Integration of Ion Exchange Resin Materials into Downstream-Processing of an Imine Reductase (IREDs)-Catalyzed Reaction

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Abstract

In this study an ion exchange resin-based downstream-processing approach for imine reductase (IRED)-catalyzed reactions was investigated. As a model reaction, 2 methylpyrroline was converted to its corresponding product (*S*)-2-methylpyrrolodine with >99% of conversion by the (*S*)-selective IRED from *Paenibacillus elgii* B69. Under optimized reaction conditions full conversion was achieved using a substrate concentration of 150 mmol·L^{-1} and 500 mmol·L^{-1} of D-glucose. Seven commercially available cation and anion exchange resins were studied with respect to their ability to recover the product from the reaction solution. Without any pre-treatment, cation exchange resins Amberlite IR-120(H), IRN-150, Dowex Monosphere 650C and Dowex Marathon MSC showed high capacities (up to >90%). A 150 mL-preparative-scale reaction was performed yielding ca. 1 g product with >99% purity. Any further purification steps, e.g. by column chromatography or recrystallization, were not required.

Keywords

enzyme, downstream-processing, ion exchange resin, process development, imine reductase (IREDs)

Introduction

Chiral amines are valuable building blocks for the synthesis of a vast selection of active pharmaceutical ingredients (APIs), agrochemicals and fine chemicals . These compounds can be obtained by chemical and biotechnological approaches, whereas biocatalytic applications frequently enable the highest stereo-, regio- and chemoselective conversion from the respective starting materials being a significant process advantage over conventional chemical approaches . In addition, in the past decades the scientific advances in protein engineering techniques have provided a better access to specifically improved enzymes for tailor-made bio-processes, also in the chiral amine synthesis . Especially amine transaminases , amine dehydrogenases , and imine reductases (IREDs) have recently attracted a lot of attention for synthesizing a wide selection of chiral amines. A special case are IREDs, which allow a direct synthesis of enantiopure secondary amines from pro-chiral imines . Within this relatively young group of enzymes, Mitsukura et al. first reported the purification and characterization of the (*R*)-IRED from *Streptomyces* sp. GF3587 reducing 2-methyl-1-pyrroline (2-MP) to (*R*)-2-methylpyrrolidine ((*R*)-2-MPN) with an enantioselectivity of 99%. The same reduction was performed using purified (*S*)-IRED from *Streptomyces* sp. GF3546 synthesizing (*S*)-2-methylpyrrolidine ((*S*

)-2-MPN) . Since then, this beneficially conversion yielding optically active secondary and tertiary amines by using nicotinamide adenine dinucleotide [phosphate] (NAD[P]H) as the cofactor has been broadened to a wide substrate scope from acyclic imines up to five and six membered heterocycles . Also, the IRED-catalyzed asymmetric reductive amination of ketones has been reported recently , boosting this asymmetric biosynthesis in the research field.

A significant amount of IRED-based reports focus on the optimization of the enzymatic reaction, either by process or reaction engineering while optimizing the relevant environmental conditions (pH, temperature, etc.), or the biocatalyst itself via enzyme engineering (K_{M}, k_{cat}, substrate scope, etc.). Unfortunately, the downstream-processing (DSP) within such biocatalytic processes is often not investigated in detail and typically limited to an initial pH-shift of the aqueous reaction medium, followed by single or even multiple extractions and final distillation or column purification steps. Alternative non-conventional reaction media such as ionic liquids , deep eutectic solvents and other could be used to simplify the DSP but may suffer from major drawbacks like activity losses of the biocatalyst and a high complexity associated with a cost-intensive system. In addition, *in situ* -product removal (ISPR) techniques such as crystallization or membranes would be also a powerful addition, but are currently not widely applicable for IRED-catalyzed reaction systems .

This study aims at presenting the utilization of ion exchange resins as an easy option to perform the DSP of IRED-catalyzed reaction systems, including its potential usage on a preparative scale. A small selection of ion exchange materials was mentioned in the past within biocatalytic processes, but their applications are still somehow limited with a few noticeable exceptions . In this study, the ion exchange resins are applied to the IRED-reaction system to selectively remove the reactants from the reaction solution without any significant changes to the aqueous reaction medium, which also minimizes the processing steps during the DSP. In addition, this strategy opens the possibility of simply reusing the biocatalyst without any potentially harmful biocatalyst immobilization steps. In contrast to classical adsorbers, ion exchange resins offer a more specific interaction with charged reactants such as ammonium- and iminium-ions. This concept was also recently reported for a decarboxylase-reaction system for the synthesis of a benzoic acid derivate using the commercially available anion exchange resin Dowex 1x2 (Cl) .

