ARTICLE

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Non-Hazardous Biocatalytic Oxidation in Nylon-9 Monomer Synthesis on a 40 g Scale With Efficient Downstream Processing

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ABSTRACT: This paper describes the development of a biocatalytic process on the multi-dozen gram scale for the synthesis of a precursor to Nylon-9, a specialty polyamide. Such materials are growing in demand, but their corresponding monomers are often difficult to synthesize, giving rise to biocatalytic approaches. Here, we implemented cyclopentadecanone monooxygenase as an *Escherichia coli* whole-cell biocatalyst in a defined medium, together with a substrate feeding–product removal concept, and an optimized downstream processing (DSP). A previously described hazardous peracid-mediated oxidation was thus replaced with a safe and scalable protocol, using aerial oxygen as oxidant, and water as reaction solvent. The engineered process converted 42 g (0.28 mol) starting material ketone to the corresponding lactone with an isolated yield of 70% (33 g), after highly efficient DSP with 95% recovery of the converted material, translating to a volumetric yield of 8 g pure product per liter.

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KEYWORDS: Baeyer-Villiger monooxygenases; process intensification; polyamide 9; medium engineering; chromatography-free downstream processing; performance material monomers

Introduction

Aliphatic polyamide materials constitute a multi-billion euro market with applications in the automotive, film and coating, and electronic devices industries (Herzog et al., 2000). With rising demand of artificial polymer materials in general, and polyamides (PAs) in particular, the industry explores bio-based production solutions to reduce dependence on fossil resources and to increase sustainability (Carole et al., 2004). The PA types PA 6 and PA 6.6 form the largest market share. Several biocatalytic processes have been established for the production of muconic acid (Suastegui et al., 2016), which can be converted via hydrogenation to adipic acid, the monomer for PA 6.6 (Sengupta et al., 2015).

Odd carbon-numbered PAs often yield materials with superior properties (Horn et al., 1963), but successful commercialization of those heavily depends on the availability of cheap precursors with an odd carbon count, for example, from ricinolic acid after oxidative cleavage (Teomim et al., 1999). Naturally, fatty acid-derived molecules with odd carbon number are scarce, although engineered microbes that overproduce them have been described (Cao et al., 2015; Wu and San, 2014). Monomers coming from cost-intensive multistep syntheses, or with high bulk material costs, limit the use of these polymers on broad scale (Finch, 2001). Biocatalytic processes toward polymer precursors can improve the economics of monomer synthesis (Schaffer and Haas, 2014), as has been demonstrated for the Nylon 12 monomer ω-aminododecanoic acid methyl ester (Ladkau et al., 2016).

Compared to PA 6 and PA 6.6, PA 9 has enhanced polyethylene-like properties (e.g., low water absorption, dimensional stability under different conditions of humidity, impact strengths, good behavior at low temperatures, low density, etc.), while maintaining some properties of polyamides (e.g., high melting points, good aesthetic properties, good processability, and resistance to hydrocarbon solvents). These physical properties make PA 9 very useful in the manufacture of precision components (Cotarca et al., 2001). In 1982, Minisci et al. (1982) developed a convergent pathway for the synthesis of monomers for PA 9, commercialized as Perlagon (Rath, 1972), starting from the readily available bulk chemicals cyclohexanone and acrylonitrile, leading to the ketone 3-(2-oxocyclohexyl)propanenitrile (OCHPN) (Fig. 1). Their process was later optimized by Cotarca et al. (2001) by

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improving the synthesis of 3-(7-oxooxepan-2-yl)propanenitrile (OOPN). Still, both routes used stoichiometric amounts of an alkylcarboxylic peracid—explicitly described as explosive—while heating above room temperature to perform the key Baeyer-Villiger oxidation step. The hazard of explosion is a clear disadvantage, and requires specific safety measures.

This paper describes an alternative protocol using aerial oxygen as terminal oxidant, operating at room temperature, and in water. The chemical Baeyer-Villiger oxidation of the process from ketone OCHPN to lactone OOPN was substituted by a 4 L whole-cell biotransformation on the multi-dozen gram scale. Subsequently, an efficient DSP method was developed to obtain lactone OOPN in high isolated yields and purity without chromatographic purification.

