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COMMUNICATION

Cascade biotransformation to access 3-methylpiperidine in whole cells

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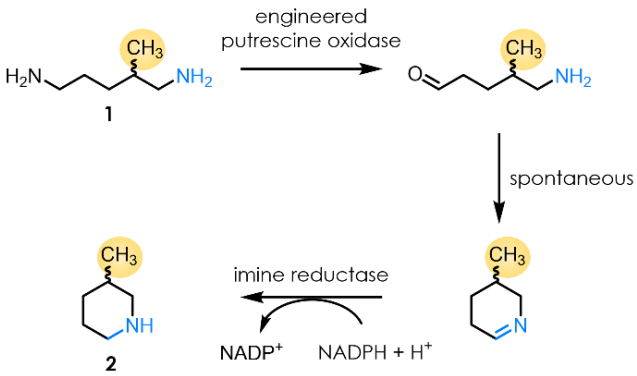
Abstract: Synthesis of 3-methylpiperidine from 1,5-diamino-2-methylpentane in preparative scale is reported by using recombinant *Escherichia coli* cells expressing a variant of the diamine oxidase from *Rhodococcus erythroprolis* and an imine reductase from *Streptosporangium roseum.* Optimization of process parameters for cultivation and bioconversion led to substantial improvements in the initial laboratory procedure. The transformation of the methyl-substituted diamine substrate to the *N*-heterocyclic product was successfully scaled-up from shake-flask to a 20 L bioreactor with increased substrate concentrations. Remarkably, we obtained 67% of 3-methylpiperidine product from 140 g substrate within 52 h.

The development of cascade reactions for industrially relevant compound classes has recently gained increasing interest since intermediate purifications and isolations can be avoided.[1–5] Biocatalytic reactions are generally performed under mild reaction conditions, like ambient temperature and physiological pH. For that reason, enzymes are usually compatible with each other, opening the possibility of combining several reactions in the same reaction vessel. In the last years, enzymatic cascades have reached a remarkable level of complexity allowing the one-pot preparation of valuable organic molecules. Nitrogen-containing heterocycles are privileged structures that are ubiquitously found in nature and in various drug candidates.[6] Consequently, the development of synthetic methods for the preparation of these compounds constitutes an area of current interest.[7] Piperidines, in particular, are among the most common heterocyclic building blocks in approved pharmaceuticals.[8] Conventionally such compounds have been prepared by reduction of the corresponding pyridines[9], dinitriles[10], isoxazolinones[11] and heteroaromatic *N*-oxides[12], alkylation and reduction of bicyclic lactams, hydroaminations[13,14], ring-closing metathesis[15] or cyclization of linear diamines.[16] Biocatalytic cascade reactions have begun to provide alternative strategies for the synthesis of substituted pyrrolidines and piperidines from diketones, ketoaldehydes, diamines, and ketoacids by combining transaminases[17–24], amine oxidases[25–28], carboxylic acid reductases[29,30] with imine reductases[31–38]. The diastereoselective synthesis of disubstituted pyrrolidines and piperidines has also been reported with transaminases and chemical reducing agents.[39–41] Although several methods are presented for the asymmetric synthesis of 2- as well as 2,6-disubstituted piperidines, the synthesis of 3-substituted has been subject to little scientific research. 3-Methylpiperidine is an intermediate in the industrial production of nicotinic amide and nicotinic acid, which is an essential vitamin (vitamin B_3).[42] In this regard, the synthesis of 3-substituted piperidines is of considerable importance.

Recently, we reported an enzymatic cascade for the preparation of *N*-heterocyclic pyrrolidines and piperidines from the corresponding diamine substrate. Substituted pyrrolidines and piperidines were obtained with up to 97% product formation in a one-pot reaction directly from the corresponding diamine substrates.[43] Our approach relies on the application of the engineered putrescine oxidase (PuO) from *Rhodococcus erythropolis* for the oxidation of diamines to the amino aldehyde, which undergoes spontaneous cyclization to the corresponding imine. Subsequent reduction mediated by the imine reductase from *Streptosporangium roseum* (IRED_*Sr*) provides access to enantioenriched *N*-heterocycles (Scheme 1).



