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Aldolase catalyzed L-phenylserine synthesis in a slug-flow microfluidic system – Performance and diastereoselectivity studies

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HIGHLIGHTS

- L-phenylserine was produced by threonine aldolase in slug-flow microfluidic system.
- Substrate benzaldehyde was continuously supplied from the organic phase.
- TBME solvent provides mild conditions and high product concentration.
- MeTHF solvent provides high concentration of L-*syn*-phenylserine.
- Toluene provides low value of *syn-anti* forms ratio in short residence time.

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ABSTRACT

We study synthesis of L-phenylserine catalyzed by the enzyme L-threonine aldolase in a slug-flow microfluidic system. Slug-flow arrangement allows for the continuous refilling of sparingly soluble substrate (benzaldehyde) into an aqueous reaction mixture. We identified suitable composition of an organic phase to provide stable slug-flow in a wide range of operational parameters. Solvent screening revealed that tert-butyl methyl ether (TBME) as the organic solvent provides the most friendly environment for L-phenylserine synthesis due to a low degree of enzyme deactivation and high benzaldehyde concentration in the reaction mixture. The effects of substrate concentrations, enzyme concentration, and dimethylsulfoxide (DMSO) concentration on the L-phenylserine concentration in the product stream were examined and proper reaction conditions were identified. Experimental results on the L-phenylserine diastereoselectivity demonstrate that the amount of *syn*-conformation of L-phenylserine increases with the reaction time. High *syn*- to *anti*- concentration ratio is achieved with 2-methyltetrahydrofuran (MeTHF) solvent in a system with long residence time and, finally, low *syn*- to *anti*-concentration ratio is provided by toluene environment and short residence time.

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1. Introduction

Threonine aldolases (TA) (EC 4.1.2.5.) represent a class of enzymes that catalyzes aldol reactions of glycine and variety of aromatic and aliphatic aldehydes (Steinreiber et al., 2007a). Genes encoding TAs have been found in plants, vertebrates, several bacteria, and fungi (Liu et al., 2000). Various β -hydroxy- α -amino acids can be formed in the aldol reaction under mild conditions. These products are widely used as pharmaceutical or pesticide precursors. TAs divides into L-threonine aldolases (LTA) and D-threonine aldolases (DTA) according to their specificity at the

α -carbon. Diastereoselectivity at the β -carbon strongly depends on reaction conditions (Steinreiber et al., 2007a). Longer reaction time usually leads to higher yields but reduces diastereoselectivity. Therefore, products with high diastereoselectivity are obtained in so called kinetic regime with short reaction time. The conversion of reactions catalyzed by TAs is limited by reaction equilibrium, which can be partly overcome by ten-fold excess of glycine (Steinreiber et al., 2007b). Alternative non-enzyme ways of the synthesis of optically pure β -hydroxy- α -amino acids such as the Sharpless reactions (Alonso and Riera, 2005; Kim and Kirk, 2001), addition of imides to aldehydes (Willis et al., 2005) or exploiting aziridinium intermediates (Davies et al., 2014) also suffer from several disadvantages, e.g. expensive chiral precursors, severe conditions or low stereoselectivity.

Enzyme reactions are carried out in many types of chemical reactors. Small characteristic dimensions of microreactors bring

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many benefits resulting in higher reaction yield or selectivity (Jahnisch et al., 2004) and open novel process windows which may give access to new products (Hessel et al., 2013). Microreactors for the use in biochemical applications have been recently reviewed, e.g., in Bolivar et al. (2011), Dencic et al. (2013), Hessel et al. (2014), Munirathinam et al. (2015), Wohlgemuth et al. (2015). Despite of the fact that the use of microreactors in the production of fine chemicals is still limited, significant intensification of various chemical syntheses has been achieved. Many progressive solutions of microreactor systems such as reversible enzyme binding to microfluidic structure, the integration of separation and analytical steps on a chip or implementation of multiple step processes are currently investigated to make the microtechnology more attractive for industrial purposes (Bolivar et al., 2011).

One type of microfluidic devices exploits multiphase flow, particularly slug or segmented flow (Köhler and Cahill, 2014). The slug flow is typically formed at T or Y microchannel bifurcations. Two immiscible fluids (liquid–liquid or gas–liquid) are introduced into the bifurcation by two separate channels. At the place of the bifurcation, one fluid wets the channel walls and forms a continuous phase. The other fluid forms droplets or slugs that are dispersed in the continuous phase. In a regular regime, slugs of approximately uniform size are continuously produced and travel with constant velocity in the downstream channel. Slug flow arrangement provides plug-like behavior of bioreactors with uniform residence time of all segments of a reaction mixture as well as great control of a reaction process (Janous et al., 2014). Moreover, mass and heat transfer can be tremendously intensified due to the short transport distances among slugs, existence of thin boundary layer of the continuous phase, and internal circulation in slugs (Jovanovic et al., 2011).

