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Using Spinchem® RBR Technology for Immobilized Enzymatic Reactions: A Case Study

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KEYWORDS.

Biocatalysis, enzyme reuse, enzyme recycling, immobilized enzymes, rotating bed reactor.

ABSTRACT

The practically and financially viable use of immobilized enzymes in the production of pharmaceutical and bulk chemicals requires highly catalytically active enzymes and efficient mass transfer of substrate to the active site. The substantial cost of immobilized enzymes further mandates extended re-use of the enzymes to keep operating expenditures down. The extent of re-use of immobilized enzymes depends on parameters such as enzyme long-term stability under process conditions, carrier integrity and attrition due to mechanical stress, ease of work-up and recovery on scale.

Herein, we report on the use of SpinChem® rotating bed reactor (RBR) for the Novozyme 435 mediated kinetic resolution of (*cis*)-isopropyl 3-aminocyclohexanecarboxylate as an alternative to traditional reaction using free flowing immobilized lipases in an ordinary batch reactor. Reusability was studied in addition to other parameters such as type of immobilized enzyme, loading, rate of agitation, temperature and scalability. By using SpiChem® RBR technology we found that recycling of the immobilized enzyme was easy with preserved enantioselectivity and catalytic activity. The final optimized process was successfully demonstrated on a 1kg scale with 39% isolated yield and 98.8% enantiomeric purity.

INTRODUCTION

Enzymes are widely used as biocatalysts in the pharmaceuticals,[1] fine chemicals,[2] flavor and fragrances,[3] vitamins,[4] food and textile industries.[5] The use of biocatalysts has become popular in the last few decades due to its environmentally friendliness, safety, excellent chemo, regio, and stereoselectivity.[6] Despite of their excellent catalytic capabilities, industrial applications of enzymes faces severe challenges in many cases. Some issues dealing with enzymes on an industrial scale are long-term operational stability, activity, inhibition by reaction products, selectivity towards chemical reactions, recovery, reusability, ease of workup and cost.

To overcome these issues various techniques have been developed such as immobilization,[7] new biocatalyst design by protein engineering, reaction engineering, reactor engineering etc.[8] For example, recent immobilization techniques[9] of enzymes onto the solid support such as adsorption on different carrier types,[10] covalent binding, affinity, and entrapment greatly enhanced process control, stability, storage, operational conditions and greatly simplifies the recovery process leading to reduced operational costs.

For the large-scale commercial and profitable utilization of immobilized enzymes, it is not only essential to keep the immobilized enzyme activity and stability, but depending on the cost of the enzyme,[11] it should also be possible to recover and reuse the enzyme. Tailor-made expensive immobilized enzymes are still challenging and are typically constituting a significant operational cost for industrial processes. Therefore, they must be recovered and reused to ensure economic feasibility of the process without compromising the quality of the product.

Recent advances in new immobilization techniques, together with improved reaction conditions and reactor engineering have played an essential role in keeping the stability of the enzymes, facilitating their re-use on an industrial scale. For example, reactor engineering modifications on stirred tank batch reactor (STR) such as continuous flow stirred tank reactor (CSTR), membrane slurry reactor (MSR), continuous flow membrane reactor (CMR), packed bed reactor (PBR), plug flow reactor (PFR), fluidized bed reactor (FBR) and integrated product removal are used as technologies to improve the performance of enzyme use and downstream processing.

Even though STRs are widely used on industrial scale, a problem that is often encountered in the use of STRs is mechanical attrition of the immobilized enzyme caused by the impeller stirrer agitation. This mechanical grinding effect can lead to attrition from their support, forming pulverized particles which are difficult to remove and leads to loss of enzyme when recycling the beads[12]. In that event, technical complexity might arise and affect reproducibility of the reaction on an industrial scale. To avoid mechanical stress, MSR,[13] PBR, bubble column reactor (BCR)[14] or FBR reactors which are minor modifications to existing STR reactors, can be applied. Using flow chemistry is another alternative[15] or the use of a “tea bag” construction of immobilized enzyme wrapped in chemically inert first aide gauze demonstrated by Houghten[16] and also recently reported by Turner et al[17] for enantioselective hydrolysis of an ester in biphasic media.