Scheme 1. Enzyme cascade for the synthesis of 3-methylpiperidine **2**

In continuation with our recent studies on the synthesis of -heterocycles, we became interested in optimization of our enzymatic cascade for the production of 3-methylpiperidine **2** as

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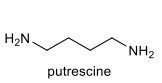
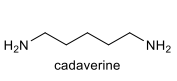
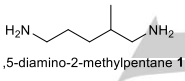
Supporting information for this article is given via a link at the end of the document.

well as transferring this cascade to an *Escherichia coli* (*E. coli*) whole cell system with facilitated up-scaling.

We began by examining the combination of IRED_*Sr* and triple variant $PuOM_1$ (L200I/E203S/I206L). In our previous study, the directed evolution of PuO has been conducted. From the random mutant library created by error-prone PCR, we obtained a single variant E203G, which exhibited increased oxidation activity of non-natural diamines compared with the PuO wild-type.[43] This result suggests that glutamic acid at position 203 may play an important role in terms of substrate specificity and prompted us to consider whether PuO activity in the oxidation of 1,5-diamino-2-methylpentane **1** can be further improved by semi-rational mutagenesis. Amino acid residues located on a loop adjacent to residue E203 were modified to improve the substrate acceptance. Four variants $PuOM_1$ – $PuOM_4$ were constructed with the variant $PuOM_1$ indeed being more active than variant E203G in the oxidation of C5 diamine substrates cadaverine and **1** (Table 1 and Table S1 in the Supporting Information).Using $PuOM_1$ a 2-fold increased activity for the oxidation of **1** (Table 1) and a 3-fold increased k_{cat} (Table S2, Figures S1 and S2) compared to the PuO wild-type was observed.

Table 1. Comparison of activity of putrescine oxidase wild-type (WT) with single variant E203G and triple variant $PuOM_1$[a]

	 putrescine	 cadaverine	 1,5-diamino-2-methylpentane **1**
	TON [min^{-1}]		
WT	1128 ± 47	98 ± 9	47 ± 8
E203G	940 ± 42	105 ± 2	43 ± 1
$PuOM_1$	509 ± 8	121 ± 11	82 ± 3

[a] Formation of oxidized 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) was followed spectrophotometrically for 15 min at 405 nm wavelength.

The cascade coupling of IRED_*Sr* and $PuOM_1$ was used to transform **1** to 3-methylpiperidine (**2)** in one-pot (Scheme 1). At 10 mM substrate concentration, **1** was converted with excellent product formation of 90.4 ± 4.7 % within 5 h. Buoyed by the compatibility of IRED_*Sr* and $PuOM_1$, we intended to transfer the *in vitro* enzymatic cascade into whole cells to achieve product formation in multi-gram scale. The bioconversion system which involves a three-reaction, two-enzyme, one-pot process was established by using recombinant *E. coli* expressing genes of engineered $PuOM_1$ and imine reductase. The optimization of fermentation and bioconversion of the cascade was investigated to achieve considerable product formation. The design and construction of the cascade started with evaluating suitable gene expression systems to produce the chosen biocatalysts. For the generation of $PuOM_1$ and IRED protein production in the arabinose-deficient strain *E. coli* JW5510, two different approaches were applied: (i) the combination of two compatible plasmids pBAD18_$PuOM_1$ and pBAD33_IRED and (ii) the generation of a single plasmid pBAD18_$PuOM_1$_IRED (Figures S3-S6). An alternative approach is the mixing of whole cells producing $PuOM_1$ with whole cells producing IRED.

SDS-PAGE analysis confirmed the synthesis of both enzymes. It further suggested that the whole cells with the single plasmid showed reduced production level of imine reductase compared to the cells with two compatible plasmids (Figure S4). The mixing of cells producing either $PuOM_1$ or IRED displayed the lowest activity, even if the amount of cells was varied at different ratios (data not shown). Methylpiperidine formation of the recombinant whole cells was further evaluated and optimized. Also, to assure efficient production of **2** and to abolish the need for exogenous NADPH cofactor supplementation, the cascade was performed at different buffer and glucose concentrations. Highest product formation was obtained within 48 h with 10 mM glucose in a 50 mM Tris-HCl buffer pH 7.5 (82.5%, Figure 1, shake flask). This result corresponds with recent observations on the formation of *N*-heterocycles combining a 6-hydroxy-D-nicotine oxidase with an IRED, which revealed that glucose concentration could have a significant impact on the product formation.[25] When more than 10 mM glucose has been applied the formation of **2** was decreased.