Many works dealing with multi-phase microreactors with free or immobilized enzymes were reviewed in Bolivar et al. (2011) and Bolivar and Nidetzky (2013). The most frequently studied systems exploit liquid–liquid slug flow. An enzyme can operate either in an aqueous phase with one or more substrates supplied from organic phase (Fisher et al., 2013; Karande et al., 2011; Salic et al., 2011; Tomaszewski et al., 2014), on the liquid–liquid interface (Cech et al., 2012; Pohar et al., 2009) or at the place of immobilization (He et al., 2010). Particularly, Fisher et al. (2013) supplied 2-cyclohexen-1-one (substrate) from an organic phase to an aqueous phase containing pentaerythritol tetranitrate reductase. Thermophilic alcohol dehydrogenase in an aqueous phase was used to convert 1-heptaldehyde to 1-heptanol with the substrate supplied from an organic phase and the product collected in the same phase (Karande et al., 2011). Alcohol dehydrogenase present in an aqueous phase was used for the oxidation of hexanol supplied from an organic phase to the product hexanal (Salic et al., 2011). 3-phenylcatechol was synthesized in aqueous phase containing 2-hydroxybiphenyl 3-monooxygenase with 2-hydroxybiphenyl as a substrate transferred from an organic phase (Tomaszewski et al., 2014). Enzyme reactions catalyzed by lipases such as hydrolysis (Cech et al., 2012) or esterification (Pohar et al., 2009) typically operate at the water–oil interface, thereby the slug-flow regime provides both large interfacial area and intensive mass transfer, see e.g. (Dessimoz et al., 2008; Di Miceli Raimondi et al., 2008; Chai et al., 2010; Tsaoulidis and Angeli, 2015; Woitalka et al., 2014).

There are more complex arrangements of phases in enzyme microreactors. Gas–liquid two-phase system enables controlled dosing of oxygen substrate to an enzyme dissolved in an aqueous phase (Kawakami et al., 1989). Cech et al. (2013) used third gaseous phase (nitrogen) to regularly separate two-phase segments of an oil–aqueous mixture to provide uniform residence time in an enzyme microreactor. Tomaszewski et al. (2014) employed a gas phase as an oxygen reservoir in three phase reaction system for

Fig. 1. L-phenylserine is synthesized from benzaldehyde and glycine in the presence of LTA.

enzyme synthesis of 3-phenylcatechol.

Enantioselective enzyme syntheses using slug flow (Koch et al., 2008a, 2008b) or single-phase flow (Bartha-Vari et al., 2015; Kataoka et al., 2010; Kawakami et al., 2012; Tibhe et al., 2013) arrangements have been carried out in microreactors. Almost enantiopure cyanohydrins were synthesized by hydroxynitril lyase in a slug flow microreactor (Koch et al., 2008a, 2008b). Lipase immobilized in monolith and tubular microreactors allowed for significant increase of enantioselectivity of transesterification reactions (Kataoka et al., 2010; Kawakami et al., 2012). Recently, Tibhe et al. (2013) carried out synthesis of L-phenylserine from glycine and benzaldehyde catalyzed by LTA (Fig. 1) in two types of microreactors. They used either LTA immobilized on Eupergit support (Katchalski-Katzir and Kraemer, 2000) in a packed bed arrangement or free LTA in a capillary microreactor with laminar one phase flow and gas–liquid slug flow. Reaction yield less than 40% was achieved because of the limitation by reaction equilibrium and the existence of product inhibition. Diastereomeric excess of about 20% was observed.

This paper examines the idea to use slug-flow micro-bioreactors for stereoselective aldol reactions. According to our best knowledge, this type of enzyme reactions has not been carried out in any similar arrangement. Diastereoselective production of the L-forms of phenylserine catalyzed by LTA was selected in this case study. The slug-flow arrangement represents an alternative to single-phase bioreactors due to continuous refilling of the reaction mixture by one substrate from an organic phase. The effects of substrate concentrations, organic solvent selection, enzyme concentration, and dimethylsulfoxide (DMSO) concentration on the composition of product stream are systematically studied. Various organic solvents are tested in the view of low enzyme deactivation. Regimes leading to high L-phenylserine concentration in the product stream or to the preferential formation *syn/anti* forms are examined.

