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Communication

Process Development and Protein Engineering Enhanced Nitroreductase-Catalyzed Reduction of 2-Methyl-5-nitro-pyridine

Amin Bornadel,* Serena Bisagni, Ahir Pushpanath, Iustina Slabu, Jacques LePaih, Alan H. Cherney, Steven M. Mennen, Simon J. Hedley, Jason Tedrow, and Beatriz Dominguez*

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ABSTRACT: Reduction of aromatic nitro compounds to anilines is of great interest to the chemical industry. Biocatalytic reduction of nitroarenes has made it possible to effectively produce anilines by applying nitroreductase enzymes (NR) in combination with vanadium pentoxide. Herein, the NR-catalyzed reduction of 2-methyl-5-nitro-pyridine (**2**) to give the desired aniline (**1**) was studied as a model reaction. It demonstrates the importance of process development and enzyme engineering as key approaches to overcome scale-up issues and improve yield and productivity. Moving to fed-batch allowed controlling the feeding rate of **2** to prevent the accumulation of intermediates and formation of undesired side products. Starting with a substrate (**2**) concentration of 200 mM (28 g/L) and enzyme loading of 5 mg/mL (18% w/w), it was possible to achieve complete conversion and **1** in 95% yield by high-performance liquid chromatography (89.1% isolated yield) over 18 h, whereas, with 500 mM (69 g/L) **2** and an enzyme loading of 10 mg/mL (14.5% w/w), the same conversion and yield were achieved in 26 h. A rational engineering of NR-4 yielded faster variants, including NR-5, in only one round. The improved rate of the new variants allowed increasing the feeding rate of **2** to shorten the reaction time to less than a day as well as decreasing the enzyme loading to 3.6%.

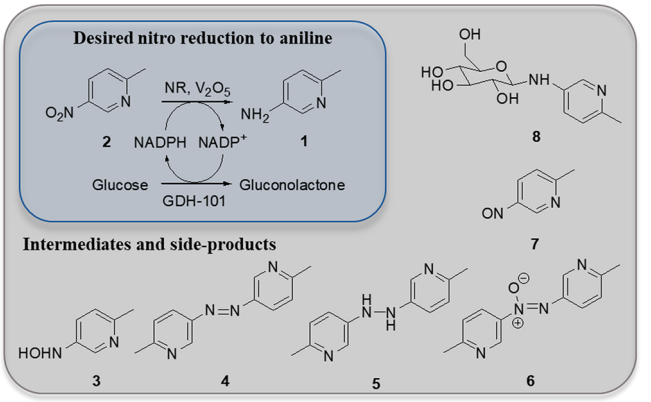
KEYWORDS: *NR catalyzed reaction, reduction of nitro aromatics, enzyme engineering, fed-batch reaction, biocatalytic process development*

Anilines are present in a number of active pharmaceutical ingredients (APIs) and agrochemicals.[1−4] They are generally produced by a reduction of the corresponding nitrobenzene precursors, either following catalytic methods involving the use of metals such as palladium or platinum[4−8] or more traditional methods using stochiometric reducing agents.[1]

We recently developed an approach to the reduction of nitrobenzene derivatives that allows for robust chemoselectivity under mild conditions.[9,10] This methodology relies on the use of flavin mononucleotide (FMN)-dependent nitroreductases (NRs) in combination with a transition metal to catalyze the formation of the hydroxylamine intermediate and its subsequent disproportionation to form the corresponding aniline and the nitroso intermediate as a side product. The nitroso compound formed is efficiently recycled in the reaction by the NR catalysis. This system overcomes previous limitations encountered in biocatalytic nitroreductions[11−16] and enables the effective conversion of nitro compounds to their desired anilines. The enzymatic catalysis is advantageous owing to its highly tunable reactivity that would avoid over-reduction pathways. Furthermore, the terminal reductant, glucose, would not require the intensive specialized infrastructure that is needed for handling hydrogen.

