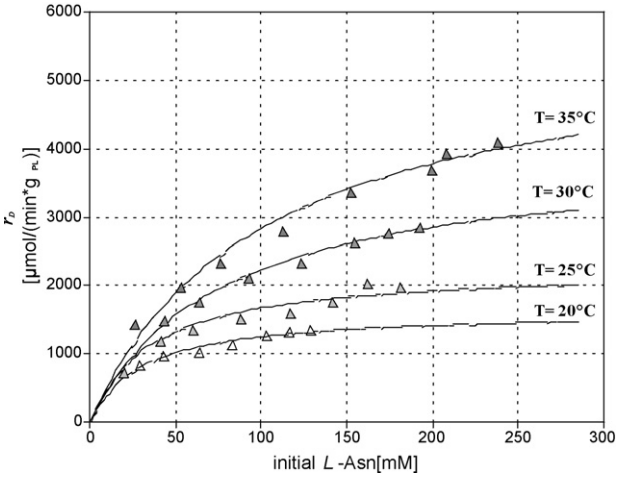


**Fig. 6.** Experimental data for *L*-asparagine racemization with *PL* shown by symbols. Lines show data calculated by non-linear fitting for racemization of *L*-Asn using $K_m$ and $V_{max}$ shown in Table 4. Solvent: 100% $H_2O$.

8 $V_{max(D\text{-}Met)}$ is significantly higher (23%) than $V_{max(L\text{-}Met)}$ (Table 3). $K_m$ values increase only slightly when rising the temperature from 20 to 40 °C (follow *van't Hoff* relation, [25]) and fell with risen pH values.

### 3.2.2. Asparagine

Kinetic parameters were determined for the racemization of *D*- and *L*-Asn with *PL* in $H_2O$ at $T = 20$–$40\,^{\circ}C$ (with step of 5 °C). Figs. 5 and 6 show the results of the kinetic studies for *D*- and *L*-Asn racemization which were determined as described for methionine. Similar to methionine racemization $V_{max}$ values increase with higher temperatures (about 3.5-fold for *L*-Asn from 20 to 35 °C and 5.2-fold for *D*-Asn from 20 to 40 °C). $K_m$ values also increase significantly with rising temperatures which indicates a reduced substrate affinity (follow *van't Hoff* relation). Values for $V_{max(L\text{-}Asn)}$ and $V_{max(D\text{-}Asn)}$ strongly differ from each other. This apparent imbalance should be adjusted by a consistent difference of $K_m$ values (Eq. (2)). Here this requirement can only be met (excluding values determined at 30 °C) when considering the broad error ranges (Table 4). A reasonable explanation for this might be the relatively

**Table 3**
Kinetic data for racemization of *D*- and *L*-methionine with *CL* at different temperatures and pH values. $K_m$ and $V_{max}$ were calculated by non-linear regression. Solvent: 95 vol.% 100 mM $KP_i$-buffer, 5 vol.% MeOH.

| Temperature [°C] | pH | $K_{m(D\text{-}Met)}$ [mM] | $V_{max(D\text{-}Met)}$ [U/$g_{CL}$] | $K_{m(L\text{-}Met)}$ [mM] | $V_{max(L\text{-}Met)}$ [U/$g_{CL}$] |
|---|---|---|---|---|---|
| 20 | 7 | 25 (±13.0%) | 1696 (±3.1%) | 31 (±17.0%) | 1889 (±4.5%) |
| 25 | 7 | 22 (±11.5%) | 1892 (±2.6%) | 23 (±14.7%) | 2150 (±3.3%) |
| 30 | 7 | 23 (±10.6%) | 2075 (±2.4%) | 30 (±15.7%) | 2296 (±4.0%) |
| 35 | 7 | 30 (±8.9%) | 2512 (±3.9%) | 39 (±11.6%) | 2833 (±3.5%) |
| 40 | 7 | 32 (±8.4%) | 2668 (±2.3%) | 35 (±11.4%) | 2847 (±3.2%) |
| 25 | 6 | 42 (±14.6%) | 1160 (±4.5%) | 30 (±22.3%) | 1174 (±5.9%) |
| 25 | 8 | 28 (±4.5%) | 3627 (±1.1%) | 20 (±8.7%) | 2951 (±1.9%) |

**Table 4**
Kinetic data for racemization of *D*- and *L*-asparagine with *PL* at different temperatures. $K_m$ and $V_{max}$ were calculated by non-linear regression. Solvent: 100% $H_2O$.