2. Material and methods

2.1. Materials

LTA (EC 4.1.2.48) having concentration 2.3 mg ml^{−1} extracted from *Thermomicrobium roseum* was donated by the Industrial Biotechnology Group (University of Leipzig, Germany). L-threonine and β-nicotinamide adenine dinucleotide disodium salt (NADH) were purchased from AppliChem GmbH (Darmstadt, Germany). Glycine, benzaldehyde, tert-butyl methyl ether (TBME), 2-methyltetrahydrofuran (MeTHF), Toluene, pyridoxal-5-phosphate (PLP), dimethyl sulfoxide (DMSO), mono- and di- potassium phosphate, fluorescein isothiocyanate (FITC), and the enzyme

Fig. 2. Reaction setup for enzyme production of L-phenylserine in a slug flow system. Syringes A and B contain organic and aqueous phases, respectively. Syringe C contains solution for the termination of the enzyme reaction.

alcohol dehydrogenase were purchased from Sigma-Aldrich. Polyglycerol polyricinoleate (PGPR) was donated by Danisco company (Czech Rep.).

2.2. Reaction setup

The enzyme reaction was investigated in a capillary microfluidic reactor, see Fig. 2. Teflon capillary (i.d. of 500 μm , length of 2.8 m) was placed into tempered water bath (50 $^{\circ}\text{C}$). PEEK three-way connector with i.d. of 1 mm or 0.5 mm was used as a slug flow generator. Another three-way connector was placed at the end of the reaction capillary. It served for the introduction of a termination agent into the reaction mixture. All solutions were dosed by syringe pumps (neMESYS, cetoni, Germany).

Aqueous and organic phases were prepared in 10 ml flasks. 10 ml of the aqueous phase contained 0.75 g of glycine (reaction substrate), 100 μl of PLP (enzyme cofactor), 1 ml of the stock solution of LTA (enzyme catalyst), and 2.5 ml of DMSO; everything dissolved in a phosphate buffer (pH=8; 0.05 mol l^{-1}). DMSO increases the solubility of benzaldehyde in the aqueous phase. 10 ml of the organic phase contained 4.244 g benzaldehyde (reaction substrate with the final concentration of 4 mol l^{-1}) and 500 μl of PGPR (emulsion stabilizer) dissolved in an organic solvent (MeTHF or TBME or Toluene). Some of these parameters were changed in parametrical studies. Organic and aqueous phases were mixed in 1:1 vol ratio to provide the total flow rate of 18.32 $\mu\text{l min}^{-1}$ in the reaction capillary with the mean residence time of 30 min. 40% solution of trichloroacetic acid was used as the agent for the termination of the enzyme reaction.

Reference experiments were carried out in a batch arrangement in 10 ml flasks. The composition of both phases was same as in the slug flow arrangement. The total amount of the reaction solution was 5 ml. The batch reactor was stirred with the frequency of 400 rpm. The reaction time ranged from 30 min to 6 h.

2.3. Determination of enzyme activity

To evaluate the denaturation effect of selected organic solvents in both the batch and slug-flow two-phase arrangements, enzyme activity of LTA was determined. LTA catalyzes the cleavage of L-threonine (the product in our study) to glycine and acetaldehyde. The assay exploits coupling with the reduction of acetaldehyde by the enzyme alcohol dehydrogenase (ADH) (Marcus and Dekker, 1993), see Fig. 3. The aqueous solution for the enzyme assay contained potassium phosphate buffer (0.05 mol l^{-1} , pH=8), NADH (200 $\mu\text{mol l}^{-1}$), L-threonine (50 mol l^{-1}), PLP (50 $\mu\text{mol l}^{-1}$), 2 mg ADH, and LTA solution in the final volume of 1 ml. Either 50 μl of LTA stock solution or 500 μl of the aqueous reaction mixture (contains 10 vol% of the original LTA stock solution) was used. The assay was carried out in 1 ml stirred cuvette under 37 $^{\circ}\text{C}$. The enzyme activity was evaluated from the slope of decrease of NADH absorbance at 340 nm (spectrophotometer Avantes).

Fig. 3. Reaction scheme of LTA activity assay.

Samples of the reaction mixture were taken periodically from the aqueous phase at the outlet of the capillary reactor or from the control batch reactor.

2.4. Analysis of product streams

Because pure standards of *syn*- and *anti*-forms of L-phenylserine were not available, the total concentration of L-phenylserine was determined indirectly from the stoichiometry of the enzyme reaction (Fig. 1), i. e., the total L-phenylserine concentration is given by the difference between the benzaldehyde concentrations in samples collected before and after the enzyme reaction.