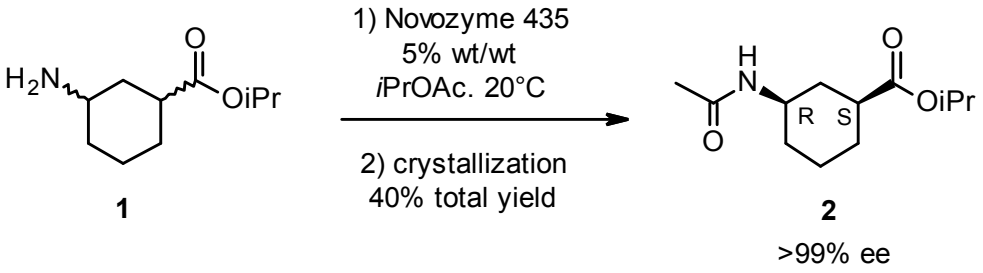
Another recent variation of which combines the advantages of FBR and STR reactors is the rotating bed reactor (RBR or spinning basket reactor) consisting of a catalyst containing compartment attached to a stirrer shaft, which was developed by SpinChem®(Figure 1).[18] Its four-compartment rotating flow cell filled with enzymes ensure enzyme carrier stability by protecting it from shear forces and grinding and also result in efficient recycling opportunities. The reactants are repeatedly passed through the immobilized enzyme due to centrifugal forces, and packed bed allows for efficient mass transfer. Recycling of enzyme can simply be performed by a drain out-fill in principle and no filtration is necessary.

Figure 1: SpinChem rotating bed reactor for ca 70 mL immobilized enzyme.

For example, ease of convenience for the reuse of enzyme by the application of rotating bed reactor technology was demonstrated by Bornscheuer and coworkers in a reaction catalyzed by an immobilized transaminase.[19] Other successful examples of the use of SpinChem technology have been reported such as a lipase catalyzed intramolecular amination reaction[20] and an invertase enzymatic reaction.[21]

Following these successful examples, we wished to compare and validate here the use of a rotating bed reactor in our process development and manufacture of pharmaceutical building blocks and active pharmaceutical ingredients. As a model reaction for this study, we chose the recently published Novozyme 435 mediated acetylation of (*cis*)-isopropyl 3-aminocyclohexanecarboxylate

1 (Scheme 1) which was reported to give the amide **2** in both a high yield and enantioselectivity in batch mode.[22]



Scheme 1: Novozyme 435 mediated acetylation of (*cis*)-isopropyl 3-aminocyclohexanecarboxylate **1**

Using rotating bed reactor technology for this reaction instead, we wished to investigate the effect of rate of agitation and temperature as well as ease of recycling of the enzyme. Next, using optimal conditions, we also wished to evaluate the scalability of the reaction.

RESULTS AND DISCUSSION

Below is presented the results of our study of the following reaction parameters: type of enzyme, loading, temperature, and rate of agitation. Following optimization of the conditions we investigated the reusability of immobilized enzyme and reaction scalability. All reactions were monitored by inline FT-IR and off-line ^{1}H-NMR and reactions were stopped at about 50% conversion.

Screening of enzymes: Initially, we first screened various immobilized lipases for the acetylation of **1** with respect to conversion rate and enantiomeric selectivity. A pre-selection was done with respect to the rate of reaction by screening 20 different immobilized enzymes for the acetylation of **1** on 100 mg scale. Additionally, vinyl acetate and ethyl acetate as acetate donors as well as

heptane and ethyl acetate as alternative solvents were added to the screening matrix. Previously identified isopropyl acetate as combined acetyl donor and solvent remained the better choice. The six immobilized enzymes giving the highest rate of reaction were selected for further study. For this purpose, pre-packed cartridges with 1.5 g each of the six top-ranking immobilized enzymes were used to catalyze the reaction of **1** with *i*PrOAc on 15 g scale. In addition to Novozyme 435, ChiralVision Immozyme IMMCALB-T2-150XL, Purolite Lifetech CalB Immo 5572, 5872, 8285, and CalB ImmoPlus were studied using 10% wt/wt loading at 20 °C and iPrOAc as the solvent. As shown in Table 1, all of these enzymes resulted in a similar ee but differed in the reaction time to reach 45-50% conversion. Novozyme 435, CalB Immo 8285, CalB ImmoPlus, and IMMCALB T2-150XL gave the desired ~50% conversion after 6 h but the lipases CalB Immo 5587 and 5872 gave slower rate of reaction. Given similar performances for the first four mentioned immobilized lipases, Novozyme 435 was chosen due to wide availability and familiarity with previous studies.[22]