The present study focuses on the application of this new technology utilizing 2-methyl-5-nitro-pyridine (**2**) as a model substrate (Scheme 1) to demonstrate the importance of simultaneously addressing both reaction engineering and enzyme development to enhance the overall productivity and

Scheme 1. NR Catalyzed Reduction of 2-Methyl-5-nitro-pyridine (2) to the Corresponding Aniline (1), where GDH-101 Is Used to Catalyze the Oxidation of Glucose for the Regeneration of the NADPH Cofactor[a]



[a]The nitro reduction intermediates and side products (**3** to **8**) shown in the outer box should be minimized to enhance the yield of **1**.

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Table 1. Summary of the Main Reaction Parameters and Results Obtained for the Experiments Performed on a Preparative Scale for the Process Development of NR-Catalyzed Nitro Reduction of 2-Methyl-5-nitro-pyridine (2) to the Corresponding Aniline (1)

entry[a]	**2** (mM)	F_2[b] (mM/h)	NR type	NR (mg/mL)	NR loading (NR:**2** w/w %)	time (h)	conversion (%)	**8** (%)	**6** (%)	**5** (%)	**3** (%)	**1** (mM)	$Y_{1/NR}$[c] (**1**/NR g/g)
1	200[d]	12	NR-14	5	18.1	18	100	5.1	0	0	0	189.7	4.1
2	400[e]	16.5	NR-14	10	18.1	17	68.7	7.8	0.7	0	1.5	234.5	2.5
3	400	16.5	NR-4	10	18.1	24	100	9.8	0	0	1.4	355.2	3.8
4	400	16.5	NR-4	10	18.1	24	96.1	11.7	0	0	0.2	336.8	3.6
5	500[f]	15	NR-4	10	14.5	24	76.0	10.4	0	0	0.8	324.1	3.5
						40	100	14.3	0	1.0	0.2	422.5	4.6
6	500	19	NR-4	10	14.5	26	100	5.1	0	0	0	474.7	5.1
7	500	19	NR-4	5	7.2	19	72.3	6.6	0.8	0.3	0.5	320.7	7.0
8	500	23	NR-5	5	7.2	16	77.1	5.2	0	0	1.2	353.9	7.7
						21	95.0	7.6	0.9	0	0.8	428.5	9.3
9	500	30	NR-5	2.5	3.6	18	68.7	2.1	2.4	0.8	0	317.0	13.7

[a]All reactions were performed on a 25 mL scale in fed-batch under the conditions specified in Table S2. [b]Feeding rate of **2** applied from the start of the reaction in fed-batch. [c]Yield of **1** in gram per gram of NR used. [d]28 g/L. [e]55 g/L. [f]69 g/L.

yield of the reaction. The choice of substrate **2** was based on its application for the synthesis of a desired API, as well as safety considerations taking into account the outcomes of process hazard analysis for **1** and **2** as included in the Supporting Information. Moreover, the nitropyridine compound **2** is an interesting precursor owing to a structural homology to some substituted nitroarenes commonly found in APIs, such as Crizotinib[17] or Merestinib.[18] Herein, the reactor design and reaction parameters optimization were applied to increase the substrate concentration and minimize the accumulation of intermediates and side products, while enzyme engineering and high-throughput screening were employed to design and obtain improved enzymes for achieving higher productivities.

Scale-up studies performed following the proof of concept[9,10] showed that increasing the substrate concentration in batch resulted in higher formation of the undesired side products, particularly **6**. A batch reaction run at 200 mM (28 g/L) **2** catalyzed by 2.5 mg/mL NR-14 resulted in 89% **1**, 1% **5**, and 10% **6** after 4 h as measured by high-performance liquid chromatography (HPLC) (Table S4). Side-product formation was reduced by minimizing the accumulation of the hydroxylamine and nitroso intermediates **3** and **7**. When the reaction in batch was repeated under the same conditions but with portionwise addition of 200 mM **2** (50 mM every hour), the conversion to the product was improved by 4% to give 93% **1** and 7% **3**, **4**, and **6**. The portionwise addition approach was then tested at increased substrate concentration (500 mM **2**) with unsatisfactory results. The reaction was halted after 10 h, with only 62% **1**, 13% side products, and 25% unreacted **2**.