The presented ion exchange resin-based DSP-concept involves a simple addition of the ion exchange resin to the reaction medium for selectively capturing the reactants. This is followed by a filtration step to remove the resin particles and a final release of the product from the resin into ether (Figure 1). Due to the ubiquitous use of 2-methyl-1-pyrroline as a model compound in IRED-catalyzed reactions it was specifically chosen for this study and used for the preparative synthesis and isolation of the corresponding enantiopure product (S)-2-methylprrolidine. The highly (S)-selective IRED from*Paenibacillus elgii* B69 was applied in whole cells as a model biocatalyst.

[**Figure 1**]

Experimental Section, Materials and Methods

General Information

All solvents, reactants and starting materials were received from commercial suppliers (Sigma-Aldrich, Chem-Pur, Thermo Fischer Scientific (Acros Organic)) and used as received. Ultrapure water (UPW, 0.06 µS[?]cm^{-1}) was produced with an Ultra Clear Reinstwassersystem by SG Water (now Evoqua, Guenzburg, Germany) and used throughout this study. All experiments were carried out at atmospheric conditions. An IKA HS 260c shaker (IKA-Werke, Staufen, Germany) tempered with a Huber CC-K6 thermostat (Peter Huber Kältemaschinenbau AG, Offenburg, Germany) was used throughout all experiments for tempering and shaking of the reaction vessels at 180 rpm if not mentioned otherwise. Recombinant (S)-selective IRED from*Paenibacillus elgii* B69 was transformed, cloned and overproduced in *E. coli* BL21 (DE3) as previously described .

Gaschromatography

The Conversion of all reactions was measured by gas chromatography with a Trace 1310 gas chromatograph from Thermo Scientific (Dreieich, Germany) with a flame ionization detector equipped with a HP-5 column from Agilent Technologies (30 m x 0.250 mm, 0.25 µm, 19091J-433, SN: USF724723H). Helium was used as the carrier gas (purity: 99.999%) with a flow rate of 0.75 mL[?]min^{-1} was used for all measurements. Temperatures of the injector and detector were set to 250 °C. Temperature program: 40 °C for 10 min, followed by a heating rate of 125.0 °C[?]min^{-1} to 250 degC and a hold time for 5 min. The sample was injected within split-mode with a split flow of 7.5 mL[?]min^{-1} and a purge flow of 3 mL[?]min^{-1}. Authentic reference material was used for external quantification (see Figure S 1 to 4 for further information). All measurements were carried out in triplicate and the calculated standard deviations are shown.

General Procedure for Biotransformations

50 mg lyophilized whole *E. coli* cells containing the corresponding overproduced enzyme were rehydrated in 400 µL of a 100 mmol[?]L^{-1} sodium phosphate buffer (NaP_i) pH 7.5 and shaken at 30 °C and 180 rpm in a TS-100 thermos shaker from Biosan. After 30 min, 100 µL of a 2-MP substrate stock solution (250 mmol·L^{-1} in NaP_i) and 50 mg of d-glucose were added successively to the cell suspension (100 mmol[?]L^{-1}final concentration of the substrate and 500 mmol*$L^{-1}$of d-glucose). A blank sample without the whole cell biocatalyst was prepared to ensure the absence of undesired reactions. The vials were tightly sealed and shaken at 30 degC and 180 rpm for 24 h.

After 24 h the reaction vessels were centrifuged for 5 min at 14000 rpm and 100 µL of the supernatant was transferred to 900 µL DCM together with 30 µL of a 10 m NaOH. The basified extraction was promoted by vigorous shaking, 250 µL of the DCM phase were transferred to GC Vials together with 25 µL of a solution containing *n* -heptane as a standard and afterwards analyzed by gas chromatography.

For the evaluation of the maximal substrate concentration, desired volumes of the substrate either from stock solution or the pure substance were mixed together with buffer and d-glucose in a 500 µL-scale as mentioned above. Samples for GC were taken to investigate the reaction system at definite time intervals (conversion vs. time) and the final concentration of the substrate was set to 150 mmol·L^{-1}.

Adsorption- and Desorption-Studies

The specific characteristics of the commercially available resins are shown in Table S1 (see SI). The exchange resins were used as received in their native ionic form.