Table 1: Screening of lipases for acetylation of **1**

Entry	Conversion[a] (%)	Time in hours	optical purity [%ee]
Novozyme 435	51	6	90
Purolite Lifetech CalB Immo **8285**	50	6	92
Purolite Lifetech CalB Immo **5587**	50	24	89
Purolite Lifetech CalB Immo**Plus**	49	6	90
ChiralVision **Immozy**me IMMCALB-**T**2-**150**XL	52	6	92
Purolite Lifetech CalB Immo **5872**	50	24	90

[a]Reactions were performed in SpinChem RBR S2 in iPrOAc with 10% wt/wt loading of enzyme at 20 °C and 500 rpm.

Loading of Novozyme 435:

The effect of loading of Novozyme 435 was studied to find optimal utilization of the enzyme. The time required was decreased with increasing enzyme loadings from 5% to 10% wt/wt but no significant rate increase was observed at higher loadings (Table 2). To minimize the production cost, we chose to use 10% loading which was sufficient to achieve 45-50% conversion within 6 h.

Table 2: Effect of Novozyme 435 loading (wt/wt) on rate of acetylation of **1** and ee. Reactions at 20 °C, 500 rpm.

Entry	Loading (wt %)	Conversion (%)	Time in hours	optical purity [%ee]
1	5	50	24	85
2	10	51	6	90
3	20	50	6	87

[a]Reactions were performed in a SpinChem RBR S2.

Effect of rate of agitation:

By increasing agitation speed from 100 rpm to 500 and 750 with 10% loading of the enzyme, reaction time is decreased significantly from 11 to 6 hours but similar ee is observed (90% ee). By increasing rate of agitation further to 1000 rpm no improvements were observed. Due to technical difficulties with higher vortexing and shaking, 500 rpm was selected for further studies.

Effect of Temperature:

The reaction temperature in general significantly influences both the rate of reaction and the stability of the enzyme. On one hand, an increase in temperature normally lead to an increase in

reaction rate but on the other hand, the higher temperature may denature the protein, leading to rapid enzyme inactivation and a decreased enantioselectivity. With the aim to increase the rate of acetylation reaction of **1** and to facilitate subsequent recycling experiments, the role of temperature was explored (Figure 2). We found that the time to reach around 45-50% conversion was significantly reduced with increasing reaction temperature without effecting the enantioselectivity. We selected a temperature of 50 °C as the optimal temperature for further recycling experiments as a good compromise between long-term enzyme stability and reaction rate.