A set of reaction experiments in single phase was realized for this purpose. The reaction mixture contained 0.053 g (0.1 mol l^{-1}) of benzaldehyde, 0.375 g (1 mol l^{-1}) of glycine, 0.5 ml of LTA, 50 μl of PLP, 1 ml of DMSO, and 3.5 ml of phosphate buffer solution (pH=8, 0.05 mol l^{-1}). The reaction was carried out in a batch reactor and the reaction times were set to 0, 15, 30, 45, and 60 min. The reaction was terminated by adding a trichloroacetic acid.

The benzaldehyde concentration was then determined by gas chromatography. The device GC-FID (Varian 430-GC) having a CP-Sil 5 CB column of 30 m (i.d. of 0.25 mm with film thickness of 1 μm) with a flame ionization detector were used. The carrier gas was helium (1 ml min^{-1} , split ratio 100). Oven temperature was held at 250 $^{\circ}\text{C}$. Injector and detector temperature were fixed at 260 $^{\circ}\text{C}$.

Once the total concentration of L-phenylserine in a sample was known, the ratios of the *syn*- and *anti*- forms of L-phenylserine in the same sample were determined by HPLC. A chiral column (Chirex 3126 (D)-penicillamine Column 250 $\times$ 4.6 mm) was used to obtain two signal peaks corresponding to the *syn*- and *anti*-forms of L-phenylserine at the retention times 15 and 30 min, respectively. The mobile phase consisted of 75 vol% of 2 mmol l^{-1} CuSO_4 in demi water and 25 vol% of methanol. The flow rate was set to 1 ml min^{-1} . The column temperature was kept at 40 $^{\circ}\text{C}$. Both optical forms of L-phenylserine were detected spectrophotometrically at 254 nm (Tibbe et al., 2013).

Each point reported in parametrical studies was calculated as an arithmetic average of three values coming from three independent experiments. Corresponding 95% confidence intervals typically do not exceed 6% of the absolute value of the average.

2.5. Enzyme labeling and adsorption studies

To optically monitor the location of LTA in the slug flow system, the enzyme was labeled by FITC. Protein concentration in the stock solution was determined to be 2.3 mg ml^{-1} by the Bradford protein assay (Berges et al., 1993). The enzyme solution was purified in a desalination column PD-10 (GE Healthcare) by washing with a carbonate buffer (100 mmol l^{-1} , pH = 9). The enzyme concentration after the purification was 1.4 mg ml^{-1} . 1 ml of the purified enzyme solution was mixed with 3.84 mg of FITC. The mixture was stirred for 2 h (300 rpm). The labeled enzyme and free FITC molecules were separated through the desalination column. The enzyme concentration after labeling remained unchanged (1.4 mg ml^{-1}). The catalytic activity of the labeled enzyme decreased only about 5% if compared to the stock enzyme.

It suggests that the enzyme structure was not significantly changed by the labeling process.

The distribution of LTA in the reaction mixture (without benzaldehyde) was investigated in two types of experiments. Fluorescence emission of the labeled enzyme was recorded by a confocal microscope (Olympus Fluoview FV-1000) at wavelength 495 nm in both experiments. The first method was carried out as follows. Droplet of the aqueous phase with the labeled LTA was put on a glass plate and completely covered by TBME. Fluorescence images of the interface at one focusing plane were recorded in time. Changes in the spatial distribution of fluorescence emission were investigated.

The other experimental method is based on the evaluation of fluorescence intensity in the bulk of the aqueous phase at one focusing plane before and after an experiment. The aqueous phase was mixed with organic phase and intensively stirred for 30 min. The enzyme has a chance to be trapped on the interface. Possible decrease of fluorescence emission after phase separation reflects enzyme adsorption to the interface.

2.6. Benzaldehyde partitioning

One of the LTA substrates, benzaldehyde, is well miscible with many organic solvents and sparingly miscible with water. Benzaldehyde partitioning between aqueous and organic phases significantly depends on the composition of both phases. The information about benzaldehyde solubility and partitioning were obtained in a set of batch experiments.

Chosen amount of benzaldehyde was added to an organic solvent (TBME, 2MeTHF, Toluene). 10 ml of buffered aqueous phase (phosphate buffer pH=8, 0.05 mol l⁻¹) contained 0.75 g of glycine and chosen volume of DMSO. Organic and water phases were mixed in the volume ratio of 1:1. The mixture was stirred for 30 min. After phase separation, the aqueous phase was mixed with ethyl acetate in the volume ratio of 1:2 to extract benzaldehyde.

Benzaldehyde concentration in the extract was obtained by GC analysis of the ethylacetate-benzaldehyde mixture, see the Section 2.4 for details.