To evaluate the capability for a DSP of the case studied biocatalytic IRED-catalyzed reaction, the capacity of the resins with respect to substrate and product were determined. A 100 mmol·L^{-1}test solution of product and substrate in 100 mmol·$L^{-1}$$NaP_i$ buffer solution together with 500 mmol·L^{-1} of d-glucose was prepared for the adsorption experiments. 4 mL of this test solution were then added to 0.1 g, 0.5 g and 1.0 g of each resin, respectively. The shaking-flask assays were performed at 30 degC and 180 rpm for 30 min. The adsorption of the substrate and the product were determined by analyzing the residual in the solution before and after adding the adsorbent resins via GC. 100 µL of the supernatant were transferred to 900 µL DCM together with 30 µL of a 10 m NaOH and further processed for the GC as mentioned above. For calculations of the adsorption, the measured concentration of the prepared test solution was set at 100% and compared to the amount of substrate after the adsorption.

After the successful adsorption of 2-MP and 2-MPN desorption experiments were executed with Amberlite IR-120(H), Dowex Monosphere 650C and Marathon MSC(H) cation exchange resins. Therefore, 0.5 g of each resin was loaded with the substrate and the product as mentioned above and after a filtration-step rinsed in 4 mL of a two phase system composed of 5% NaOH and cyclopentyl methyl ether (CPME) at a volumetric ratio of 1:1 at 30 °C and 180 rpm for 30 min. Afterwards, the concentrations of the substrate and the product in the ether phase were determined via GC as mentioned above.

Preparative Scale Experiment

For the preparative scale experiment, 15 g lyophilized whole *E. coli* cells containing the corresponding overproduced enzyme were rehydrated in 150 mL of a 100 mmol·L^{-1} sodium phosphate buffer (NaP_i) pH 7.5 and stirred at 30 °C. After 30 min, 2.13 mL of 2-MP and 15 g of d-glucose were added successively to the cell suspension (150 mmol*L^{-1}final concentration of the substrate and 500 mmol*$L^{-1}$of d-glucose). The reaction mixture was stirred at 30 degC for 24 h. The conversion of the reaction was tracked after {0; 6; 17 and 24} h by removing 500 µL from the reaction broth and centrifuging it at 14000 rpm for five minutes. 100 µL of the supernatant were then proceeded and analyzed via GC as mentioned above.

After reaching a maximum conversion, 30 g Dowex Monosphere 650C cation exchange resin (0.2 g·mL^{-1}) were added to the reaction broth and stirred for 30 min at 30 °C. A 500 µL-sample was taken and analyzed as mentioned before to check the full adsorption onto the resin. The cation exchange resin beads were filtered out of the reaction broth, and the remaining resins were desorbed in 75 mL of 5% NaOH and 75 mL of CPME at 30 °C for 30 min. To promote the phase separation, the reaction mixture was centrifuged for 5 min at 4000 rpm. The ether phase was dried with Na_2SO_4 for 2 h. Afterwards, the amine was oiled out with *in situ* generated HCl gas as amine hydrochloride and the remaining solvent CPME was removed by evaporation at 80 mbar. The purity of the viscous oil was checked by NMR (see SI).

Results and Discussion

Optimization of the Enzymatic Imine Reductase Reaction

Initial experiments targeted an increase of the substrate concentration to facilitate higher productivities within the biocatalytic process using the highly enantioselective imine reductase from*Paenibacillus elgii* B69. Considering the chosen conditions, the starting substrate concentration leads to a full conversion in the range of 25 mmol·L^{-1} to 150 mmol*L^{-1}, but a deactivation of the catalyst was found at even higher substrate concentrations (see Figure 2A). This is solely caused by a substrate inhibition and not a pH-shift due to the cofactor regeneration-based formation of gluconic acid by glucose dehydrogenase. Noteworthy, attempts using an excessive pH control with a 1 mol*L^{-1} phosphate buffer pH 7.5 were also not successful due to a complete deactivation of the biocatalyst. A full conversion was reached within 24 h under the chosen reaction conditions (Figure 2B). The final optimized reaction conditions facilitated full conversions at 30 degC, a substrate concentration of 150 mmol*L^{-1} and 500 mmol*L^{-1} of d-glucose. These experiments clearly indicate that further improvements of the catalytic robustness towards higher substrate loadings and higher specific activities are required to improve the overall process productivity, which was not the main aim of this study. Other noticeable examples of imine reductase-catalyzed reactions at larger scale were recently reported for the biocatalytic reductive amination reaction between cyclohexanone and cyclopropylamine with an excellent volumetric productivity of 12.9 g*L^{-1}*h^{-1} and a TON above 48000