To improve control over the rate of substrate addition and minimize the accumulation of intermediates, the reaction was next attempted in fed-batch. Toluene was the cosolvent of choice based on screening results performed on a small scale (Table S1). With log *P* (logarithm of octanol to water partition coefficient) of 2.5 and solubility in the region of 70 g/L, toluene offers a good compromise between facilitating the mass transfer by solubilizing **2** and enzyme deactivation due to the detrimental effect of organic solvent on protein stability.[19] Nevertheless, when the reaction is run in fed-batch with 200 mM **2** in toluene, the feedstock (0.5 M solution of **2** in toluene) corresponds to 40% of the reaction volume. Adding this large proportion of feedstock to the reaction caused severe enzyme deactivation, biphasic inhomogeneity, and, consequently, incomplete conversion. To overcome this problem, **2** was protonated in acidic water (predicted pK_a = 1.9), where it became readily soluble.

The reaction was repeated in fed-batch with 200 mM **2** and 5 mg/mL (18% w/w) NR-14 following the experimental procedures described in the Supporting Information. The reaction was run at pH 8.0 (controlled using an automated pH-STAT titrator) and was monitored based on the rate of addition of NaOH (45%), which progressed on a straight line following the feeding rate of **2** set at 12 mM/h. An HPLC analysis of the reaction after 18 h showed significant results including complete conversion to mainly **1** and absence of side-products **3**−**7**. In addition, the HPLC traces showed an impurity of polar character (**8**) in 5% with a UV−vis absorbance spectrum resembling that of **1**. ^{1}H NMR, two-dimensional (2D) NMR, and liquid chromatography-mass spectrometry (LCMS) data (Figures S2 and S3) confirmed the identity of **8** being the *N*-glucoside[20] of **1**. Electrospray ionization shows ES+ at m/z = 271, consistent with Mw = 270 Da. The reaction's yield of **1**, estimated by HPLC, was 94.9% based on the formation of 189.7 mM **1** (Table 1, Entry 1). Workup of the reaction was attempted through liquid−liquid extraction, where 482 mg of **1**, equivalent to an isolated yield of 89.1%, was achieved, following the procedure presented in the Supporting Information. It was observed during the workup that the first extraction recovered **1** in high purity as analyzed by HPLC (Figure S5). Side-product **8** was not extracted, as it is quite polar, which facilitates product purification.

Following the breakthrough achieved when the reaction was run with 200 mM **2**, for the next attempt the substrate concentration was doubled while maintaining the enzyme loading constant. Therefore, the reaction was run in fed-batch with 400 mM (55 g/L) **2** and 10 mg/mL NR-14 (Table 1, Entry 2). The feeding rate of **2** was increased to 16.5 mM/h to achieve completion in 24 h. However, in the presence of a higher substrate concentration, after 15 h, the NR activity was completely depleted. As a result, the reaction reached 68.7% conversion and yielded in 234.5 mM **1**. In addition, only 1.5% **3** and 0.7% **6** were detected, which shows the fed-batch strategy employed is successful in minimizing the accumulation of the undesired compounds **3**−**7**. The amount of **8** increased

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to 7.8% due to a higher concentration of **1** formed in the presence of the remaining glucose.

Meanwhile, through screening of additional NRs from our enzyme collection, NR-4 was identified to outcompete NR-14 for the biotransformation of **2**. Repeating the reaction in fed-batch with 400 mM **2** and 10 mg/mL NR-4 allowed the reaction to proceed to complete conversion in 24 h (Table 1, Entry 3). The yield of **1** increased to 88.8% based on 355.2 mM **1** formed according to the HPLC data. There was also 9.8% *N*-glucoside product (**8**) and 1.4% **3** in the reaction mixture. The effect of increasing temperature on side-product formation was studied by repeating the reaction at 40 °C. The higher temperature resulted in faster rates; hence, almost no accumulation of intermediates **3**−**7** (Table 1, Entry 4). Nevertheless, higher temperatures also increased the rate of enzyme deactivation due to thermal denaturation, which, in turn, led to a complete loss of the NR activity and incomplete conversion (96.1%). It was observed that the formation of **8** increased with temperature (11.7% after 24 h).