3. Results and discussion

3.1. Slug flow stability

Initially tested reaction conditions led to the formation of unstable slug flow in the reaction capillary irrespective of selected organic solvent (Fig. 4a). To provide reproducible reaction conditions, it was necessary to guarantee stable slug flow for at least 60 min. For the purpose of this paper, we define stable slug flow as flow with regular slugs of the aqueous phase regularly separated by the organic phase. The lengths of segments of both phases have to be approximately equal (given by 1:1 vol ratio) and not significantly exceed the capillary diameter.

Several parameters were tested to increase the slug flow stability. When a high concentration of benzaldehyde in the organic phase was used, stable slug flow with toluene or TBME was observed, see Fig. 4b. However, slug flow with MeTHF was irregular. The use of slug flow generator with i.d. of 1 mm always led to stable slug flow profile (Fig. 4c) even with low benzaldehyde concentration in organic phase. Unfortunately, the produced segments with the length about 20 mm were too large to provide high interfacial area that is necessary for intensive mass transfer.

The addition of 5 vol% of PGPR to organic solvent led to the stabilization of slug flow profile (Fig. 4d) for all three organic solvents even when 0.5 mm capillary was used. PGPR is usually used as the stabilizer of water in oil emulsions (Knoth et al., 2005; Vladisavljevic et al., 2000). For example, Vladisavljevic et al. (2000) prepared stable water in oil emulsions with a narrow droplet size distribution with the oil phase containing 2.5–10% PGPR. PGPR typically decreases the viscosity of an oil phase (Knoth et al., 2005)

Fig. 4. Influence of several factors on the stability of the slug flow. (a) benzaldehyde concentration $c_B=1$ mol l⁻¹, generator i.d. of 0.5 mm, PGPR not present; (b) $c_B=4$ mol l⁻¹, the other parameters as in (a); (c) generator i.d. of 1 mm, the other parameters as in (a); (d) 5 vol% of PGPR in the organic phase, the other parameters as in (a).

Fig. 5. Enzyme deactivation in the presence of selected organic solvents. (a) Time courses of relative LTA activity (A_{LTA}) in the reference batch reactor. The lines help to lead an eye among the experimental points. (b) Relative LTA activity in the batch and slug flow arrangement after 30 min experiments.

and forms an energy barrier that inhibits the coalescence of particular droplets (Dedinaite and Campbell, 2000). Based on these findings, the reaction experiments were carried out with the organic phase containing 5 vol% of PGPR.

3.2. Enzyme deactivation

One of the key parameters negatively affecting the efficiency of an enzyme synthesis is the tendency of an enzyme to undergo

denaturation. The dependence of relative LTA activity (A_{LTA}) on the residence time in the reference batch reactor was studied in the presence of the selected organic solvents, see Fig. 5a. The obtained results show that enzyme activity monotonously decreases with growing residence time. Enzyme activity ranges from 83% to 90% and from 50% to 75% of the original value for one and six hours of the residence time, respectively. TBME provides the most friendly environment for the enzyme synthesis.

The comparison of the degree of enzyme inactivation in the slug flow microreactor and reference batch reactor revealed that the enzyme denaturation is less intensive in the slug flow arrangement regardless of the used solvent, see Fig. 5b. The most probable reason for this finding is the denaturation of the tertiary LTA structure due to either high shear forces localized at the mechanical stirrer (Thomas et al., 1979) or protein unfolding at the liquid-liquid interface of fine emulsion (Donaldson et al., 1980). LTA activity in the slug flow regime decreases only by several percent within 30 min reaction period. As in the batch reactor, the lowest degree of LTA denaturation is observed for TBME.

For comparison, Karande et al. (2010) observed 46–81% inactivation of soluble thermophilic alcohol dehydrogenase within 2.5–8 min time interval in a slug-flow bioreactor. They showed that addition of a surfactant (Tween 20) avoids the enzyme adsorption to water-oil interface. The degree of inactivation was then only several percent within 5 min. This result is in agreement with our findings for LTA with PGPR used as an interface stabilizer.

3.3. Enzyme location

Globular proteins contain hydrophilic and hydrophobic domains in their structure. It can be thermodynamically favorable for the enzyme to adsorb on the interface formed by aqueous and organic phases (Hickel et al., 1998). Some enzymes such as lipases operate on the interface taking one substrate from one phase and the other substrate from the other phase (Jurado et al., 2008; Tsai and Chang, 1993). In this study, LTA is considered to be a bulk catalyst in the aqueous phase and the organic phase serves only as a substrate reservoir. However, internal mixing inside slugs and diffusion itself take enzyme molecules into the contact with the interface, where they can adsorb. Corresponding protein unfolding can lead to a decrease of enzyme activity (Hickel et al., 1998; Straathof, 2003). Hence the possibility of LTA adsorption on the interface is examined in this paragraph.