[Figure 2]

Selection of Ionic Exchange Resins

The efficiency and selectivity of ion exchange resins is specifically controlled by their chemical structure. Noteworthy, the choice of operating conditions has a substantial directional effect on the overall process using such ion exchange resins, incl. its respective productivity, and should always be investigated in detail. This is particularly relevant for an imine reductase-catalyzed reaction, as significant differences between the substrates (imines/primary amines) and the final product (secondary amines) are found. Unfortunately, to date, no effective ion exchange resin-based recovery method has been reported for imine reductase-catalyzed reaction in the scientific literature. Most reports involve classical extraction and distillation steps, which require an additional effort during the DSP.

Thus, different commercially available cation- and anion- exchange resins were screened for their ability to remove the reactant's product (*S*)-2-MPN and the possibly remaining substrate 2-MP from the reaction solution. Therefore, test solutions containing 100 mmol*L^{-1} of the product and the substrate together with 500 mmol*L^{-1} d-glucose in 100 mmol*L^{-1} NaP_i buffer pH 7.5 were prepared and three different doses (0.02 g, 0.1 g and 0.2 g per mL test solution, respectively) were analyzed. The residual concentration in solution was measured via GC and the results are shown in Figure 3. Figure 3A presents the remaining concentration after 30 min and Figure 3B after 24 h. For all entries, equilibrium concentrations are almost achieved after 30 minutes, but a longer adsorption time should be executed to improve yields. Values greater than 100% were the result of the swelling degree of the polymeric material. Here, the resins soak up the solvent water and consequently this increases the residual concentration in solution.

[**Figure 3**]

While comparing the overall data set presented in Figure 3 it is clear that cation exchange resins clearly result in a stronger removal (entries 1-4) from the aqueous reaction medium in comparison to the anion exchange resins (entries 5-7), which is expected since the substrate and product are both positively charged (Figure 4). However, the hydrophobic backbone of the tested anion exchange resins still provides a residual, relatively weak reactant adsorption that slightly favors the product (*S*)-2-MPN from the imine reductase-catalyzed reaction.

[**Figure 4**]

The cation exchange resins Amberlite IR-120(H) (entry 1), IRN-150 (entry 2), Dowex Monosphere 650C (entry 3) and Dowex Marathon MSC (entry 4) remove the reactants relatively similarly, while a stronger adsorption of the product and the substrate can be recognized with respect to an increase of the ion exchanger's doses. All cation exchange resins used in this study are composed of a styrene/divinylbenzene-based matrix with sulfonic acid moieties (Figure 5).

[**Figure 5**]

In doses of 0.2 g*mL^{-1}, Amberlite IR-120(H), Dowex Monosphere 650C and Dowex Marathon MSC adsorb >90% of 2-MP and (*S*)-2-MPN from the test solution. In the smallest dose of 0.02 g*mL^{-1} a selective adsorption of about 40% of the product to the substrate is observed regarding of Amberlite IR-120(H), Dowex Monosphere 650C and Dowex Marathon MSC. The highest removal efficiency was found with Dowex Marathon MSC, which was used during the scale-up and the DSP of (*S*)-2-MPN. A remarkably high selectivity towards the product (*S*)-2-MPN over the substrate 2-MP was observed using the ion exchange resin IRN-150 making it also a perfect candidate for a potential *in situ* product adsorption (ISPA) process (entry 2 with 0.2 g resin per mL reaction medium). Due to this type of procedure, the ion exchange resin could be dispersed in the reaction solution while the biocatalytic reaction takes place. A resulting product would be directly adsorbed and thus the reaction equilibrium pushed to the products' side according to the Le Chatelier's principle. Unfortunately, all our attempts using this cation exchange resin within an imine reductase-catalyzed reaction were unsuccessful due to a full deactivation of the biocatalyst by the resin material. The reason for this behavior is unknown and will be investigated thoroughly in a future study.

Scale-up and Downstream-Processing of (*S*)-2-MPN

After establishing the successful adsorption procedure, the development of an efficient adsorption-desorption methodology was crucial. A 150 mL-preparative-scale IRED-catalyzed reaction was performed. The reaction was checked for a full conversion (see Figure 6) and after 24 h, 30 g Dowex Monosphere 650C cation exchange resin (0.2 g*mL^{-1}) were added to the reaction broth and stirred for 30 min at 30 degC. The adsorption of the product was checked and confirmed to be >95% (see Figure S 5 and 6).