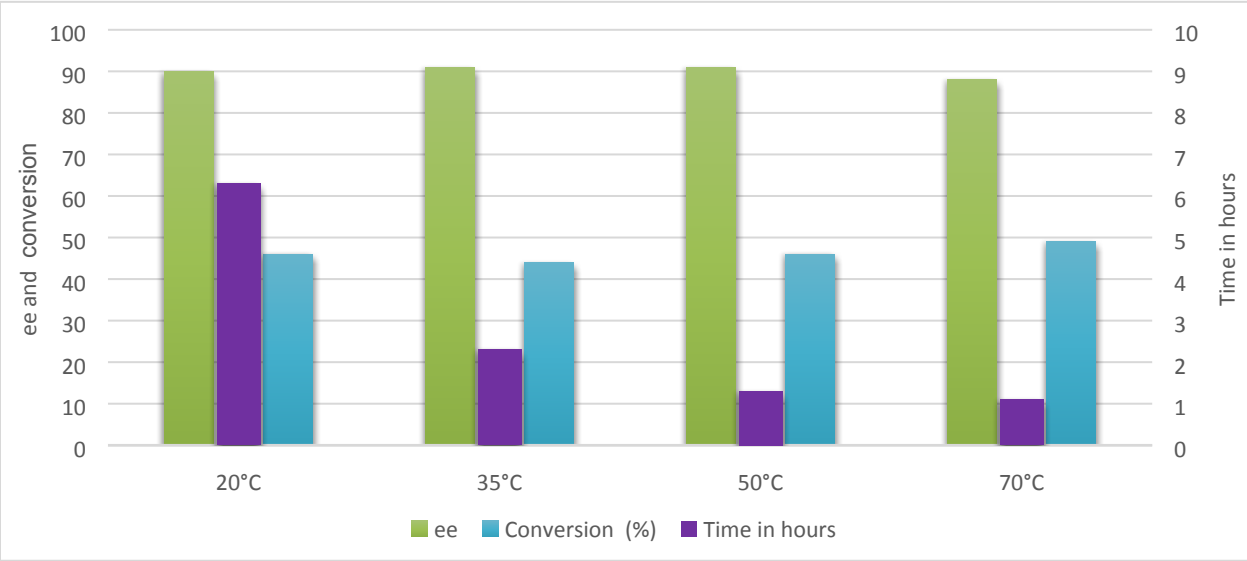


Figure 2: Effect of temperature on the rate of acetylation of **1** and enantiomeric purity. Reactions were performed in a SpinChem RBR S2.

Observation:

In our initial attempts using the rotating bed reactor technology for the reaction depicted in Scheme 1, a minor amount of white precipitate was observed which remained with the enzyme beads after the reaction was complete (Figure 3a). Even though it was not a significant amount it coated the surface of the enzyme beads and caused blockage of the flow resulting in a rate decrease of reactions. After analysis by ^{1}H-NMR and ^{13}C-NMR the precipitated salt was identified as the

acetate salt of the amine **1**. The formation of the acetic acid was confirmed as originating from a hydrolysis of the *i*PrOAc solvent mediated by the enzyme. To avoid the formation of the acetic acid, anhydrous solvent (< 200 ppm water) was used instead. Although this measure did significantly reduce the amount of acetate salt formed, traces of insoluble carbonate salt of **1** was also formed as a result of exposure of starting amine to air. To avoid the formation of this salt, the system was rigorously degassed three times with nitrogen before substrate addition. With these measures taken, we did not observe any problem with salt formation as shown in Figure 3b.

Figure 3: Formation of acetate salts due to hydrolysis of isopropyl acetate.

a) Using non-anhydrous solvent in RBR

b) With anhydrous solvent in RBR

Recycling experiments:

With the optimized reaction conditions in hand, the reusability of the immobilized enzyme was evaluated through recycling experiments. The reactions were monitored continuously by in-line FT-IR (Figure 5). Thus, with a reaction temperature of 50 °C, a loading of Novozyme 435 of 10% and rate of agitation of 500 rpm, 10 cycles were performed with the same enzyme. After each cycle, the reaction mixture was simply drained out and the rotating bed reactor was primed with *i*PrOAc prior to the next cycle experiment. We found that the immobilized enzyme was surprisingly stable under these conditions and we could run 10 consecutive recycling experiments

with preserved rate of reaction and enantioselectivity of the enzyme (~ 90% ee, Figure 4 & 5). It is worth to mention that even after 10 cycles the Novozyme 435 was still highly catalytically active and gave as good enantioselectivity as that obtained after first cycle.