Materials and Methods

Cloning of CPDMO

The CPDMO (*cpdB*) gene, originally from *Pseudomonas* sp. strain HI-70 (Iwaki et al., 2006), which we obtained as an *E. coli* TOP10 expression system with a pBAD vector, was cloned into pET26b(+) in *E. coli* BL21(DE3) (details in the supporting information).

Determination of Equilibrium Load Constants for SFPR

Lewatit VP OC 1163 resin was suspended in medium in a shake flask and the substrate was added neat to the suspension, followed by incubation at rt for 2 h (ratios from 0.1 to 1.0 resin/ketone). The supernatant was extracted with ethyl acetate and analyzed by GC-FID (details in the supporting information).

Cultivation Media

Standard M9 cultivation medium and variations thereof (different carbon sources and ammonium levels) were used in this study. The composition can be found in the supporting information.

Preculture Cultivation

Precultures were inoculated (1:100) from permanent cultures (harvested in the late exponential phase, stored at −80°C with 25% (v/v) glycerol) in M9 medium with either glucose or glycerol as carbon source and the appropriate antibiotic. They were grown for 16 h in a volume of 10 mL in non-baffled 250 mL Erlenmeyer flasks for shake flasks experiments, and in a volume of 40 mL in non-baffled 500 mL Erlenmeyer flasks for reactor experiments, at 37°C and 350 rpm in an orbital shaker (InforsHT Multitron 2 Standard, Bottmingen, Switzerland), and were used for the inoculation of the main culture (1:100).

Cultivation Experiments for the Characterization of CPDMO Constructs on the Shake Flask Scale

Both above-mentioned expression systems were cultivated in M9 minimal medium with glucose or glycerol as carbon source, supplemented with either kanamycin (100 $\mu g\,mL^{-1}$) for the *E. coli* BL21(DE3) pET26b(+)::*cpdmo* construct or ampicillin (100 $\mu g\,mL^{-1}$) and L-leucine (4 $g\,L^{-1}$) for the *E. coli* TOP10 pBAD::*cpdmo* construct (details in the supporting information).

The main culture on the shake-flask scale was cultivated in 10 mL in a non-baffled 250 mL Erlenmeyer flasks at 37°C and 350 rpm until an $OD_{590} = 1$. Subsequently, the culture was cooled to 24°C and induced with IPTG for the pET26b(+)::*cpdmo* or rhamnose for the pBAD::*cpdmo* system. One hour after induction, the ketone was