To intensify the reaction further, the substrate concentration was increased to 500 mM (69 g/L) **2** using the same loading of 10 mg/mL NR-4 (Table 1, Entry 5). The feeding rate of **2** was 15 mM/h, and the addition of substrate was completed in 32 h. Analysis of the reaction after 24 h, when the feeding was still progressing, showed there was no accumulation of **2**, as no trace of **2** was detected by HPLC. At this point 380 mM **2** had been consumed by the reaction, whereas the concentration of **1** was 324.1 mM, and the rest was **3** and **8**. The reaction was continued overnight, and analysis of a sample taken after 40 h showed full conversion and 85% (422.5 mM) **1**. The remaining 15% was mostly *N*-glucoside product **8** (14%). To minimize **8**, it was anticipated that increasing the pH of the reaction would reduce the acid-catalyzed formation of the Schiff base derived from the aniline and *β*-D-glucose.[20,21] Therefore, the reaction with 500 mM **2** catalyzed by NR-4 (10 mg/mL) was run under pH 8.0. Furthermore, the feeding rate of **2** was increased to 19 mM/h to make up for faster nitro reduction rates achieved at the higher pH.[9] The new conditions improved the reaction's outcomes significantly. Analysis of the reaction after 26 h showed 100% conversion and 95% yield (474.7 mM **1**) had been achieved (Table 1, Entry 6). There were no traces of **3**−**7** detected by HPLC, and the amount of **8** formed was reduced from 14.3% to 5.1%. Reducing the NR-4 concentration to half (5 mg/mL) under the same conditions resulted in the reaction stopping after 18 h due to enzyme deactivation. Analysis of the reaction showed the conversion was 72.3%, and 320.7 mM **1** was formed (Table 1, Entry 7).

Rational enzyme engineering was employed to overcome the activity and stability limitations of the wild-type enzyme.[22] Driven by proprietary computational methodologies at JM, the SmartScaffold platform was used to design reduced alphabet, multisite saturation libraries of the chosen scaffold.[23] Enzyme engineering was fundamentally driven by computational studies. Initially, a decision was made on which enzyme to use as a scaffold for engineering, that is, either NR-4 or NR-14. This was decided primarily based on expression data, which clearly pointed toward enhanced soluble expression of the former. The enzymes that are more soluble during recombinant expression in *Escherichia coli* are usually preferred scaffolds for engineering, because they ensure reproducible expression during library generation.[24] In silico structural modeling of NR-4 was performed, with SmartScaffold applying docking and binding energy calculations to identify hotspots in the active site that, if mutated to more favorable residues, would enhance binding of **2** in the active site in a more productive conformation (Figure 1). Of the 16 residues

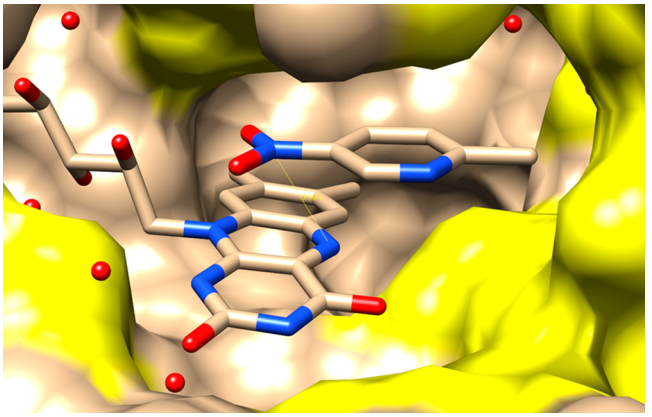

Figure 1. Homology structural model of NR-4 showing the docking of **2** in the active site based on the highest-scoring docked pose and the distance to the catalytic N5 on the FMN cofactor.