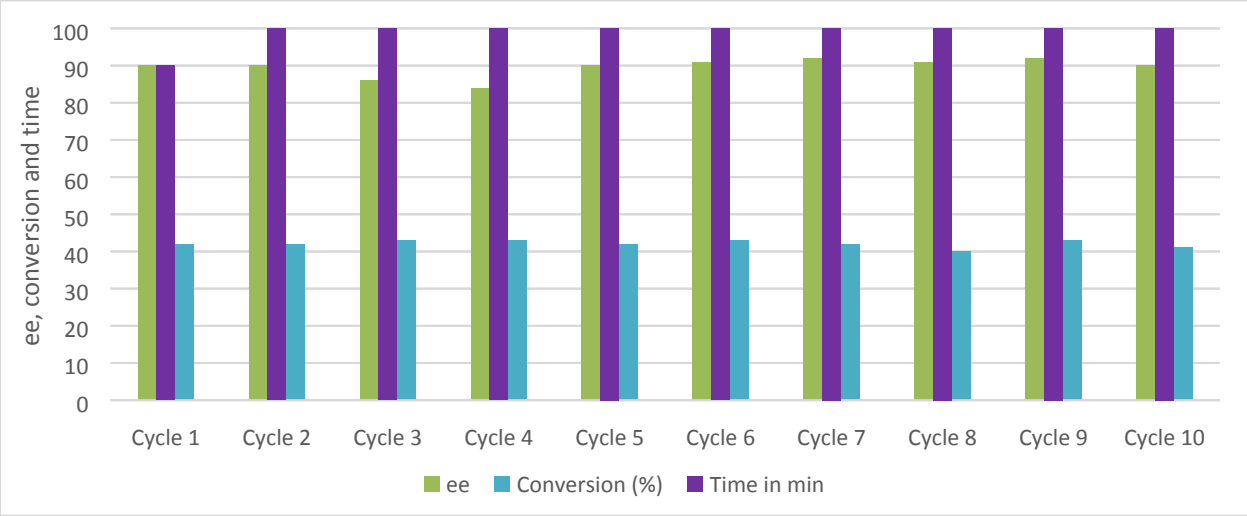


Figure 4: Recycling experiments using Novozyme 435 on the rate of acetylation of **1** and ee over 10 cycles. The reaction was conducted in SpinChem RBR S2 as described in the Experimental Section.

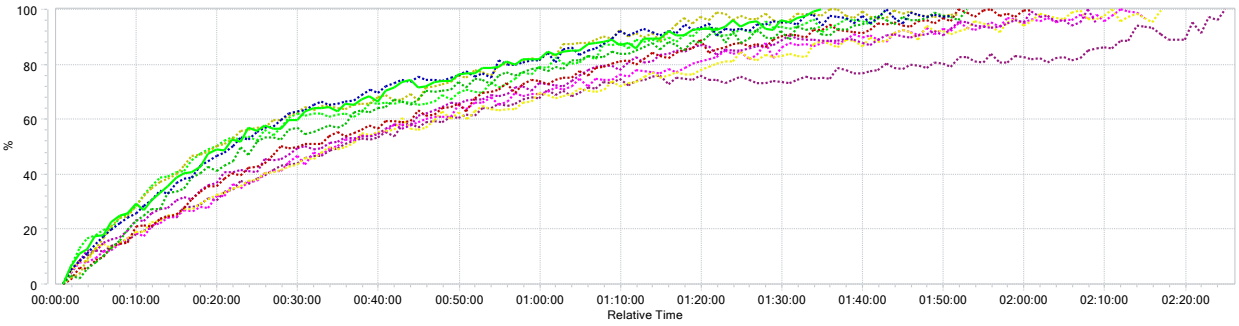


Figure 5: FT-IR monitoring (1680 cm^{-1}) of the formation of **2** as a function of time and overlay of recycling experiments 1-10 (dissolved in 10 relative volumes of isopropyl acetate). 10% wt/wt of Novozyme 435 was used and at 50 °C. Absolute time on X-axis is given in hours and peak height on Y-axis is given as relative absorption %.

Scalability: Using the optimized conditions, we wished to evaluate the scalability of the reaction. Thus, starting from a small-scale experiment using the 150 mL SpinChem RBR reactor, the reaction was further scaled-up to 1 L and 10 L (Figure 6). Satisfyingly, highly consistent reaction rates and enantioselectivity was observed at all three scales (Figure 7).

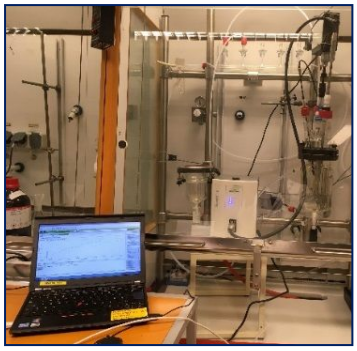


Figure 6: (Left) Reactor setup with in-line FT-IR monitoring and (Right) Reactor set-up for 10 L.

On a 1000g scale (=10L), the reaction was successfully scaled up with an enzyme loading of 10% wt/wt and with a substrate loading of 100g/L. The reaction reached 46% conversion within 90 min at 50 °C and after an aqueous acidic workup and crystallization the desired compound was obtained with a 39% overall yield with 98.8 % ee.