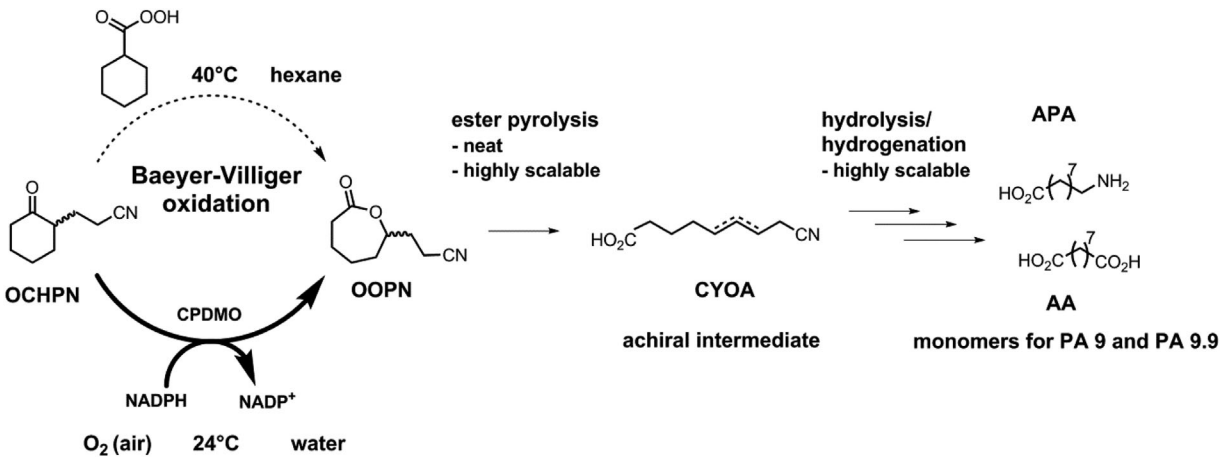

Figure 1. Schematic comparison of the chemical Baeyer-Villiger process by Cortaca et al. (2001) (dashed arrow) vs the enzymatic BVMO transformation described in this paper (solid arrow). The process by Cotarca et al. (2001) contained a problematic H_2O_2—mediated ring cleavage of ketone OCHPN via Baeyer-Villiger oxidation with alkylcarboxylic peracid to lactone OOPN and subsequent ester pyrolysis to the unsaturated achiral 8-cyanooct-6-enoic acid (CYOA) and further modification via hydrolysis and hydrogenation to 9-aminoperlagonic acid (APA) and azelaic acid (AA), which are the monomers for PA 9 and PA 9.9 Cotarca et al., 2001.

added as a 2.5 M solution in dioxane to a final concentration of 5 mM.

Conditions for Scale-Up Reactor Experiments

For the reactor experiments, biotransformation was performed in a 7 L RALF bioreactor (Bioengineering, Wald, Switzerland) in M9 minimal medium (upscaling experiments) or modified M9 minimal medium (modification: 3× nitrogen and magnesium sources; SFPR experiment) with an operational volume of 4 L. The aeration was set to 1 vvm, controlled by a MX4/4 gas module (DASGIP, Jülich, Germany). The stirring frequency was 1000 rpm with Rushton turbines and the pH was manually adjusted to 6.5 with 3 M NaOH and 3 M H_3PO_4. Off-gas analysis was performed with a GA4 off-gas analyzer (DASGIP). Antifoam (O-30, Sigma–Aldrich, St. Louis, MO) was added in 0.1 mL batches whenever necessary. The culture was initially incubated at 37°C. At $OD_{590} = 1.0$ (see optimization experiments) or $OD_{590} = 2.6$ (biotransformation with resin), the bioreactor was cooled to 24°C and CPDMO expression was induced with 0.2 mM IPTG. The resin material Lewatit® VP OC 1163 (177.1 g, $X_{eq} = 0.40$) was suspended in sterile deionized water (approx. 250 mL) in a baffled shake flask. Then the ketone (42.5 g, 281 mmol) was added and the mixture was shaken overnight (200 rpm, 24°C). The resin slurry, containing the adsorbed ketone, was added to the bioreactor at 2.5 h after induction of CPDMO expression.

CPDMO Activity Measurement After the SFPR Biotransformation

For the activity assay after the reactor experiment, 10 mL of culture broth without resin were withdrawn from the reactor before DSP. The satellite culture was incubated at 24°C and 350 rpm in a 250 mL unbaffled shake flask. The ketone was added as a 2.5 M solution in dioxane to a final concentration of 5 mM.

Downstream Processing

The bacterial culture-resin suspension was pumped to a flask. The bioreactor was rinsed with water to collect the remaining resin; the two suspensions were combined. This suspension was coarse-filtered; the filtrate was centrifuged and the resin was washed and triturated with MTBE. This organic extract was washed with brine. The resin itself was transferred to a Soxhlet continuous extraction apparatus using methyl *t*-butyl ether (MTBE). After 20 cycles, the extract was combined with the resin wash solution and concentrated under reduced pressure, yielding 39.0 g of crude mixture of ketone and lactone. Further extraction of the resin with fresh MTBE provided additional 3.0 g crude reaction product.

The supernatant of the centrifuged, coarse-filtered filtrate was decanted from the cell pellet, and equally distributed to a separation funnel and a continuous extractor. Manual extraction with MTBE yielded 0.9 g crude product; continuous extraction ran for 15 h, yielding 2.2 g crude product. All fractions were combined after quantification of the composition via calibrated GC.

Remaining starting material was recovered as colorless liquid by vacuum short-path distillation. Various lactone fractions were obtained as colorless oils at higher temperature and lower pressure. The separate fractions were then recrystallized from EtOAc/MTBE. The colorless crystals were separated from the yellow supernatants by decantation, then filtered and washed with cold heptane. All obtained fractions were analyzed by GC and combined for drying. MTBE from all extraction steps was recovered from rotary evaporation in sufficient spectroscopic purity and good efficiency (84%; details in the supporting information).