delineating the active site, 5 residues, namely, Ile41, Ala136, Tyr224, Tyr229, and Tyr230, were selected as hotspots for mutagenesis based on sequence conservation analysis of the entire NR protein family and structural evidence (Figure 2). A

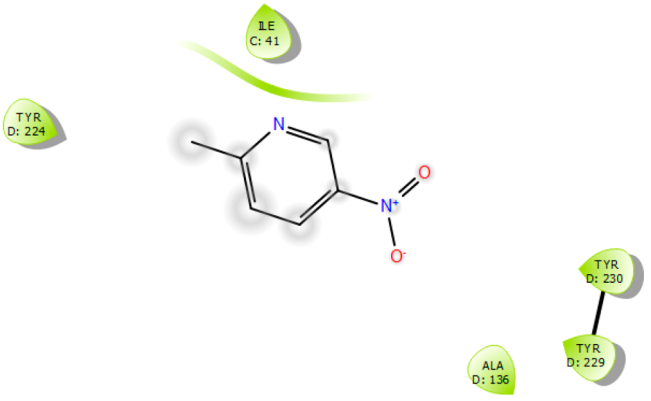

Figure 2. A 2D depiction of the five residues delineating the active site, selected within close proximity to the bound **2**, which could elicit a positive effect if mutated.

reduced alphabet approach was chosen to do a combinatorial mutagenesis of all the sites together. A reduced alphabet approach was adopted, with the five residues being mutated simultaneously. Although this methodology is usually more successful, as it accounts for synergistic effects, a second, more conservative strategy was also attempted, where each of the residues was mutated individually to all other possible amino acids. By applying these parallel approaches, four sets of NR-4 mutant libraries were generated, providing an adequate and tailored mutant-sequence space for exploration.

The libraries containing ∼2000 variants were screened, as presented in the Supporting Information, initially with a spectrophotometric assay measuring the depletion of reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm for the conversion of **2**. A consolidated library was created with the best hits (ca. 200) identified in the previous stage, which were screened for the reaction of **2** analyzed by

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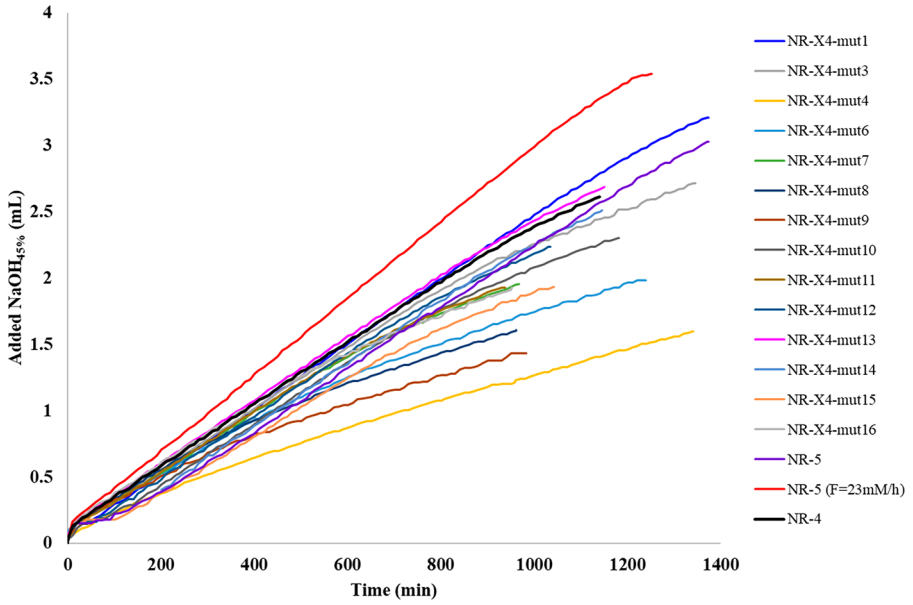

Figure 3. Titration profiles for the fed-batch (19 mM/h) reactions run on a large scale to evaluate the activity and in-process stability of the top 15 mutants selected. A control experiment with NR-4 was run under similar conditions. An additional run with NR-5 (red) under a higher rate of feeding **2** (23 mM/h) was performed.