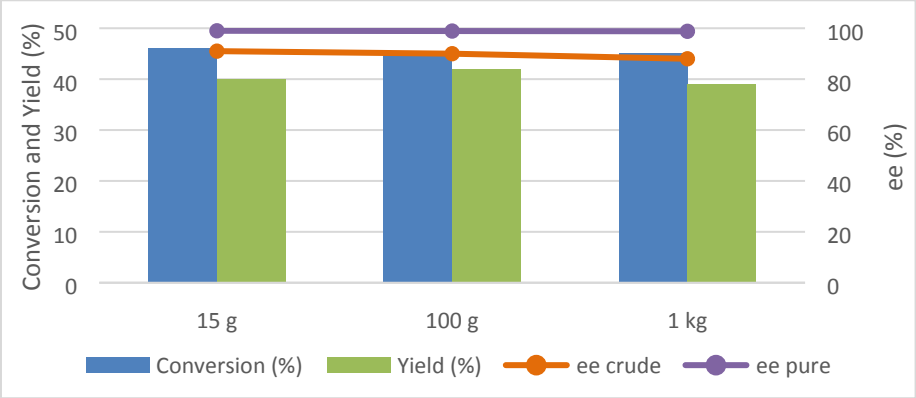


Figure 7. Reaction scaling behavior at 15, 100, and 1000 g substrate: Blue bars show conversion and green bars show isolated yield after work-up and crystallization (left axis). Orange line shows

crude enantiomeric purity while the purple bar shows enantiomeric purity after crystallization (right axis). All reaction scales were run for 90 minutes under equivalent conditions such as concentrations, temperature and enzyme loading.

CONCLUSION

The feasibility of the Novozyme 435 mediated acetylation of **1** using SpinChem RBR technology was demonstrated. Moreover, this technology is more convenient for process chemistry due to reusability of the same enzyme for up to 10 cycles with minimal loss of catalytic activity. Also, we have shown that this technology is scalable from 100 ml to 10 L scale and give similar good results. Using this technique multiple recycling experiments can be performed with ease of operations and preserved enantioselectivity and rate of reaction.

Materials and methods

General. All the materials and solvents were purchased from commercial suppliers and used as-is without further purifications. Novozyme 435 was purchased from Novozymes A/S Denmark (activity 9000 PLU/g). Spinchem RBR S2 (up to 28 mL resin volume), RBR S3 (up to 69 mL resin volume), RBR S4 (up to 0.8 L resin volume, 3D printed custom made) reactors, Lifetech CalB Immo 5587, 5872, 8285, and CalB ImmoPlus, and Immozyme IMMCALB-T2-150XL immobilized lipase resins were produced by Purolite Life Sciences and ChiralVision B.V. respectively and provided in cartridges by SpinChem AB. All the reactions were performed under nitrogen. Optimization study was performed on 150 mL scale using SpinChem RBR S2 reactor equipped with an overhead stirrer. Scale up reactions were performed on 1000 ml scale using a SpinChem RBR S3, and on 10L scale using a SpinChem RBR S4 (3D printed custom made) reactor. All conversions were recorded using ^{1}H NMR analysis of the crude reaction mixtures.

Enantiomeric purity of compound **2** was determined using chiral HPLC [Lux C2 column (4.6 × 150 mm), 15% 2-propanol in CO_2 120 bar as eluent]. NMR measurements were performed using a Bruker Avance III spectrometer. FT-IR measurements were performed using a Mettler Toledo React IR 15 instrument integrated with a DiComp (diamond) FT-IR probe.

EXPERIMENTAL SECTION

(*1S,3R*)-Isopropyl 3-Acetamidocyclohexanecarboxylate 2 (Recycling experiments 15g scale)

Novozym 435 (Lipase B of Candida antarctica immobilized on a macroporous acrylic resin, (activity 9000 PLU/g) was purchased from Novozymes. Novozyme 435 cartridges (containing a total of 1.5 g of enzyme) was assembled to a Spinchem RBR S2 equipped with temperature and FTIR probes. The catalyst chamber was flushed with nitrogen and anhydrous *i*PrOAc (150 ml) was charged at room temperature. The reactor system was degassed and back filled with N_2 three times then heated to 50°C before addition of (*cis*)-isopropyl 3-aminocyclohexanecarboxylate (**1**) (15 g, 77.73 mmol) via syringe under nitrogen atmosphere. The reaction mixture was stirred at 50 °C with 500 rpm and the progress of the reaction was monitored by ^{1}H NMR and inline FT-IR. After reaching the desired conversion, the reaction mixture was simply drained out from the reactor with vigorous stirring and the Novozyme 435 enzyme chamber was rinsed with anhydrous *i*PrOAc (50 ml). This reaction was repeated another 9 cycles using the same procedure as described above but using the re-used Novozyme 435.