Snapshot series of the aqueous-organic interface is depicted in Fig. 6. The enzyme is homogeneously dissolved in the water phase at the beginning of the experiment (0 min). Certain part of the enzyme molecules is trapped at the interface after 20 or 30 min.

Fig. 6. Fluorescence detection of enzyme close to liquid-liquid interface.

The snapshots show that the enzyme molecules aggregate to visible lumps at the interfacial area. There is a visible stripe of lower enzyme concentration between the interface and the bulk of aqueous phase. The stripe extends with increasing time (compare the snapshots for 20 and 30 min) as the enzyme molecules diffuse to the interface where they are trapped. No fluorescence signal was detected in the organic phase, which indicates none or negligible mass transfer of LTA molecules to the organic phase.

The measurements of fluorescence intensity in the bulk of the aqueous phase after intensive stirring and phase separation showed 51% decrease of signal if compared to signal before stirring. This result again indicates extensive adsorption of LTA to the liquid–liquid interface. As the enzyme keeps its activity for long time period (shown in previous chapter), we can conclude that the process of interfacial adsorption does not lead to any fatal denaturation; however, the total rate of the L-phenylserine synthesis is then necessarily affected by the mass transfer in the vicinity of the interface.

3.4. Benzaldehyde partitioning

We tested different compositions of both liquid phases to enhance substrate miscibility (benzaldehyde) with the aqueous phase. Addition of DMSO to the aqueous phase significantly increases the benzaldehyde content, see Fig. 7a. If TBME is selected as the organic solvent, it provides the highest benzaldehyde concentration in the aqueous phase. Benzaldehyde concentration in the aqueous phase with 25% content of DMSO is almost three times higher than in DMSO free environment.

Another set of experiments deals with benzaldehyde partitioning between the two liquid phases. Fig. 7b shows that the benzaldehyde concentration in the aqueous phase monotonously increases with growing benzaldehyde concentrations in the organic phase. The highest concentration of benzaldehyde achieved in this study exceeds 0.14 mol l^{-1} .

3.5. Parametrical studies

Reaction conditions allowing for high L-phenylserine concentration in the product stream of the slug flow microreactor were examined in parametric space. The effects of DMSO, enzyme, and glycine concentrations in the aqueous phase and the effects of solvent and benzaldehyde concentration in the organic phase were studied. The basic composition of both phases is defined in Section 2.2. The results described below lead to the definition of reaction conditions that are further used in performance and diastereoselectivity studies in Section 3.6.

Influence of various DMSO concentrations on L-phenylserine concentration in the product stream is shown in Fig. 8a. It was expected that higher amount of DMSO will lead to higher product concentration due to higher benzaldehyde (substrate) concentration in the aqueous phase (Fig. 7a). However, the results show that the highest product concentrations were achieved in experiments with 12.5 vol% instead of 25 vol% of DMSO in the aqueous phase. There are at least two reasons for the observed behavior: (i) destabilization of slug flow caused by high concentration of DMSO; (ii) substrate or product inhibition of free LTA or LTA trapped at the interfacial area.

We observed that 25 vol% content of DMSO eventually leads to the destabilization of slug flow in the reaction capillary because such great addition of DMSO cosolvent significantly decreases the interfacial tension (Foucault et al., 1993; Li et al., 1996). Irregular slug flow is coupled with the decrease of interfacial area.

Experiments with labeled enzyme proved that LTA is partially trapped at the interfacial area. DMSO cosolvent provides higher benzaldehyde concentration at the enzyme binding sites, which

Fig. 7. (a) Dependence of benzaldehyde concentration in the aqueous phase (c_B^w) on DMSO concentration (X_{DMSO}^w) and used organic solvent. Initial benzaldehyde concentration in the organic phase was set to 1 mol l^{-1} . (b) Dependence of benzaldehyde concentration in aqueous phase on benzaldehyde concentration in TBME (c_B^o). DMSO content in the aqueous phase is 25 vol%.

can directly lead to substrate inhibition of the biocatalyst as observed in Contestabile et al. (2001). Alternatively, the increased rate of product formation can result to the product inhibition of adsorbed/immobilized LTA, which has been proved in Tibhe et al. (2013).