HPLC. The top 15 mutants selected were expressed on a large scale to evaluate their activity and in-process stability in large-scale reactions.

The reactions performed to assess the mutants were all run under similar conditions including 500 mM **2** in fed-batch (19 mM/h) catalyzed by 5 mg/mL NR at pH 8.0. They were monitored following the profile of NaOH added for the titration to maintain the reactions' pH constant (Figure 3), as well as by HPLC. Provided that the reaction progressed to complete conversion, ~3.5 mL of base (45% NaOH) was required to maintain the pH at 8.0. According to the results obtained, NR-X4-mut1 and NR-5, a single mutant (Tyr224-His) and a triple mutant (Ala136Gly/Tyr224Lys/Tyr229Asn), respectively, topped the enzymes tested by demonstrating improved activity. The improved activity of the mutants is hypothesized to be due to better coordination of the substrate in the active site. Often, a higher activity by enzyme engineering is achieved at the expense of reduced enzyme stability.[25] However, histidine is seen to offer a good compromise at this position and is less destabilizing than lysine. Additional mutations (Ala136Gly and Tyr229Asn) to partially offset the loss of stability appear to be required if Tyr224 is mutated to lysine. In hindsight, an inclusion of histidine in the reduced alphabet for position Tyr224 might have led to further improved variants, which opens up opportunities for further engineering.

The fed-batch nitro reduction of 500 mM **2** catalyzed by NR-5 (5 mg/mL) was repeated at a higher feeding rate of **2** (23 mM/h) to achieve higher space-time yields. After 16 h, when there was still 112 mM **2** remaining to be fed, the conversion was 77% and 70.8% (354 mM) **1** formed. An HPLC analysis showed that **2** was mostly consumed, and 1.2% **3** and 5.2% **8** were also detected. After 21 h, when the entire substrate was fed, the conversion was 95% and 85.7% (428.5 mM) **1** formed. The reaction was progressing sluggishly at this point, as most of the enzyme was deactivated. Formation of **6** (0.9%) was observed, possibly because of the loss of NR activity; hence, the accumulation of **3** and **7** forming **6**. Being able to reduce the reaction time from 40 h (Table 1, Entry 5) to 21 h (Table 1, Entry 8) by replacing NR-4 with the faster, engineered NR-5, allowed reducing the formation of **8** from 14.3% to 7.6%. Furthermore, the volumetric productivity was doubled from 1.1 to 2.2 g L^{-1} h^{-1} based on the formation of 428.5 mM **1** in 21 h.

The effect of different additives, including poly(ethylene glycol) (PEG) and polyethylenimine (PEI) of different molecular weights, on the enzymatic stability was studied next.[26] PEI was observed to be a good enzyme stabilizer and showed a significant effect on retaining the enzymatic activity. When PEI (branched, $M_n \approx 10\,000$) was used in 3:1 (w/w) to NR-5, the initial activity of the enzyme was improved by 10%, and ca. 94% of the activity was retained after 24 h (Table S3). The effect of using PEI as an additive was tested on a 25 mL scale for a run with 500 mM **2** (Table 1, Entry 9). The NR-5 concentration was reduced to half (2.5 mg/mL), and the feeding rate of **2** was increased to 30 mM/h. Analysis of the reaction after 18 h showed 68.7% conversion and 63.4% (317 mM) **1**. Moreover, using PEI lowered the amount of impurities formed over the course of the reaction, particularly **8**, which was reduced to 2.1%. By reducing the NR-5 loading to half, the yield with respect to the amount of NR used ($Y_{1/NR}$) was improved by ~47% from 9.3 to 13.7 (**1**/NR g/g).