Sampling for ee analysis: 2ml of reaction mixture from each cycle was collected, the organic layer was extracted first with aqueous HCl (2M, 2 ml) and then aqueous $NaHCO_3$ (saturated, 2 ml). The resulting organic layer was concentrated and analyzed by chiral HPLC.

(*1S,3R*)-Isopropyl 3-Acetamidocyclohexanecarboxylate 2 (1kg scale)

Novozyme 435 (100 g) was charged to a 10 L SpinChem RBR under nitrogen. The reactor was flushed with nitrogen, anhydrous iPrOAc (10 L) was charged and degassed with nitrogen three times with vigorous stirring. Then the reactor was heated to 50 °C before addition of (*cis*)-isopropyl 3-aminocyclohexanecarboxylate (1000 g, 5.18 mol) via addition funnel under nitrogen atmosphere. After stirring at 50 °C for 60 minutes, the reaction mixture was drained out and the enzyme compartment was rinsed with isopropyl acetate (4L). The combined organic layer was washed with oxalic acid solution (140 g, 0.30 eq, 1.55 mol) in water (4 L). The aqueous acidic layer was extracted with isopropyl acetate (3 × 1L), and the combined organic layer was washed with aqueous Na_2CO_3 solution (2L, saturated). The organic layer was then washed with brine (2L) followed by polish filtration (cartridge Demicap Proclear PP, 10.0 μm) and concentration to give the crude title compound as a white solid (530 g, 98.7% w/w, 2.33mol, 45% corrected yield) with 88% ee. To the above crude compound was added TBME (3L) and heptane (7L). The resulting slurry was heated to 70-80 °C and stirred until fully dissolved. The clear solution was allowed to crystallize by allowing the solution to cool to room temperature slowly (5 °C /h) with continuous stirring overnight. The resulting slurry was then filtered, and the crystalline white solid was washed with cold TBME/heptane mixture (3:7 mixture, 200 mL). After drying under reduced pressure at 40 °C, the title compound **2** was obtained as white solid (460 g, 99% w/w, 2.02 mol, 39.1% yield), ee >98.8%. Analytical data of **2** were in accordance with those given in reference 21.

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Conflict of interest statement: C. T. Öberg is an employee of SpinChem AB.

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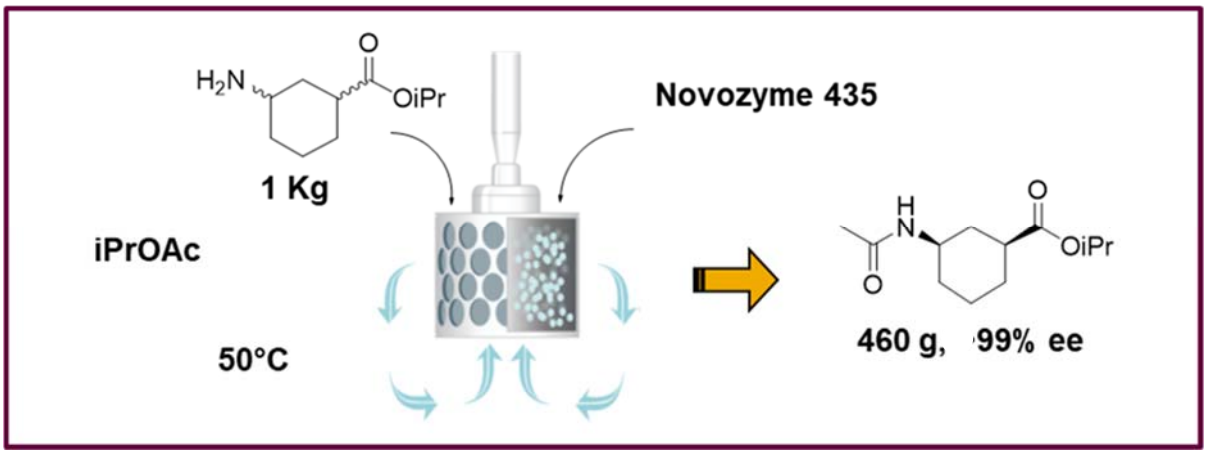
H2N
O
OiPr
1 Kg
iPrOAc
50°C
Novozyme 435
H
N
O
O
OiPr
460 g, 99% ee