| Temperature [°C] | $K_{m(D\text{-}Asn)}$ [mM] | $V_{max(D\text{-}Asn)}$ [U/g$_{PL}$] | $K_{m(L\text{-}Asn)}$ [mM] | $V_{max(L\text{-}Asn)}$ [U/g$_{PL}$] |
|---|---|---|---|---|
| 20 | 18 (±26.1%) | 770 (±4.8%) | 30 (±19.3%) | 1626 (±5.6%) |
| 25 | 50 (±33.3%) | 1299 (±11.4%) | 47 (±19.6%) | 2427 (±6.3%) |
| 30 | 62 (±8.9%) | 1881 (±3.1%) | 76 (±10.0%) | 3930 (±3.9%) |
| 35 | 66 (±8.2%) | 2585 (±2.8%) | 101 (±17.5%) | 5683 (±7.1%) |
| 40 | 103 (±8.8%) | 4032 (±3.3%) | [a] | [a] |

[a] Due to the high racemization activity for *L*-Asn at 40 °C it was not possible to detect an activity at a substrate concentration where it is close to the expected $V_{max}$ (at least 70%) and therefore was not determinable with a tolerable error.

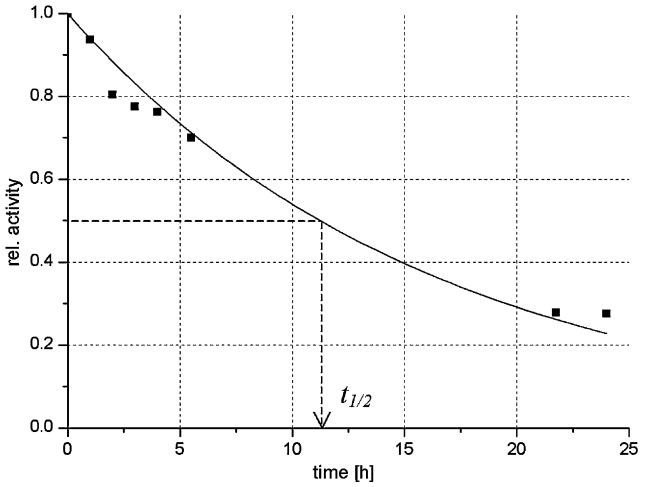


**Fig. 7.** Relative activity of *CL* in 95 vol.% 100 mM $KP_i$-buffer (pH 7.0), 5 vol.% MeOH at 35 °C over a period of 24 h.

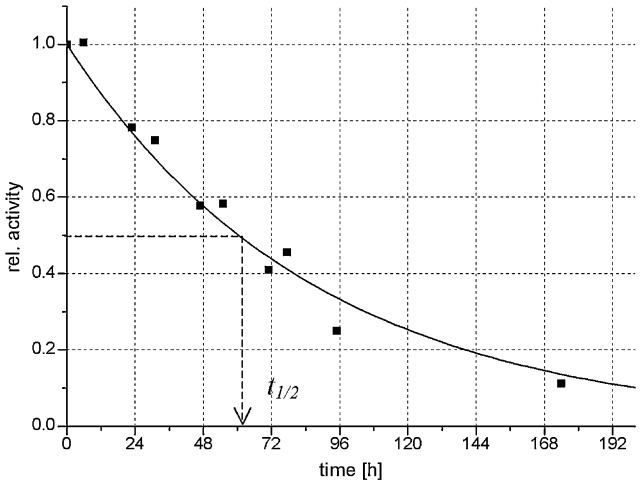


**Fig. 8.** Relative activity of *PL* in 500 mM *DL*-Asn solution at 35 °C over a period of 7 days.

poor solubility of *D*-/*L*-Asn (ca. 290 mM each at 35 °C). Due to this, kinetic measurements could not be carried out under substrate saturation conditions ($V_{max}$ region) which lead to wide error ranges. Due to the very high racemization activity for *L*-Asn at 40 °C (about 5000 μmol/(min $g_{PL}$) at initial substrate concentration of 260 mM) it was not possible to perform a measurement at a substrate concentration where the activity is even close to the expected $V_{max}$ (at least 70% of $V_{max}$). In this case determined data was afflicted with an intolerable error and therefore is not included in the data analysis. Another reason for aforementioned broad error ranges, namely side reactions that might have led to an enantiospecific degradation of one enantiomer, could be ruled out due to constant mass balances. Therefore the calculated kinetic parameters can only be considered as approximate values. All kinetic parameters are summarized in Table 4.