[**Figure 6**]

After the filtration of the cation exchange resin beads, the desorption was carried out using a mixture of

5% NaOH and cyclopentyl methyl ether (CPME) in a volumetric ratio of 1:1. After the phase separation the product amine was oiled out from the ether phase as the hydrochloride salt using gaseous HCl. Herein, CPME offers a relatively low water content with the organic layer, which is highly important to precipitate the hydrochloride-salt of the amine. After evaporating the residual CPME, ca. 1 g of a brown-orange oil was obtained with a purity of >99% (see Figure S 7 to Figure S 10 for further information). Any further purification steps, e.g. by column chromatography or recrystallization, were not required highlighting the applicability of this method.

Summary and Conclusion

In this contribution we presented the process development of the IRED-catalyzed synthesis of (*S*)-2-methylpyrrolidine with a special emphasis on an ion exchange-based downstream-processing approach. The biocatalytic reaction was partly optimized resulting in a substrate concentration of 150 mmol*L^{-1} and 500 mmol*L^{-1} of d-glucose at 30 degC for 24 h. Additionally, a variety of adsorbent resins for the effective removal of both product and substrate from the reaction solution were investigated and Amberlite IR-120(H), IRN-150, Dowex Monosphere 650C and Dowex Marathon MSC were found to be the most effective cation exchange resins. After further studies, the resin Dowex Monosphere 650C was successfully used for the downstream-processing of a scaled up reaction to produce highly pure (>99%) (*S*)-2-methylpyrrolidine without any further purification steps. The developed process concept in this study, namely the integration of ion exchange resin materials into downstream-processing, is efficient and very simple to perform.

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Conflict of Interest

The authors declare no conflict of interest.

Figure legends

Figure 1. General scheme for the imine reductase-catalyzed reduction of 2-methyl-1-pyrroline (2-MP) to (*S*)-2-methylpyrrolidine ((*S*)-2-MPN) and the developed downstream-processing with ion exchange resins including its subsequent desorption and release into ether to yield the pure product.

Figure 2. A) Conversion versus initial substrate concentration to determine the maximal substrate loading for the IRED-catalyzed synthesis. B) Time course of the imine reductase-catalyzed reaction using a substrate concentration of 150 mmol*L^{-1}. All experiments were carried out with 100 mg*mL^{-1} whole cell catalyst, sodium phosphate buffer (NaP_i) pH 7.5 and 500 mmol*L^{-1} of d-glucose in 500 µL-scale experiments at 30 °C. The dashed lines are a guide to the eye.

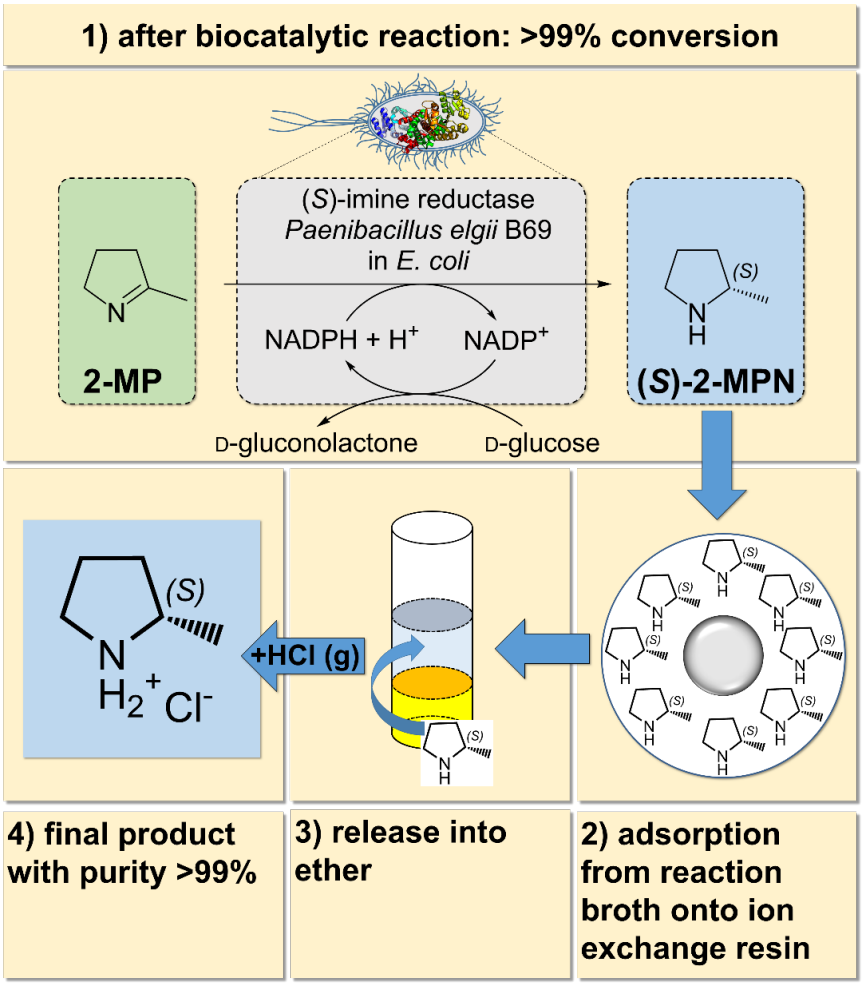
Figure 3. Adsorption after 30 min (A) and 24 h (B) of the imine-substrate 2-MP (grey) and of the amine-product (*S*)-2-MPN (black) onto seven different commercially available cation- and anion-exchange resins at 30 °C and 180 rpm from a test solution containing 100 mmol·L^{-1} either of the substrate and product together with 500 mmol·L^{-1} d-glucose in a NaP_i buffer solution at pH 7.5.