Analytical Techniques

Optical density was measured at 590 nm in 10 mm cuvettes with a Colorwave CO7500 colorimeter (Biochrom, Cambridge, UK): values over OD = 1.5 were determined from 1:10 dilutions with sterile, medium at rt. The OD-g Cell Dry Weight (gCDW) relationship was determined from biological triplicates, with three technical replicates each, as $1\ OD = 0.447\ gCDW\ L^{-1}$.

The biotransformation process (ketone and lactone concentrations) was monitored via GC-FID (Thermo Finnigan Focus GC/DSQ II, Thermo Fisher, Waltham, MA) equipped with a standard capillary column (BGB5, 30 m × 0.32 mm ID, BGB Analytik, Schloßböckelheim, Germany). A total of 100 μL sample were extracted with 200 μL ethyl acetate, containing 1 mM methyl benzoate as internal standard. GC temperature protocol: 200°C for 1 min, ramp 40°C/min, 280°C for 1 min. The concentrations of ketone/lactone were calculated from calibration curves relative to the internal standard (linear range: ketone 0.2–12.8 mM/lactone 0.2–7.7 mM).

In the SFPR experiments, the samples were taken from the liquid phase and from the resin, and extracted separately.

The supernatant analysis was performed via HPLC using refractive index detection and a photodiode array detector for detection of glucose, acetate, pyruvate, and ethanol. Separation was achieved on an ROA-Organic Acid H+ column with isocratic elution (5 mM H_2SO_4 in water; details in the supporting information).

During the stationary phase of the bacterial culture, approximate glucose concentrations were determined with glucose strips (Akku-Chek, Roche, Basel) by centrifuging the sample, diluting the supernatant 1:10 and use the supernatant according to the instructions of the manufacturer.

Fitting of the Biomass Concentration Curve

The fitting of the biomass concentration was performed according to a logistic function for description of substrate-independent modeling of bacterial growth (He et al., 2008; Lan et al., 2006; Vinayagam et al., 2015) (details in the supporting information).

OTR/CTR Calculations via the Gas-Balancing Method and Carbon Balancing

The oxygen transfer rate (OTR) and carbon dioxide transfer rate (CTR) were calculated according to the following equations (Garcia-Ochoa et al., 2010; Royce, 1992; Suresh et al., 2009):

$$\mathrm{OTR} = \frac{Q}{V}\left(C_{O_2}^{\mathrm{in}} - C_{O_2}^{\mathrm{out}}\right)$$

$$\mathrm{CTR} = \frac{Q}{V}\left(C_{\mathrm{CO_2}}^{\mathrm{out}} - C_{\mathrm{CO_2}}^{\mathrm{in}}\right)$$

With Q being the oxygen gas flow, V being the volume of the bioreactor and C_{in} and C_{out} being the carbon and oxygen concentrations measured at bioreactor inlet and outlet (details are given in the supporting information).

Carbon balancing was performed according to the following equation

$$c(C_{\mathrm{glucose}}) - c(C_{\mathrm{biomass}}) - c(C_{\mathrm{CTR}}) = c(C_{\mathrm{unbalanced}})$$

Results and Discussion

Selection of a Suitable Biocatalyst

Two BVMOs were already known to accept the OCHPN ketone, but the described catalysts preferentially converted the (*R*)-enantiomer, leaving the (*S*)-ketone untouched, or oxidizing it at a very low rate (Berezina et al., 2007). We found this trend to hold true for many other BVMOs (Fink et al., 2015).

Since any chiral information is lost in the pyrolytic ring-opening elimination of the OOPN lactone to the unsaturated acid 8-cyanooct-6-enoic acid 8-cyanooct-5-enoic acid (CYOA), the Baeyer-Villiger oxidation (BVOx) should proceed non-selectively to increase both reaction rate and yield. Therefore, it was important to find a non-selective enzyme. We initially screened a previously designed library of variants of cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871 and cyclopentanone monooxygenase from *Comamonas* sp. NCIMB 9872 (Clouthier et al., 2006; Reetz et al., 2004). Unfortunately, both enzymes were selective for one stereoisomer, or the conversion was not complete after 24 h, even at the low substrate loading used in these screening reactions (4 mM; Supplementary Fig. S1). In the course of a systematic study on BVMO-catalyzed oxidations of nitriloketones (Fink et al., 2015), experiments on an analytical scale showed that a cyclopentadecanone monooxygenase (CPDMO) from *Pseudomonas* sp. HI-70 (Iwaki et al., 2006) catalyzed the transformation of both starting material enantiomers with low selectivity ($E = 5$–16) and at a high rate. Another BVMO, 2-oxo-Δ3-4,5,5-trimethylcyclopentenylacetyl-CoA monooxygenase (OTEMO) from *Pseudomonas putida* (Leisch et al., 2012) was even less selective ($E = 2$) in our experiments (Supplementary Fig. S2), but due to its reportedly poor stability (Kadow et al., 2012) we chose CPDMO as a candidate for further optimization.