### 3.3. Enzyme stability

To investigate the enzyme stability in the presence of 5 vol.% MeOH, 5 mg *CL* were incubated at 35 °C in 1 mL 95 vol.% 100 mM $KP_i$-buffer (pH 7), 5 vol.% MeOH over a period of 24 h. At defined times 100 μL of this solution were added to 100 μL of 300 mM *L*-

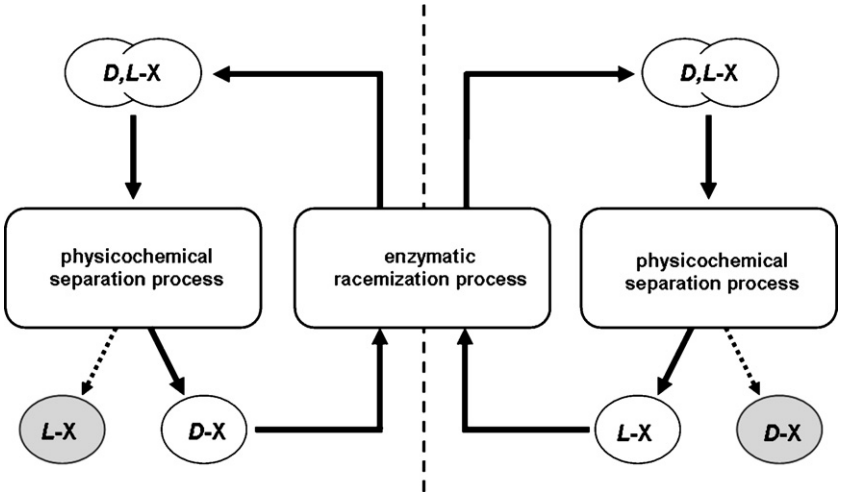


**Fig. 9.** Combination of a physicochemical separation process with enzymatic racemization of the unwanted counterenantiomer. Either *L*-enantiomers (left side) or *D*-enantiomers (right side) can be obtained from this process. A cyclic operation enables a theoretical yield of 100% of the target enantiomer (grey).

Met in 95 vol.% 100 mM $KP_i$-buffer (pH 7), 5 vol.% MeOH and initial reaction rates were determined by HPLC. Fig. 7 shows the relative activities fitted by non-linear regression using Eq. (3) where $r_{rel}$ is the relative reaction rate giving an inactivation constant $k_{time}$ of 0.0614 $h^{-1}$ (±8%) and a half-life time $t_{1/2}$ of 11.3 h (Eq. (4)).

$$r_{rel}(t) = r_{rel}(0) \cdot e^{(-k_{time} \cdot t)} \quad (3)$$

$$t_{1/2} = \frac{\ln(0.5)}{-k_{time}} \quad (4)$$

To investigate the influence of highly concentrated Asn solutions on the enzyme activity 1 mg *PL* was incubated at 35 °C in 1.5 mL aqueous 500 mM *DL*-Asn solution over a period of 7 days. Initial reaction rates were determined as described above but with 100 μL of 200 mM aqueous *D*-Asn solution as substrate (Fig. 8). Non-linear regression gave a $k_{time}$ of 0.0115 $h^{-1}$ (±5%) and a half-life time of 60.3 h.

The medium composition and temperature of both stability experiments were chosen in accordance to the planned reaction design for each enzyme preparation. Since *CL* will be applied in a process which implies chromatography for enantioseparation of methionine we determined the stability in the mobile phase (which also has to serve as reaction medium). An according experiment was performed with *PL*. In this case the reaction medium will be a highly concentrated asparagine solution with only a very small enantiomeric excess. We simulated this by storing the racemase in an aqueous 500 mM *DL*-Asn solution.

## 4. Conclusions

A plasmid containing an amino acid racemase gene was cloned into *E. coli* BL21 (DE3) and overexpressed during a 20-L fed-batch fermentation. The produced protein shall be used as biocatalyst in an integrated physicochemical separation process with enzymatic racemization. Two differently pure enzyme preparations were prepared from cell extract of which one was sufficient for methionine racemization but still had impurities which led to enzymatic asparagine degradation. Further purification yielded in a pure racemase lyophilizate with no side reactions concerning asparagine as substrate. We have investigated the kinetic parameters $K_m$ and $V_{max}$ for the racemization reactions of methionine in 95 vol.% 100 mM $KP_i$-buffer, 5 vol.% MeOH and of asparagine in $H_2O$ at different temperatures and pH values with these two preparations. We showed that $V_{max}$ and $K_m$ values for both reaction systems increased with temperature. During stability studies we have determined half-life times of crude lyophilizate in 95 vol.% 100 mM $KP_i$-buffer (pH 7), 5 vol.% MeOH and of pure lyophilizate in 500 mM *DL*-Asn (each at 35 °C). These reaction media and temperature were chosen for prospective reactor design. Further studies will concentrate on the reactor design for a combined enzymatic racemization and physicochemical enantioseparation process as depicted in Fig. 9. One major benefit will be the theoretical possible yield of 100% of one enantiomer out of a racemic mixture.