Figure 4. A) Protonated form (brown) and non-protonated form (blue) of the imine substrate species and B) protonated form (red) and non-protonated form (grey) of the total amine product species with respect to the pH. Diagrams and pK s values calculated with "chemicalize" (www.chemicalize.com).

Figure 5. General presentation of styrene (blue)/divinylbenzene (red)-based cation exchange resin structure with sulfonic acid functional groups.

Figure 6. Tracking of the preparative-scale reaction from the reaction start (t = 0 h) to the end (t = 24 h). Retention times were verified by commercially available authentic reference substances (see SI for further information). Retention times: internal standard: 6.8 min, substrate 2-MP: 9.2 – 9.4 min, product (*S*)-2-MPN: 7.7 – 7.8 min.

Figure 1:



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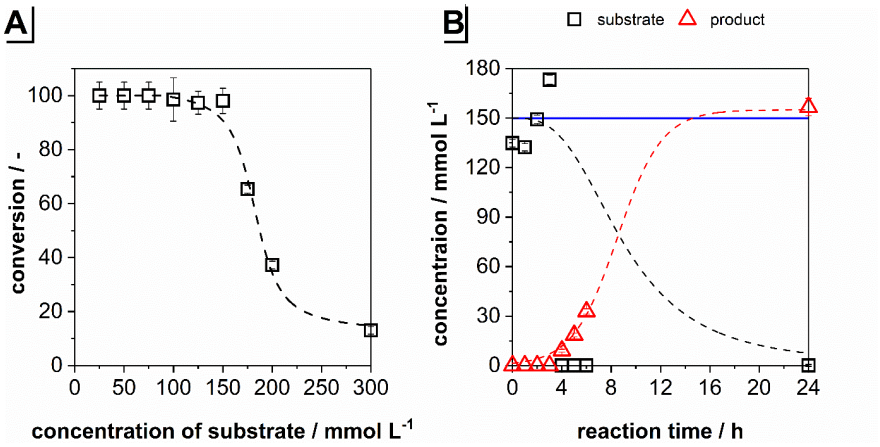
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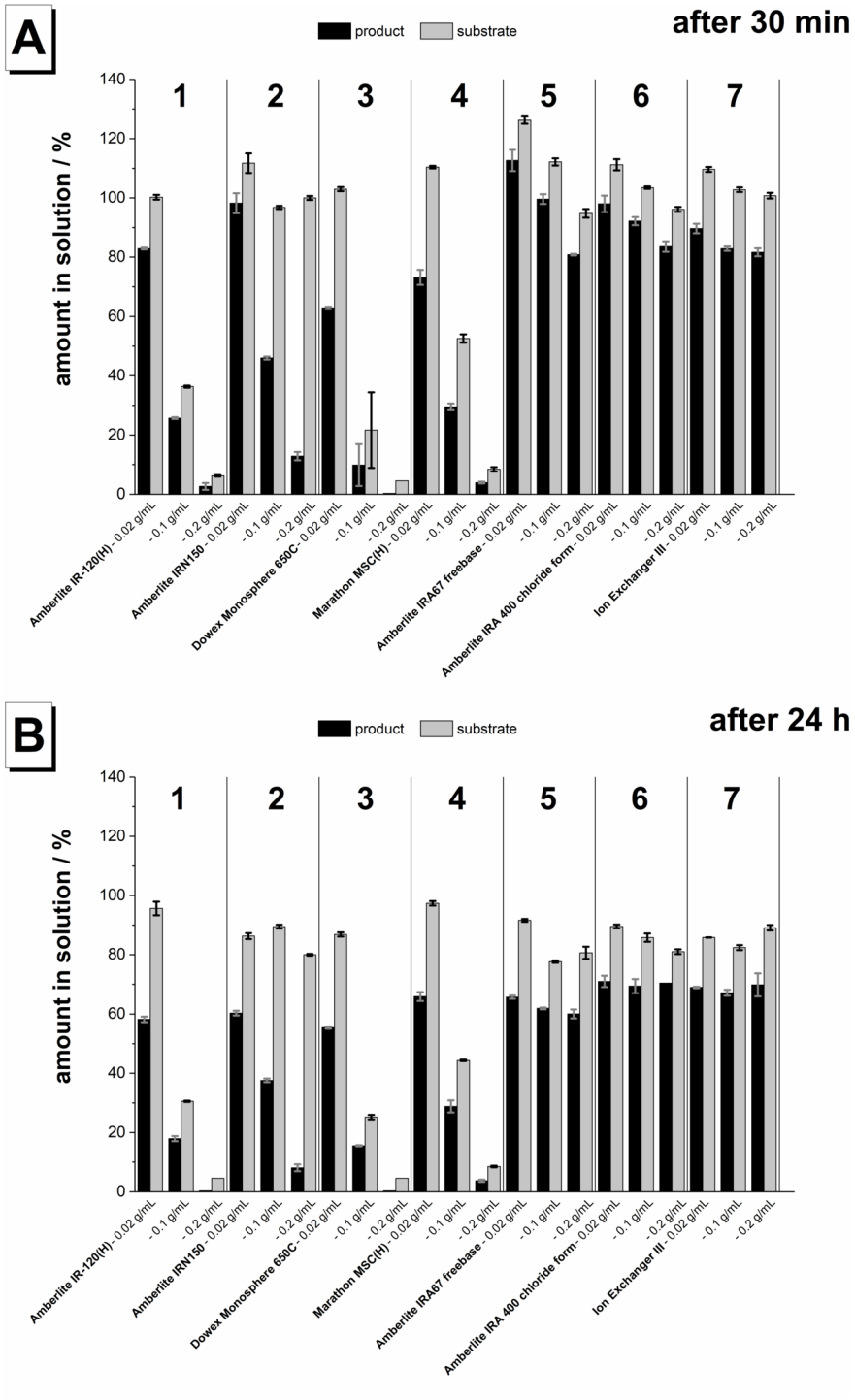
Figure 3: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Dowex Monosphere 650C - 0.02 g/mL
- 0.1 g/mL
- 0.2 g/mL
Marathon MSC(H) - 0.02 g/mL
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Amberlite IRA67 freebase - 0.02 g/mL
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Dowex Monosphere 650C - 0.02 g/mL
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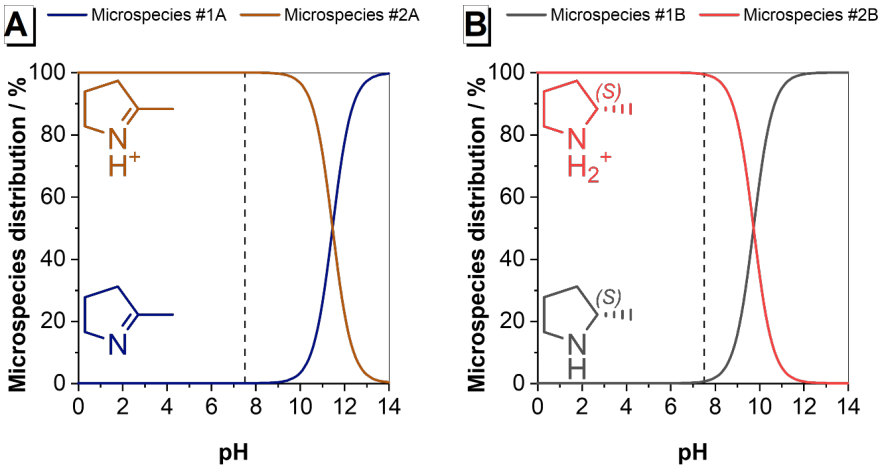
Figure 4:



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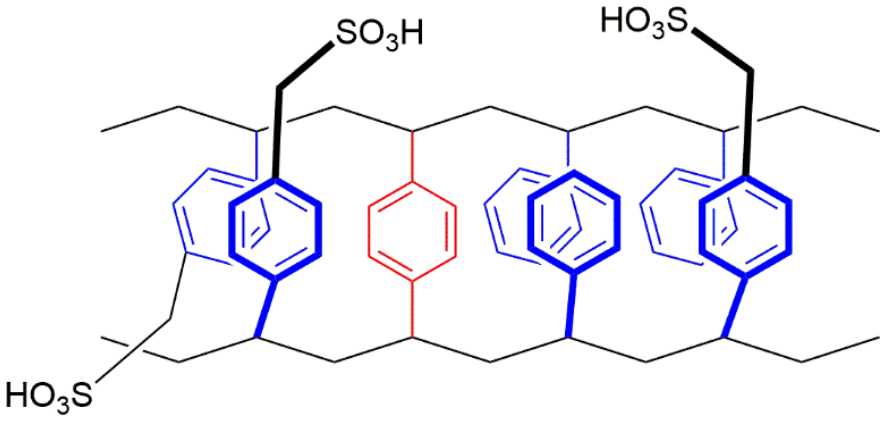
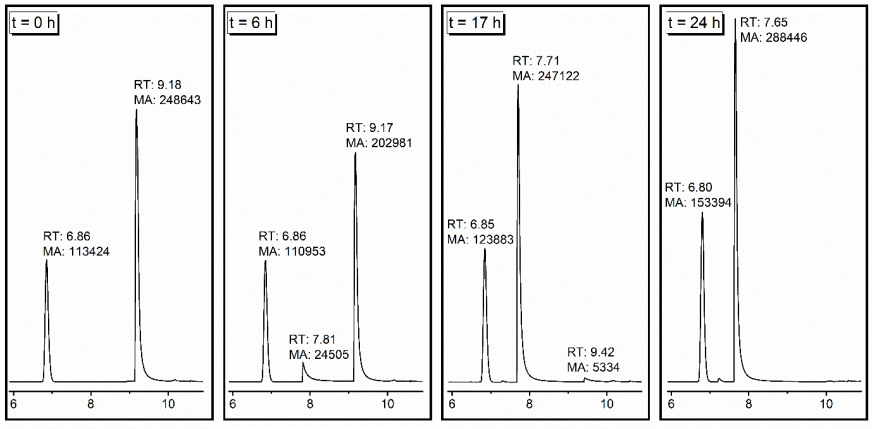


Figure 6:



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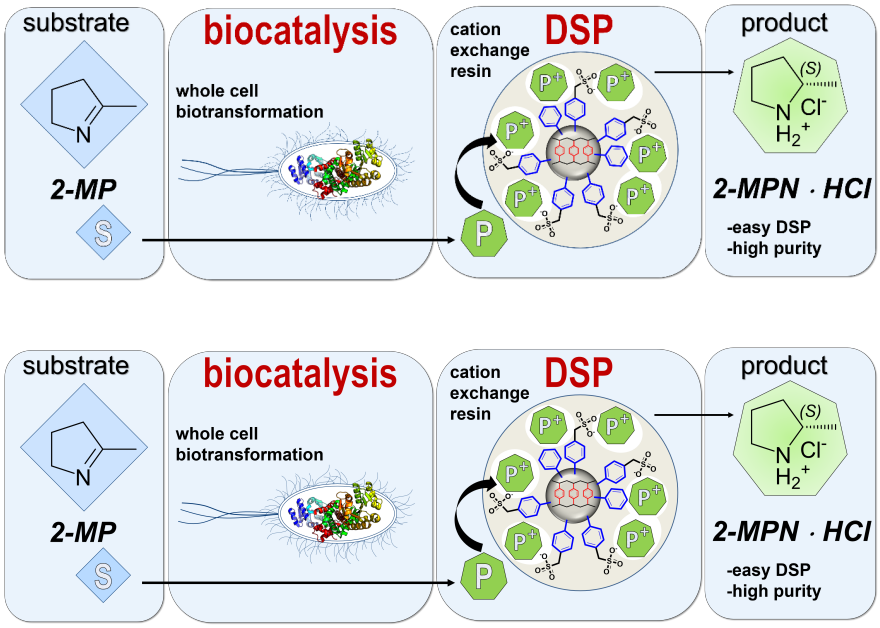

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-easy DSP
-high purity