Integration of ion exchange resin materials for a downstream-processing approach of an imine reductase-catalyzed reaction

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Abstract
In this study, an ion exchange resin-based downstream-processing concept for imine reductase (IRED)-catalyzed reactions was investigated. As a model reaction, 2-methylpyrrole was converted to its corresponding product ((S)-2-methylpyrrolidine 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 and 500 mmol/L 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 pretreatment, cation-exchange resins Amberlite IR-120(H), IRN-150, Dowex Monosphere 650C, and Dowex Marathon MSC showed high recovery capacities (up to >90%). A 150-ml preparative scale reaction was performed yielding 1 g hydrochloride salt product with >99% purity. Any further purification steps, for example, by column chromatography or recrystallization, were not required.

KEYWORDS
downstream-processing, enzyme, imine reductase, ion exchange resin, process development

1 | INTRODUCTION

Chiral amines are valuable building blocks for the synthesis of a vast selection of active pharmaceutical ingredients, agrochemicals, and fine chemicals. These compounds can be obtained by chemical and biotechnological approaches, whereas biocatalytic applications frequently enable the highest stereo-, regio-, and chemo-selective 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 bioprocesses, also in the chiral amine synthesis. Especially amine transaminases and amine dehydrogenases and imine reductases (IREDs) have recently attracted a lot of attention for synthesizing a wide selection of chiral amines. A special case is IREDs, which allow a direct synthesis of enantiopure secondary amines from prochiral 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 (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-MPN). Since then, this beneficial conversion yielding optically active secondary and tertiary amines by using nicotinamide adenine dinucleotide (phosphate) 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, further 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 nonconventional reaction media, such as ionic liquids, deep eutectic solvents, and others, could be used to simplify the product isolation 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 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 product isolation from 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 eventually minimizes the processing steps during a potential 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 derivative using the commercially available anion-exchange resin Dowex 1×2 (Cl). In this study, the presented ion exchange resin-based concept involves a simple addition of the ion exchange resin to the reaction medium for selectively capturing the reactants. This is followed by a sieving step to remove the resin particles and a final release of the

1) after biocatalytic reaction: >99% conversion

2) adsorption from reaction broth onto ion exchange resin

3) release into ether

4) final product with purity >99%

FIGURE 1  General scheme for the imine reductase-catalyzed reduction of 2-methyl-1-pyrroline to (S)-2-methylpyrrolidine and the developed downstream-processing approach with ion exchange resins including its subsequent desorption and release into ether to yield the pure hydrochloride salt product.
product from the resin into ether (Figure 1). Due to the ubiquitous use of 2-MP 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-MPN. The highly (S)-selective IRED from Paenibacillus elgii B69 was applied in whole resting cells as a model biocatalyst. This study focuses on the development of a concept and does not represent a fully optimized DSP process. Further studies will concentrate on mathematical models and adsorption isotherms for the respective ion exchange resin, which will improve the DSP process significantly.

2 | EXPERIMENTAL SECTION, MATERIALS, AND METHODS

2.1 | General information

All solvents, reactants, and starting materials were received from commercial suppliers (Sigma–Aldrich, ChemPur, Thermo Fischer Scientific [Acros Organics]) and used as received. Ultrapure water (0.06 μS/cm) 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 P. elgii B69 was transformed, cloned, and overproduced in Escherichia coli BL21 (DE3) as previously described.52

2.2 | Gas chromatography

The conversion of all reactions was determined by gas chromatography (GC) 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 × 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 for all measurements. Temperatures of the injector and detector were set to 250°C. The specific characteristics of the commercially available resins are shown in Table 1. The exchange resins were used as received in their native ionic form.

2.3 | General procedure for biotransformations

Fifty milligrams of lyophilized whole E. coli cells containing the corresponding overproduced enzyme were rehydrated in 400 μl of a 100 mmol/L sodium phosphate buffer (NaPi), pH 7.5 and shaken at 30°C and 180 rpm in a TS-100 thermo shaker from Biosan. After 30 min, 100 μl of a 2-MP substrate stock solution (250 mmol/L in NaPi) and 50 mg of D-glucose were added successively to the cell suspension (100 mmol/L final concentration of the substrate and 500 mmol/L 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°C and 180 rpm for 24 hr.

After 24 hr, the reaction vessels were centrifuged for 5 min at 14,000 rpm and 100 μl of the supernatant was transferred to 900 μl of dichloromethane together with 30 μl of a 10 M NaOH. The basified extraction was promoted by vigorous shaking, 250 μl of the dichloromethane phase was transferred to GC vials together with 25 μl of a solution containing n-heptane as a standard and afterward analyzed by GC.