Table 2. Design of Experiment (DoE) for evaluating the influence of main process parameters during bioconversion.

Batch process #	pH	T [°C]	pO_2 [%]	Substrate [mM]	Product formation [%][a]	
					6 h	48 h
1	7.2	20	10	15	12	64
2	7.2	30	50	15	23	82
3	**7.8**	**30**	**50**	**15**	**67**	**98**
4	**7.8**	**30**	**10**	**15**	**64**	**89**
5	7.8	20	50	15	37	93
6	7.2	30	50	35	21	70
7	7.2	30	10	35	21	67
8	7.2	20	50	35	11	49
9	7.8	20	50	35	15	66
10	**7.8**	**30**	**10**	**35**	**40**	**78**
11	7.8	20	10	35	21	57

[a] A significant part of the positive effect on final product formation is the increase of the initial substrate concentration, pH and temperature.

To demonstrate the synthetic applicability of the one-pot cascade, the protein production and conversion of **1** to **2** was scaled-up to a 20 L bioreactor (Figure 1). Initial fermentation and bioconversion studies using 10 mM **1** illustrated that the activities declined for the scaled-up process with product formations of less than 30% (Figure S19, red bars). As a consequence, the process parameters of the individual process phases such as biomass production, enzyme production and bioconversion (Table S3 and

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Figure S20) have been investigated to optimize the biotransformation. Specifically, the cultivation medium has been optimized to allow significantly higher cell mass as well as key process parameters concerning both protein production and reaction conditions. The highest conversion of **1** has been obtained applying the following reaction conditions: 14 h protein production at 30°C after induction with 0.04% (*w/v*) arabinose at OD_{600} 10-15 in mineral salt medium supplemented with casamino acids and glycerol (high cell density medium), followed by whole cell bioconversion (48 h, 30°C) adding 15 mM substrate **1** and 10 mM glucose directly to the fermentation broth (see also Figures 1 as well as 2, and Figure S19, highlighted in purple).

To gain further insights into the process parameters affecting the extent of **2** productions, several process parameters, including temperature, pH, dissolved oxygen concentration and initial substrate concentration, were varied applying Design of Experiment (DoE) (Table 2, for more details see SI Tables S4-S5 and Figures S8-S13).The effects of these modifications on the whole cell bioconversion were investigated after 6 h, 24 h and 48 h reaction time. Parallel runs with the same process conditions were performed prior to DoE in 20 L and 7 L fermenters to investigate whether the scale of the bioreactor used for the bioconversion could affect the performance of production (data not shown).