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## References

[1] N.M. Maier, P. Franco, W. Lindner, J. Chromatogr. A 906 (2001) 3–33.
[2] M. Breuer, K. Ditrich, T. Habicher, B. Hauer, M. Keßeler, R. Stürmer, T. Zelinski, Angew. Chem. Int. Ed. 43 (2004) 788–824.
[3] J. Dingenen, J.N. Kinkel, J. Chromatogr. A 666 (1994) 627–650.
[4] E. Francotte, J. Chromatogr. A 666 (1994) 565–601.
[5] K. Petrusevska, M.A. Kuznetsov, K. Gedicke, V. Meshko, S.M. Staroverov, A. Seidel-Morgenstern, J. Sep. Sci. 29 (2006) 1447–1457.
[6] T. Vries, H. Wynberg, E. van Echten, J. Koek, W. ten Hoeve, R.M. Kellogg, Q.B. Broxterman, A. Minnaard, B. Kaptein, S. van der Sluis, L. Hulshof, J. Kooistra, Angew. Chem. Int. Ed. 37 (1998) 2349–2354.
[7] H. Lorenz, A. Perlberg, D. Sapoundjiev, M.P. Elsner, A. Seidel-Morgenstern, Chem. Eng. Process. 45 (2006) 863–873.
[8] M. McCoy, Chem. Eng. News 77 (1999) 10–14.
[9] S.C. Stinson, Chem. Eng. News 77 (1999) 101.
[10] M. Bechtold, S. Makart, M. Heinemann, S. Panke, J. Biotechnol. 124 (2006) 146–162.
[11] M.P. Elsner, D.F. Menendez, E.A. Muslera, A. Seidel-Morgenstern, Chirality 17 (2005) 183–195.
[12] B. Schnell, K. Faber, W. Kroutil, Adv. Synth. Catal. 345 (2003) 653–666.
[13] O. May, S. Verseck, A. Bommarius, K. Drauz, Org. Process Res. Dev. 6 (2002) 452–457.
[14] K. Goldberg, K. Schroer, S. Lütz, A. Liese, Appl. Microbiol. Biotechnol. 76 (2007) 237–248.
[15] K. Goldberg, K. Schroer, S. Lütz, A. Liese, Appl. Microbiol. Biotechnol. 76 (2007) 249–255.
[16] H. Ikeda, Y. Yonetani, S.-i. Hashimoto, M. Yagasaki and K. Soda, WO 03/074690 A1, 2003, Japan.
[17] M. Bechtold, S. Makart, R. Reiss, P. Alder, S. Panke, Biotechnol. Bioeng. 98 (2007) 812–824.
[18] H.W.B. Roozeboom, Z. Phys. Chem. 28 (1899) 494–517.
[19] J. Jacques, A. Collet, S.H. Wilen, Enantiomers, Racemates and Resolutions, Krieger, Malabar, 1994.
[20] A.A. Rodrigo, H. Lorenz, A. Seidel-Morgenstern, Chirality 16 (2004) 499–508.
[21] V.M. Profir, M. Matsuoka, Colloids Surf. A 164 (2000) 315–324.
[22] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[23] A.C. Eliot, J.F. Kirsch, Annu. Rev. Biochem. 73 (2004) 383–415.
[24] T. Yoshimura, N. Esaki, J. Biosci. Bioeng. 96 (2003) 103–109.
[25] A. Cornish-Bowden, Fundamentals of Enzyme Kinetics, Portland Press Ltd, London, 2004.
[26] K.E. Nelson, Environ. Microbiol. 4 (2002) 777–778.
[27] D.M. Woodcock, P.J. Crowther, J. Doherty, S. Jefferson, E. Decruz, M. Noyer-weidner, S.S. Smith, M.Z. Michael, M.W. Graham, Nucleic Acids Res. 17 (1989) 3469–3478.
[28] F.W. Studier, B.A. Moffatt, J. Mol. Biol. 189 (1986) 113–130.