For the evaluation of the maximal substrate concentration, desired volumes of the substrate either from stock solution or from the pure substance were mixed with buffer and D-glucose in a 500-μl scale as mentioned above. Samples for GC were taken after 24 hr to investigate the reaction system.

2.4 | Adsorption and desorption studies

The specific characteristics of the commercially available resins are shown in Table 1. The exchange resins were used as received in their native ionic form.

To evaluate the capability for product isolation from the IRED-catalyzed reaction, the capacity of the resins with respect to substrate and product was determined. A 100 mmol/L test solution of product and substrate in 100 mmol/L NaPi buffer solution together with 500 mmol/L of D-glucose was prepared for the adsorption experiments. Four milliliters of this test solution was then added to 0.1, 0.5, and 1.0 g (dry mass) of each resin, respectively (0.02, 0.01, and 0.2 g/ml test solution, respectively). The shaking-flask assays were performed at 30°C and 180 rpm for 30 min. The adsorption of the substrate and the product was determined by analyzing the residual in the solution before and after adding the adsorbent resins via GC. One hundred microliters of the supernatant was transferred to 900 μl of dichloromethane 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 to 100% and compared with the amount of substrate after the adsorption.

2.5 | Preparative scale experiment

For the preparative scale experiment, 15 g of lyophilized whole E. coli cells containing the corresponding overproduced enzyme were rehydrated in 150 ml of a 100 mmol/L NaPi, pH 7.5 and stirred at 30°C. After 30 min, 2.13 ml of 2-MP and 15 g of D-glucose was added
successively to the cell suspension (150 mmol/L final concentration of the substrate and 500 mmol/L of \(\text{D}-\text{glucose}\)). The reaction mixture was stirred at 30°C for 24 hr. The conversion of the reaction was tracked after (0, 6, 17, and 24 hr) by removing 500 \(\mu\)l from the reaction broth and centrifuging it at 14,000 rpm for 5 min. One hundred microliters of the supernatant was then proceeded and analyzed via GC as mentioned above.

After reaching a maximum conversion, 30 g of Dowex Monosphere 650C cation-exchange resin (0.2 g/ml) was added to the reaction broth and stirred for 30 min at 30°C. A 500-\(\mu\)l sample was taken and analyzed as mentioned before to check the full adsorption onto the resin. The cation-exchange resin beads were sieved out of the reaction broth. A sieve with fine sieve openings was chosen so that the macroscopic exchange resins were easily retained and could thus be separated from the used microscopic cells. Afterward, the remaining resins were desorbed in 75 ml of 5% NaOH and 75 ml of cyclopentyl methyl ether (CPME) at 30°C for 30 min. To promote the phase separation, the reaction mixture was centrifuged for 5 min at 4,000 rpm. The ether phase was dried with \(\text{Na}_2\text{SO}_4\) for 2 hr. Afterward, the amine was oilied 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 nuclear magnetic resonance spectroscopy (see SI).

![FIGURE 2](Figure 2 Image)

**FIGURE 2** Conversion versus initial substrate concentration to determine the maximal substrate loading for the imine reductase-catalyzed synthesis. All experiments were carried out with 100 mg/ml whole cell catalyst, sodium phosphate buffer (pH 7.5), and 500 mmol/L of \(\text{D}-\text{glucose}\) in 500-\(\mu\)l scale experiments after 24 hr at 30°C. The dashed line is a guide to the eye.