Benzaldehyde concentration in the organic phase represents another parameter here examined, see Fig. 8b. The dependence of product concentration on benzaldehyde concentration in the organic phase is nonmonotonous with single maximum at $c_B^o \approx 2 \text{ mol l}^{-1}$. Higher benzaldehyde concentrations or even pure benzaldehyde as the organic phase negatively affect the product formation. Benzaldehyde (substrate) inhibition of the free enzyme or enzyme trapped at the interfacial area explains this behavior, which is supported by our own experiments with DMSO (Fig. 8a) and by Stöcklein and Schmidt (1985) who reported that TA binding sites are inhibited by acetaldehyde.

Another set of experiments with varying glycine concentrations was carried out; see Fig. 8c. These experiments prove previous finding by Steinreiber et al. (2007b) that tenfold molar excess of glycine over benzaldehyde ($c_G^w = 1 \text{ mol l}^{-1}$ and $c_B^w \approx 0.1 \text{ mol l}^{-1}$ because if $c_B^o = 4 \text{ mol l}^{-1}$ then $c_B^w \approx 0.1 \text{ mol l}^{-1}$ as plotted in Fig. 7b) provides the highest product yield. Glycine excess shifts the equilibrium to the L-phenylserine side. The observed decrease of the product concentration for $c_G^w = 1.5 \text{ mol l}^{-1}$ is statistically

Fig. 8. (a) Dependence of L-phenylserine concentration in the product stream (c_P^w) on (a) DMSO concentration (X_{DMSO}^w) and used organic solvent, (b) benzaldehyde concentration in the organic phase (c_B^o), (c) glycine concentration in the aqueous phase (c_G^w), and (d) volume content of the stock enzyme solution in the aqueous phase. Values of the other parameters are defined in Section 2.2. Experiments plotted in (c) and (d) were carried out only with TBME as the organic solvent.

insignificant. Three independent experiments give $c_P^w = 18.02 \pm 0.22 \text{ mmol l}^{-1}$ for $c_G^w = 1 \text{ mol l}^{-1}$ and $c_P^w = 17.2 \pm 1.0 \text{ mmol l}^{-1}$ for $c_G^w = 1.5 \text{ mol l}^{-1}$.

The higher enzyme concentration in the aqueous phase, the higher L-phenylserine concentration in the product stream is observed, Fig. 8d. The product concentration increases only about 50% for three times higher concentration of LTA. Hence, all other experiments were carried out with the basic enzyme concentration (2 ml of enzyme stock solution in 10 ml of the aqueous phase).

Upon our findings summarized in Fig. 8, the performance and diastereoselectivity studies described in the following section were carried out with $X_{\text{DMSO}}^w = 12.5 \text{ vol\%}$, $c_B^o = 2 \text{ mol l}^{-1}$, $c_G^w = 1 \text{ mol l}^{-1}$, $V_E = 2 \text{ ml}$.

3.6. Diastereoselectivity and performance studies under optimized conditions

The dependences of L-phenylserine concentration on the residence time in the slug flow and reference batch reactors are plotted in Fig. 9. The phenyl serine concentration reaches about 30 mmol l^{-1} within one hour in both slug flow and batch arrangements with TBME as the organic solvent. The highest possible concentration of benzaldehyde in the aqueous phase can be about 68 mmol l^{-1} for the chosen reaction conditions (Fig. 7). Hence the achieved product concentrations seem to be adequate to the results reported in Tibhe et al. (2013), who always observed less than 40% reaction yield due to the existence of the reaction

Fig. 9. Dependences of L-phenylserine concentration in the aqueous product on the residence time in slug flow and batch arrangements. The batch reaction was carried out with TBME as the organic solvent. The lines help to lead an eye among the experimental points.

equilibrium and product inhibition. Steinreiber et al. (2007b) achieved 85% yield of L-phenylserine after 2 h. However, they used LTA from a different organism and a single phase arrangement.

Performance of the both reactors with TBME shows that the rate of enzyme reaction is not limited by the interfacial mass

Fig. 10. (a) Time courses of the diastereoselectivity ratio, (b) dependence of the diastereoselectivity ratio on the L-phenylserine concentration in the aqueous phase. The batch reaction was carried out with TBME as the organic solvent. The lines help to lead an eye among the experimental points.

transfer and the interfacial area because the mixing regimes in both these arrangements are distinct but the observed time courses of the L-phenylserine concentration are almost the same (Fig. 9). If more LTA is used in the reaction mixture, the resulting concentration of L-phenylserine increases (Fig. 8d), which supports our finding about the kinetic limitation of the process. Significantly smaller concentrations of L-phenylserine in the product stream (only about 20 mmol l^{-1}) are observed for the other organic solvents, which is attributed to less friendly enzyme environment provided by toluene and MeTHF as shown in Fig. 5a.