Comparison of Different Expression Systems for CPDMO Performance

We aimed for a process in a minimal medium to enable better scalability and control, using glucose as a defined carbon source, and implementing a secondary substrate limitation (ammonium) to avoid oxygen limitation in standard bioreactors. NADPH-dependent BVMO reactions benefit from high intracellular NADPH concentration (Walton and Stewart, 2004), supported by the proper carbon source. Since the metabolization rate of glycerol is lower than that of glucose, a cultivation of *E. coli* exclusively on glycerol as carbon source may be insufficient for cofactor recycling when high substrate amounts are applied. To this end, the CPDMO gene was recloned from a TOP10 pBAD system into an IPTG-inducible expression vector. Glucose could not be used for the rhamnose-induced pBAD expression system due to inhibited uptake of other sugars by catabolite repression from glucose (Vía et al., 1996).

The newly established expression system pET26b::*cpdmo* in *E coli* BL21 (DE3) was compared in terms of biotransformation velocity to a rhamnose-induced pBAD::*cpdmo* expression system, cloned into leucine-auxotrophic *E. coli* TOP10. Both strains were grown in shake-flask experiments in 10 mL M9 minimal medium, and then tested with 5 mM ketone. Experiments with the TOP10 strain were supplemented with L-leucine. Here, glycerol was used as carbon source for both strains to ensure comparability in metabolic rates.

The pET vector system showed slower biotransformation performance in comparison to the pBAD system (18 vs. 13 h to full conversion), since exogenous supply of L-leucine to the auxotrophic TOP10 strain likely reduced the ribosomal burden and thus led to a faster protein production. We observed that addition of L-leucine to the culture was problematic, since even trace impurities in several batches of high purity grade leucine inhibited growth of the organism significantly (data not shown).Hence, we chose the new pETexpression system with a conversion rate of 0.45 mmol $\mathrm{gCDW^{-1}\,h^{-1}}$ for further development; it did not limit the operational space for a potential process in terms of carbon source and the purity requirements for the additive.

Transfer to a Controlled Bioreactor and Optimization of the Cultivation

We set up a cultivation in a bioreactor on a 4 L scale, monitoring physiological parameters, respiratory activity, and the biotransformation efficiency to establish stable conditions. The growing phase of the culture ended after approximately 17 h, followed a by nitrogen-limited stationary phase. The biomass concentration then remained stable until the end of the experiment after 52 h (1.34 $\mathrm{gCDW\,L^{-1}}$; Fig. 2A). The expected biomass concentration with the applied ammonium concentration of 0.22 $\mathrm{g\,L^{-1}}$ was 1.07 $\mathrm{gCDW\,L^{-1}}$ (Bauer and Shiloach, 1974). According to the fit of the biomass concentration, the maximum growth rate μ_{max} was 0.63 $\mathrm{h^{-1}}$. The oxygen transfer rate (OTR) and carbon dioxide transfer rate (CTR) plots clearly indicated a decline in respiratory activity as a response to induction with IPTG, and to the addition of the ketone (Fig. 2B). The maximum transfer rates were found around the time of induction (approx. 11 $\mathrm{mmol\,L^{-1}\,h^{-1}}$ OTR, approx. 12 $\mathrm{mmol\,L^{-1}\,h^{-1}}$ CTR). The absence of a permanent maximum in the OTR plot indicated that the culture was not oxygen-limited, which would be disadvantageous for our oxygenating synthetic reaction. In the stationary phase OTR values were below 5 $\mathrm{mmol\,L^{-1}\,h^{-1}}$, with average CTR values of 6.5 $\mathrm{mmol\,L^{-1}\,h^{-1}}$.