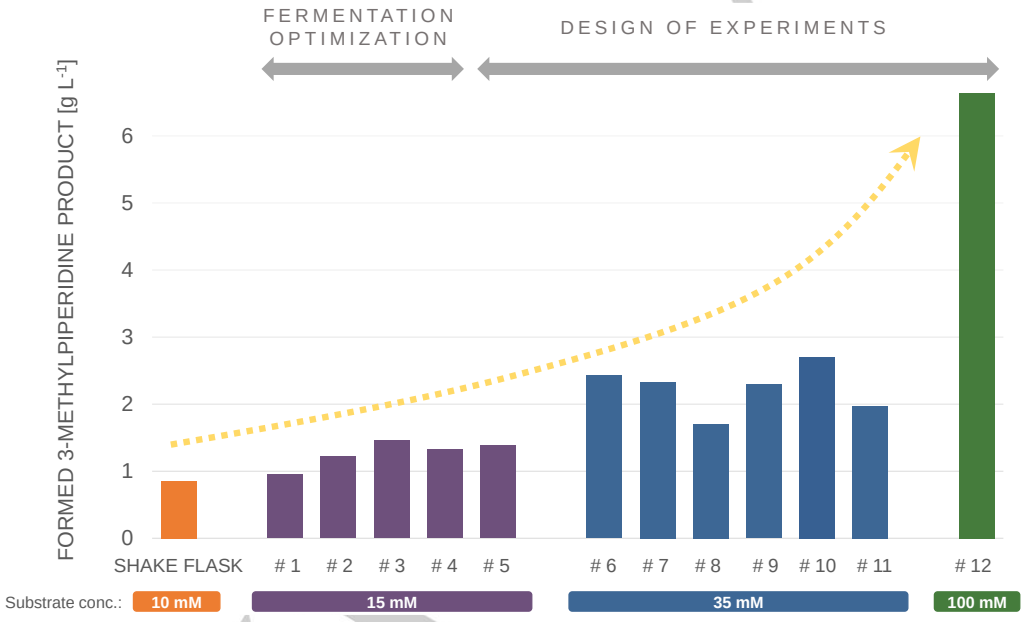

Figure 2. Evolution of process performance in the preparative-scale synthesis of **2.** Shake flask experiments of the substrate (shown in orange) were optimized through improvement of the fermentation process and use of Design of Experiments (DoE). In the course of process optimization, substrate concentrations (substrate conc.) were gradually increased from 10 mM to 100 mM. Detected product concentrations after 48 h reaction time are shown. Product formation using 100 mM of the substrate was performed for 52 h.

No differences were observed between the two applied bioconversion scales allowing the use of both 7 L and 20 L bioreactors. To demonstrate the potential for industrialization of the developed process, various 20 L scale operations were conducted using 15 mM as well as 35 mM of **1** to generate **2** (see SI Figures S14-S17). For the major part of the experiments performed with an initial substrate concentration of 15 mM, no residual substrate could be detected at 48 h bioconversion.

We observed that after 24 h reaction, the maximum titer was obtained and all substrate was converted. Batch 3 highlights the excellent conversion of **1** with 98% of formed product **2** (Table 1 and Figure 2). We thus assume that the combination of higher pH (from pH 7.2 to 7.8), as well as temperature (from 20°C to 30°C), enhanced the overall enzymatic cascade. On the basis of these promising results for the conversion of 15 mM, we investigated the whole cell cascade by using 35 mM of **1**. Highest product formation was observed in batch 8 with 78% of formed product within 48 h applying an initial substrate concentration of 35mM (Table 2 and Figure 1). The statistical analysis of DoE data further revealed that the increase of pH and temperature to 7.8 and 30°C, respectively, had a positive impact on the bioconversion reaction. Moreover, within these conditions highest product titer was observed through the increase of initial substrate concentration from 15 mM to 35 mM, which is accompanied by a lower conversion rate at high substrate concentration. Finally, optimized reaction conditions were used in the conversion of 100 mM **1** (140 g, batch 12 in Figure 1, Table S5, Figure S18) combined with additional glucose intake to improve NADPH recycling. Substrate **1** along with 100 mM glucose were directly added to the fermentation medium. The bioconversion was performed at pH 7.8 and 30°C with a cell density of 8 g L^{-1} (cell dry weight). Substrate and product concentrations were measured after 52 h, resulting in 66.7 mM **2** (see also Figure S18). Batch #12 was further applied to study the downstream processing including solid-liquid separation by centrifugation or membrane separation, distillation and liquid-liquid extraction to transfer and enrich 3-methylpiperidine **2** in organic solvent. Due to the fact that solid-liquid separation and distillation resulted in unsatisfactory product recovery, we focused our efforts on liquid-liquid extraction. The following candidate solvents were selected for extraction of **2** at alkaline conditions (pH 12), namely 1-heptanol, 1-octanol and 2-ethyl-1-hexanol. These solvents are considered as 'green solvents' contributing to a sustainable process development. They have proved suitable for the application in liquid-liquid extraction of 500 mL samples with isolated yields of up to 96% (data not shown). That allows us to conclude that liquid-liquid extraction can be applied for product purification of *N*-heterocycles in future processes.

In summary, a one-pot cascade using engineered diamine oxidase and imine reductase has been successfully implemented for the synthesis of 3-methylpiperidine from the corresponding

1,5-diamino-2-methylpentane. The compatibility of both enzymes allowed the development of a whole cell system at bioreactor scale under controlled reaction conditions eliminating the necessity of costly enzyme, coenzyme as well as cosubstrates and intermediate purification steps. Operational improvements of the vital fermentation and bioconversion parameters enabled the production of 3-methylpiperidine in high amounts. Our results demonstrate that this enzymatic cascade established as whole cell biotransformation with living cells is promising and can be applied in a large-scale format opening up the perspective towards the production of high-value piperidine compounds that might be difficult to obtain by organic chemistry.