### TABLE 1 Cation- and anion-exchange resins used in this study (see also Figure 5)

| Resin                  | Matrix            | Functional group | Total ion-exchange capacity \(q_{\text{ion}}\) (eq/L) | Moisture holding capacity (%) | Particle size                  | Uniformity coefficient | Particle diameter (μm) |
|-----------------------|-------------------|------------------|----------------------------------------------------|-------------------------------|---------------------------|-----------------------|-----------------------|
| Amberlite IR-120 (H)⁵ | PS-DVB (gel)      | Sulfonic acid    | ≥1.8                                              | 53–58                         | Uniformity coefficient: ≥1.8 Particle diameter: 620–830 μm <300 μm: 2% max |                       |                       |
| IRN-150 H/OH          | Stoichiometric equivalent mixture of Amberlite IRN77 H and Amberlite IRN78 OH (see below). |                   |                                                   |                               |                          |                       |                       |
| Amberlite IRN77 H⁵    | PS-DVB (gel)      | Sulfonic acid    | ≥1.9 (H⁺ form)                                   | 49–55                         | Uniformity coefficient: ≤1.2 Particle diameter: 650 ± 50 μm <300 μm: ≥0.2% <425 μm: ≤0.5% >1,180 μm: ≤2.0% |                       |                       |
| Amberlite IRN78 OH⁵   | PS-DVB (gel)      | TMBA             | ≥1.2 (OH⁻ form)                                  | 54–60                         | Uniformity coefficient: ≤1.1 Particle diameter: 630 ± 50 μm <300 μm: ≤0.2% <425 μm: ≤0.5% >1,180 μm: ≤2.0% |                       |                       |
| Dowex Marathon MSC (H)⁶ | PS-DVB (macroporous) | Sulfonic acid    | 1.6                                              | 50–56                         | Uniformity coefficient: max 1.1 Particle diameter: 575 ± 50 μm |                       |                       |
| Dowex Monosphere 650C (H)⁶ | PS-DVB (gel) | Sulfonic acid    | 2.0                                              | 46–51                         | Uniformity coefficient: max 1.1 Particle diameter: 650 ± 50 μm <300 μm: 0.5% >850 μm: 5.0% |                       |                       |
| Amberlite IRA67 freebase⁷ | Cross-linked acrylic gel structure | Tertiary amine   | 1.6                                              | 56–64                         | Uniformity coefficient: ≤1.80 Particle diameter: 500–750 μm <300 μm: 3.0% max |                       |                       |
| Amberlite IRA400 chloride form⁷ | PS-DVB (gel) | TMBA             | 1.4                                              | 40–47                         | Uniformity coefficient: ≤1.6 Particle diameter: 600–750 μm |                       |                       |

Note: The total exchange capacity of the resin in equivalents per liter (eq/L) is defined as the total number of counterions available for exchange per unit weight of resin.

Abbreviations: n.a. not available; PS-DVB, polystyrene/p-divinylbenzene; TMBA, trimethylbenzylammonium hydroxide.

⁵Cation-exchange resins.

⁷Anion-exchange resins.
3 | RESULTS AND DISCUSSION

3.1 | Optimization of the enzymatic IRED reaction

Initial experiments targeted an increase of the substrate concentration to facilitate higher productivities within the biocatalytic process using the highly enantioselective IRED from *P. elgii* B69. Considering the chosen conditions, the starting substrate concentration leads to a full conversion in the range of 25–150 mmol/L, but a deactivation of the catalyst was found at even higher substrate concentrations (Figure 2). This is solely caused by a substrate inhibition and not a pH-shift due to the cofactor regeneration-based formation of gluconic acid by

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**FIGURE 3** Adsorption after (a) 30 min and (b) 24 hr of the imine-substrate 2-methyl-1-pyrrolidine (gray) and of the amine-product (S)-2-methylpyrrolidine (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 either of the substrate or product together with 500 mmol/L d-glucose in a sodium phosphate buffer solution at pH 7.5
glucose dehydrogenase. Noteworthy, attempts using an excessive pH control with a 1 mol/L of phosphate buffer at pH 7.5 were also not successful due to a complete deactivation of the biocatalyst (data not shown). A full conversion was reached within 24 hr under the chosen reaction conditions. The final optimized reaction conditions facilitated full conversions at 30°C, a substrate concentration of 150 and 500 mmol/L of d-glucose. These experiments clearly indicate that further improvements of the catalytic robustness toward 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 IRED-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 48,000.⁵³

### 3.2 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 adsorption process, such ion exchange resins, including its respective productivity, and should always be investigated in detail. This is particularly relevant for an IRED-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 IRED-catalyzed reaction in the scientific literature. Most reports involve classical extraction and distillation steps, which require an additional effort during the product isolation from the reaction mixture.