Overflow metabolites (acetate, pyruvate, and ethanol) were not detected in HPLC analysis, although acetate is known to be formed under nitrogen limitation, even with low glucose consumption (Hua et al., 2004). That was beneficial, since acetate accumulation

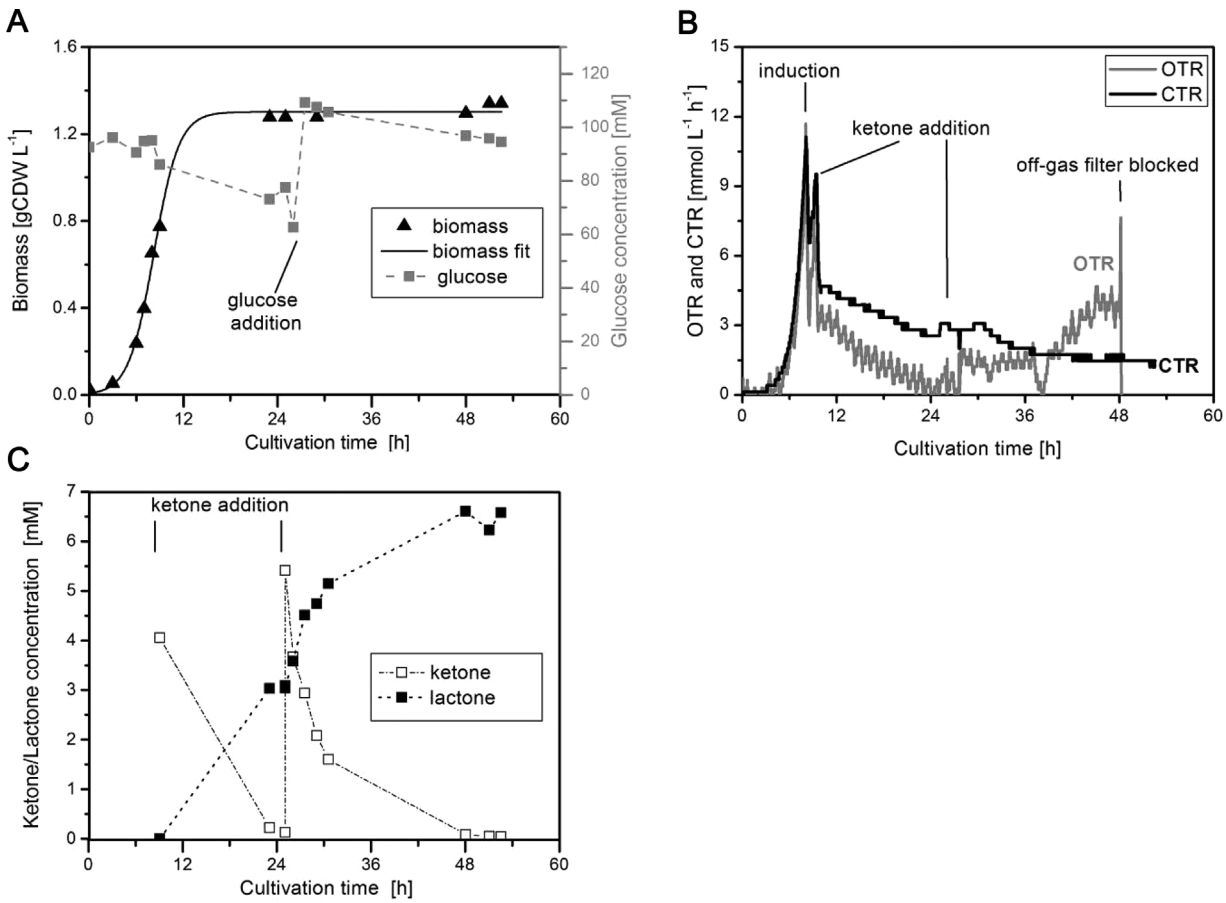

Figure 2. Biotransformation on 4 L scale with simultaneous *E.coli* BL21 (DE3) CPDMO expression during the growth phase. M9 medium with 20 g L^{-1} glucose, 1000 rpm agitation speed, 1 vvm specific aeration, $T_{initial} = 37°C$, $T_{expression} = 24°C$, induction with 0.2 mM IPTG at OD = 1.0. **(A)** Biomass formation, glucose consumption; **(B)** Off-gas analysis of the process (OTR and CTR); **(C)** ketone and lactone concentrations.