Experimental Section

Production of PuOM1 and IRED

The plasmids containing the putrescine oxidase variant PuOM1 (Leu192Ile, Glu203Ser, Ile206Leu, pITB1381) and the imine reductase (pBAD33_IRED-*Sr,* pITB1236) were transformed into arabinose deficient chemical competent *E. coli* JW5510 and plated onto selection plates containing 100 µg mL^{-1} ampicillin and 34 µg mL^{-1} chloramphenicol. The production was performed in 400 mL TB-media containing 100 µg mL^{-1} ampicillin and 34 µg mL^{-1} chloramphenicol in 2 L shake flasks, which were inoculated with an overnight culture to an OD_{600} of 0.02. The cells were grown at 37°C and 180 rpm to an OD_{600} of about 0.6 in 2 to 3h and then induced with 0.04% of L-arabinose. The production was performed at 25°C overnight. The cells were harvested by centrifugation (centrifuge Avanti J 26S XP, Beckman Coulter, Krefeld, Germany; rotor JLA 8.1) for 25 min at 4°C with 9000 g and a 400 mL culture resulted in average in a cell pellet of 5.5 g. The production of the enzymes was visualized by SDS-PAGE analysis.

Small-scale experiments

The reaction was performed in a reaction volume of 5 mL in Tris-HCl pH 7.5 with an OD_{600} of 50 (~80 mg/mL_{cww}) in 20 mL reaction vessels. The biotransformation contained 10 mM (1.162 g L^{-1}) 1,5-diamino-2-methylpentane (1M stock in DMSO) and 10 mM glucose and was run for 48 h at 25°C and 180 rpm. 1 mL samples were taken after 3 h, 6 h, 24 h, and 48 h and centrifuged. 900 µL of the supernatant was mixed with 5 µmol 2-methylpyrrolidine as internal standard and derivatized with acetic anhydride. Therefore, 250 µL of buffer (10M KOH - saturated $NaHCO_3$ volume ratio 3:17*),* 500 µL dichloromethane and 24 µL acetic anhydride was added and vortexed for 2 min. The samples were centrifuged, and the extraction was repeated. The organic phase was combined in GC vials and analyzed by GC-FID (Figure S5).

Fermentation

The culture was grown in a 20-L bioreactor fermentation with a 12-L working volume for whole-cell biocatalysts using high-cell density medium. The pH, airflow, and dissolved oxygen were adjusted to 6.6 (using NH_4OH/H_3PO_4), 12 NL min^{-1} and 50%, respectively. The temperature for cell growth was 37°C, but after induced with arabinose (0.04% *w/v*) at OD_{600} 10-15, the temperature was adjusted to 30°C temperature for enzyme production.

Production of 3-methylpiperidine at a larger scale

The conversion experiments were carried out in a 20 L bioreactor with 12 L working volume. The substrate 1,5-diamino-2-methylpentane (15 mM to 100 mM) was added into the system by one-time without prior dissolution in DMSO or other organic solvents. Glucose was added with a concentration of 20 mM followed by glucose fed-batch feeding 48 h of the bioconversion. 72 mM of glucose was converted over 48 h. NH_4OH (25%) and H_3PO_4 (30%) were used to control and maintain the pH at 7.8. The concentration of 1,5-diamino-2-methylpentane and 3-methylpiperidine was determined using the GC methods described in the SI.

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Keywords: whole cell enzyme cascade • imine reductase • upscaling • methylpiperidine • amine oxidase

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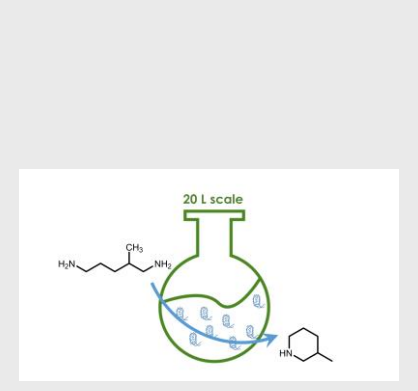
COMMUNICATION

Scaling Up! The coupling of engineered putrescine oxidase and imine reductase realizes the preparation of piperidine heterocycles. Particulary, the production of 3-methylpiperidine at 20 L scale was demonstrated using an *E. coli* whole cells system.



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