In conclusion, the NR-catalyzed reduction of nitroaromatics for the selective synthesis of anilines represents a biotransformation with substantial potential for industrial applications. The results presented in this study showcase the significance of process development in tackling issues such as low solubility or side-product formation during intensification and scale up. By switching to fed-batch to control the feeding rate of the nitropyridine substrate (**2**) solubilized in water under acidic conditions, the accumulation of the hydroxylamine (**3**) and nitroso (**7**) intermediates was prevented, which, in turn, prevented the formation of **4**, **5**, and **6**. Thus, performing the reaction in fed-batch enhanced the yield of **1**, along with process safety by preventing the accumulation of potentially toxic intermediates and side products **3**–**7**. On the other hand,

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enzyme development can boost process productivity and economy. Through the application of SmartScaffold, the discovery of promising wild-type NRs and the design of rational libraries thereof were demonstrated to afford a more robust biocatalyst. Both strategies, that is, following the best reaction conditions identified as well as shifting to the faster NR-5 designed by only one round of rational mutagenesis of NR-4, when combined, allowed us to halve the reaction time to less than 20 h and reduce the enzyme loading, fourfold, to 2.5 mg/mL.

Currently, we are working on subsequent steps to advance the biocatalytic nitro reductions further. New studies on the application of the present technology for a selective nitro reduction of various aromatic nitro compounds and on different scales have been progressing in our laboratories. Scaling up those reactions (≥1 L), introducing overhead stirring to improve mixing and mass transfer, has been observed to work extremely well with the NRs introduced in this study, which show good in-process stability. These results on substrate scope and scalability will be communicated through a follow-up report. An alternative strategy we have been looking into involves the development of immobilized NRs in the presence of the other catalytic components, that is, cofactor recycling enzymes and transition-metal additives, to facilitate performing the reaction in flow to continue enhancing the efficiency and safety of the process.[27]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.oprd.0c00464.

Experimental procedures included in the Supporting Information are as follows: (I) Studying the effect of cosolvent. (II) Scale-up of the reaction in fed-batch. (III) Identification of the polar side-product. (IV) Workup procedure. (V) Screening mutant libraries. (VI) Testing the top mutants on large scale. (VII) Effect of polyethylenimine additive on the reaction. (VIII) HPLC analysis. (IX) Process hazard analysis (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Amin Bornadel − *Johnson Matthey Plc., Cambridge CB4 0FP, U.K.;* orcid.org/0000-0002-3895-2539; Email: Amin.Bornadel@Matthey.com

Beatriz Dominguez − *Johnson Matthey Plc., Cambridge CB4 0FP, U.K.*; Email: Beatriz.Dominguez@Matthey.com

Authors

Serena Bisagni − *Johnson Matthey Plc., Cambridge CB4 0FP, U.K.*

Ahir Pushpanath − *Johnson Matthey Plc., Cambridge CB4 0FP, U.K.*

Iustina Slabu − *Johnson Matthey Plc., Cambridge CB4 0FP, U.K.;* orcid.org/0000-0003-2342-9195

Jacques LePaih − *Johnson Matthey Plc., Cambridge CB4 0FP, U.K.*

Alan H. Cherney − *Amgen, Inc., Thousand Oaks 91320-1799, California, United States;* orcid.org/0000-0001-7440-6634

Steven M. Mennen − *Amgen, Inc., Thousand Oaks 91320-1799, California, United States;* orcid.org/0000-0003-1905-5530

Simon J. Hedley − *Amgen, Inc., Thousand Oaks 91320-1799, California, United States*

Jason Tedrow − *Department of Drug Substance Technologies, Amgen Inc., Cambridge, Massachusetts 02142, United States;* orcid.org/0000-0002-5663-2360

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.oprd.0c00464

Notes

The authors declare no competing financial interest.

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