inhibits the growth of recombinant *E. coli* cells stronger than of wild-type cells in minimal media (Koh et al., 1992). Carbon balancing calculations revealed that approx. 70 mM carbon was not accounted for (total carbon: 264 mM), leading to the conclusion that overflow metabolites must have been formed, that were possibly not detectable with the applied HPLC protocol. Another possible uncertainty could derive from a different carbon content compared to the value in the literature (Bratbak and Dundas, 1984). The glucose concentration was intermittently monitored, and glucose was added to the medium shortly before the second substrate load to have comparable conditions for the two rounds of biotransformation.

The ketone was added 9 h after inoculation as a 2.5 M solution in dioxane to a final concentration of approx. 4 mM (negligible concentration of the co-solvent, see Supplementary Fig. S3). The substrate was completely consumed after 16 h, yielding exclusively the desired lactone according to GC analysis (Fig. 2C). The culture was then charged with a second load of starting material (to approx. 5 mM) to evaluate its residual activity. Full conversion to the corresponding lactone was reached again after 23 h (from the point of addition). We could not fully balance the reaction mass for ketone and lactone, and attributed that to evaporation of the ketone due to the aeration of the culture, a known issue for volatile ketones in aerated biotransformations (Rudroff et al., 2006). The reaction time for achieving full conversion was only slightly shorter in the late-exponential phase than in the stationary phase.

In summary, the cultivation in the bioreactor on a 4 L scale was successful by operating (i) without any oxygen limitation, which would reduce biotransformation velocity; (ii) without acetate formation, which would inhibit the recombinant system in minimal medium; and (iii) with similar reaction time under growing and non-growing conditions. Since most catalytic turnovers would take place in the stationary phase, after a short growth period, we continued the development for higher productivity by increasing the final concentration of biomass under controlled conditions.

Substrate-Feed-Product-Removal (SFPR) Concept Facilitates Up-Scaling to a Higher Ketone Titer

We opted to increase the reaction rate by applying a higher concentration of biomass via engineering of the cultivation medium, while mitigating negative effects of higher ketone and lactone concentrations in the medium. To tackle the first challenge, we tripled the concentrations of ammonium sulfate (sole nitrogen source) and magnesium sulfate. This way, the culture reached a

threefold higher final value (4.42 gCDW L^{-1} vs. 1.43 gCDW L^{-1} in regular M9). Triplication of magnesium only gave a final titer of 1.70 gCDW L^{-1}, indicating that the limiting secondary nutrient was nitrogen.

Next, we investigated the toxicity and growth inhibition of the ketone on the *E.coli* cells, with and without CPDMO expression, to determine the optimum concentration judged by overall biotransformation performance. In both cultures, with and without inducer, a concentration of 10 mM ketone already decreased the final biomass concentration by 30% in comparison with the reference cultivation without ketone addition. Conversely, our data did not show any acute toxicity effects, even at 20 mM ketone titer (Supplementary Fig. S4); we did not observe increased foaming from cell lysis, any other change in the appearance of the culture, or a decrease in optical density. However, we observed a decrease in growth rate with rising ketone concentrations (Supplementary Fig. S5). The product had no major influence on the growth rate of *E. coli* up to a concentration of 30 mM (data not shown). We contemplated two strategies to overcome the problematic growth inhibition: (i) to add the substrate only when the culture had grown to its final density; or (ii) to implement a concept of feeding the substrate into the biotransformation while simultaneously removing the product. We wanted to circumvent a possible product inhibition, a known obstacle for BVMO-mediated reactions (Doig et al., 2002), and also wanted to reduce the increased evaporation of the starting material due to the gassing of the liquid phase. Additionally, our goal was to enable efficient downstream processing.

We used a substrate-feed-product-release (SFPR) concept (Hilker et al., 2004, 2008; Rudroff et al., 2006), based on the equilibrium of adsorption of substrate and product to an apolar cross-linked polystyrene resin with high specific surface area (Lewatit VP OC 1163). A constant concentration of the organic reactants in the liquid phase would avoid growth-inhibiting concentrations and simplify the downstream processing, since the product would adsorb on the resin material. It was reported earlier that fed-batch operations with removal of the product by heterogeneous adsorption were not as successful as the SFPR concept due to product inhibition (Hilker et al., 2004). Even though a bioprocess without SFPR would be simpler in terms of implementation, it would complicate the downstream processing.