Evaluation of reaction data provided the information about the diastereoselectivity of the enzyme reaction (Fig. 10). The diastereoselectivity ratio ($R_{s/a}$) is defined as the L-*syn*-phenylserine to L-*anti*-phenylserine concentration ratio. The *anti*-form prevails at the start of the reaction, see Fig. 10a. The *syn*-form content increases in time in the presence of all solvents. This result is in agreement with earlier observation by Fesko et al. (2008), who found that the L-*anti*-phenylserine is preferentially produced in so called kinetic regime far from the thermodynamic equilibrium. As the equilibrium is approached, L-*syn*-phenylserine is obtained as the dominant reaction product due to higher thermodynamical stability. Fig. 10a shows that $R_{s/a}$ grow with time approximately linearly when the residence time ranges between 5 min and

60 min. The highest content of L-*anti*-phenylserine is observed if toluene is used as the organic solvent. Conversely, MeTHF provides the highest content of L-*syn*-phenylserine with $R_{s/a}$ exceeding unity at 60 min.

The basic information about the concentration of the diastereoisomers in the product stream is given in Fig. 10b. The system with TBME as the organic solvent provides the highest L-phenylserine concentration in the product stream (about 30 mmol l^{-1}). But after 60 min, $R_{s/a}$ value approaches unity, i.e., almost racemic mixture of L-*syn*- and L-*anti*-phenylserine is obtained. The lowest $R_{s/a}$ value about 0.3 is provided by toluene as the organic solvent. However, relatively low L-phenylserine concentration only about 10 mmol l^{-1} is attained. MeTHF allows for $R_{s/a}$ exceeding unity within 60 min with the total L-phenylserine concentration about 23 mmol l^{-1} . These findings show that a particular diastereoisomeric form of L-phenylserine can be preferentially produced thanks to proper choices of the organic solvent and residence time.

For comparison, we can calculate the diastereoisomeric excess of the *syn*-form $de_{syn} = (R_{s/a} - 1)/(R_{s/a} + 1) \times 100\%$ and *anti*-form $de_{anti} = (1 - R_{s/a})/(R_{s/a} + 1) \times 100\%$ by means of our $R_{s/a}$ values, respectively. We achieved $de_{anti} \sim 54\%$ after 10 min and $de_{syn} \sim 4\%$ after 60 min. In one phase reaction, Fesko et al. (2008) were able to achieve $de_{anti} \sim 70\%$ after 5 min and $de_{syn} \sim 21\%$ after 30 min with two different LTAs obtained from *Bordetella bronchiseptica* and *Pseudomonas putida*. Steinreiber et al. (Steinreiber et al., 2007a) reported $de_{syn} \sim 20\%$ after 2 h with LTA obtained from *Pseudomonas putida*. About 20% diastereoisomeric excess is reported in Ref. (Tibbe et al., 2013) with LTA from *Thermotoga maritima*. It is obvious that we were able to achieve comparable or even better diastereoisomeric excess at least for the *anti*-form of L-phenylserine.

4. Conclusions

L-phenylserine synthesis by LTA was investigated in a slug flow reactor and reference batch reactor. We observed that the rate of LTA deactivation is lower in the slug flow arrangement (a few percent within 30 min) than in the reference batch reactor (about 10% within 30 min). No significant decrease of enzyme activity coupled with the adsorption to aqueous-organic interface was confirmed.

Reaction regime providing relatively high concentration of L-phenylserine (about 30 mmol l^{-1}) was identified by means of parametrical studies. To reach this product concentration, the aqueous and organic phases have to consist of 1 mol l^{-1} of glycine, 12.5 vol% of DMSO, 20 vol% of the enzyme stock solution and 2 mol l^{-1} of benzaldehyde in TBME solvent, respectively.

High content of the L-*anti*-phenylserine form ($R_{s/a} \sim 0.3$) can be obtained with short reaction time (~ 10 min) and toluene as the organic solvent. High L-*syn*-phenylserine concentration ($R_{s/a} > 1$) is achieved with long reaction time (~ 60 min) in the presence of MeTHF. So the proper choice of organic phase is a crucial parameter in the synthesis of the diastereoisomers.

The comparison of batch and slug flow experiments in the presence of TBME reveals that there is no significant difference in the L-phenylserine yield or the concentration ratio of the stereoisomers. However, the slug flow arrangement can be preferred due to lower rate of enzyme deactivation (proved in this work) and lower demands on mechanical energy, see Čech et al. (2012) for energy analysis. We believe that intensive research of enzyme stereoselective syntheses is necessary to demonstrate possible benefits of microfluidic platforms for this type of enzyme reactions.

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