For a suitable isolation, we screened different commercially available cation- and anion-exchange resins for their ability to remove the reactant’s product (S)-2-MPN and the possibly remaining substrate 2-MP efficiently from the reaction solution. In this specific case, efficiency is defined as an adsorption of more than 95% of the product and substrate from the reaction solution together with a short period of time (30 min). In order to cover a relatively wide range of commercially available ion exchangers, exchange resins with the most varied composition possible were selected (Table 1). We assume that not all resins have an efficient removal of the product and substrate from the reaction solution and that not only the actual ion exchange but also the nonspecific binding to the respective matrix of the adsorber resins plays a decisive role in the binding of the substances. Besides the classification into cation and anion exchangers, other properties of the resins are also important criteria for the description of the adsorption and swelling effects of this study (Table 1). All used cross-linked polymer resins are gel type resins except Dowex Marathon MSC (H) which is a macroporous type resin. Noteworthy, gel type resins consist of a homogeneous matrix with a usually low surface area when dry and when brought into contact with a solvent, for example, water, the resins can swell because of the elastic properties of the polymeric network. Consequently, “solvent pores” are formed inside the resin and molecules can access the resin easier, while macroporous type resins has permanent pores is relatively stiff and shows a less dominant swelling behavior compared to a gel type resin.⁵⁴-⁵⁶ Since this study primarily aims at the conceptional removal of the product and substrate from the reaction solution, volumetric swelling tests were not performed in this work.

For testing the efficiency of adsorption of the selected resins, test solutions containing 100 mmol/L of the product and the substrate together with 500 mmol/L of d-glucose in 100 mmol/L NaPi buffer at pH 7.5 were prepared and three different doses (0.02, 0.1, 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 after 30 min (Figure 3a) and 24 hr (Figure 3b). Values greater than 100% were the result of the swelling of the polymeric material and thus concentrating the residual solution, as mentioned above.

While comparing the overall data set presented in Figure 3 it is clear that cation-exchange resins 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). Anion-exchange resins on the other hand can only bind the substance via their hydrophobic polymer backbone, which still provides a residual, relatively weak reactant adsorption that slightly favors the product (S)-2-MPN from the IRED-catalyzed reaction. All cation-exchange resins used in this study are composed of a styrene/divinylbenzene-based matrix with sulfonic acid moieties (Figure 5).

Comparing the results after 30 min (Figure 3a) in more detail, the cation-exchange resins Amberlite IR-120(H) (entry 1), Dowex Monosphere 650C (entry 3), and Dowex Marathon MSC (entry 4) remove the reactants relatively similarly (> 95%) at a dose of 0.2 g/ml. The cation-exchange resin IRN-150 (entry 2) shows a somehow selective adsorption of the product and will be therefore discussed later (see below). In general, the resins in entry 1, 2, and 4 seem to adsorb the product better than the substrate, especially at lower doses of 0.1 and 0.02 g/ml. The same trend of the results can again be discussed regarding the adsorption after 24 hr, which seems to be the chemical equilibrium (Figure 3b).

In doses of 0.2 g/ml, 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 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 Monosphere 650C, which was used during the scale-up and the isolation of (S)-2-MPN. A remarkably high selectivity toward 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 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 mixed anion-cation-exchange resin within an IRED-catalyzed reaction were unsuccessful due to a full deactivation of the biocatalyst by the resin material (data not shown). The reason for this behavior is unknown and will be investigated thoroughly in a future study.

### 3.3 Scale-up and DSP 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 (Figure 6) and after 24 hr, 30 g of Dowex Monosphere 650C cation-exchange resin (0.2 g/ml) was added to the reaction broth and stirred for 30 min at 30°C. The adsorption of the product was checked and confirmed to be >95% (Figures S5 and S6).
Due to the separation of the used cells and the cation-exchange resin beads, a sieve with a relatively open pore size can be used to easily retain the exchange resins to separate it from the applied cells. The desorption was carried out using a mixture of 5% NaOH and CPME in a volumetric ratio of 1:1. This procedure has a very positive effect on the following work up in several ways: due to the presence of the base, the product is deprotonated and simultaneously extracted into the organic solvent in one single step. In addition, the hydrochloride salt can be subsequently precipitated from the organic layer since it is not soluble in this specific ether. The product amine hydrochloride salt oiled out very easily using gaseous HCl. After evaporating the residual CPME, 1 g of oil was obtained with a purity of >99% (Figures S7–S10). Any further purification steps, for example, by column chromatography or recrystallization, were not required highlighting the applicability of this method.

4 | SUMMARY AND CONCLUSION

In this contribution we presented the process development of the IRED-catalyzed synthesis of (S)-2-MPN with a special emphasis on an ion exchange-based DSP approach. The biocatalytic reaction was partly optimized resulting in a substrate concentration of 150 and 500 mmol/L of α-glucose at 30 °C for 24 hr. 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 DSP of a scaled up reaction to produce highly pure (>99%) (S)-2-MPN without any further purification steps. The developed process concept in this study, namely the integration of ion exchange resin materials into DSP is efficient and very simple to perform.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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