We determined equilibrium load constants X_{eq} for the OCHPN ketone by incubating the resin and various amounts of ketone in medium and analyzing supernatant concentration via calibrated GC. Due to the low toxicity of the product, we did not determine X_{eq} for the product. However, from our separate extraction of the resin and the aqueous phase in downstream processing (see below), we knew that the resin's affinity for the product was equally favorable as for the ketone, or even better; only 8% of total product was recovered from the aqueous phase, corresponding to approx. 4 mM.

The optimized M9 medium in combination with the SFPR concept was applied to convert 42.5 g substrate (281 mmol), adsorbed on the resin (177.1 g, $X_{eq} = 0.40$). A constant substrate concentration of approx. 5 mM (Supplementary Fig. S6) in the liquid phase was maintained, minimizing negative effects of substrate, or product on the culture.

Biomass formed with equal rate as in the previous experiment on a 4 L scale, but reached a higher final value due to the modification of the medium composition (3.49 gCDW L^{-1}). We added a higher amount of glucose (~110 mM) at the beginning, sufficient to maintain the cofactor recycling for the first 46 h, plus two more additions in the stationary phase (Fig. 3A). The fit of the biomass concentration showed a maximum growth rate μ_{max} of 0.82 h^{-1}, higher than in the up-scaling experiment. The higher growth rate likely occurred due to a higher concentration of ammonium salt in the medium. During the stationary phase, the glucose concentration was intermittently monitored with glucose test strips (Accu-Chek, Roche, Basel), and glucose was added to the medium to keep the glucose concentration above approx. 18 mM to maintain the cells' cofactor recycling system.

OTR and CTR values were highest in the exponential phase (20 mmol L^{-1} h^{-1}). In the stationary phase rates of 10–15 mmol L^{-1} h^{-1} were reached, which directly correlated to the triplication of biomass concentration compared to the previous upscaling experiment. Still, no oxygen limitation or overflow metabolites were observed during the entire cultivation. Especially oxygen limitation can be problematic in SFPR BVMO processes at a high cell density (Hilker et al., 2006). Again, although no overflow metabolites could be detected using HPLC, carbon balancing revealed a gap of 197 mM carbon missing from the balance (total carbon: 732 mM). The unaccounted amount was directly proportional to the triplication in biomass (70 mM carbon were missing with 1.34 gCDW L^{-1} in the previous cultivation). We attributed this inaccuracy to a problem in resolution of the peak for ethanol in the HPLC chromatograms.

Directly after substrate addition, higher OTR values were observed: we could monitor the consumption of molecular oxygen by the enzymatic BVOx, and thus the reaction progress, via the OTR signal at this higher substrate load. As substrate conversion proceeded, less molecular oxygen was consumed by the CPDMO-catalyzed reaction, and the OTR values gradually converged with the CTR values; they were identical after 42 h (Fig. 3B). Measurement of the conversion progress in the culture broth medium and on the resin material also showed stagnation of the reaction rate (Fig. 3C).

The conversion in the medium was very fast (>90% after 24 h). The ratio of product to substrate on the resin increased with a slower rate, which suggested that the reaction was limited by the exchange of ketone and lactone on the adsorbed material, and not by the enzymatic reaction itself.

Throughout the reaction a considerable amount of the resin was unintentionally deposited on the upper walls and the lid of the bioreactor as a result of agitation and aeration. It could thus not directly interact with the biotransformation mixture anymore. Therefore, after 24.5 and 26 h we briefly opened the reactor and manually washed down the deposited material, showing as spikes in the OTR and CTR signals. After the first resuspension of the resin, the conversion of the starting material on the resin increased, but did not change in the liquid phase (Fig. 3C). Then, after the second resuspension, the conversion on the resin material dropped from 80% to 70%, showing that the resin deposited on the walls and lid had indeed not taken part in the reaction. The experiment was stopped after both liquid and resin analyses showed a conversion of >90% after 52 h.

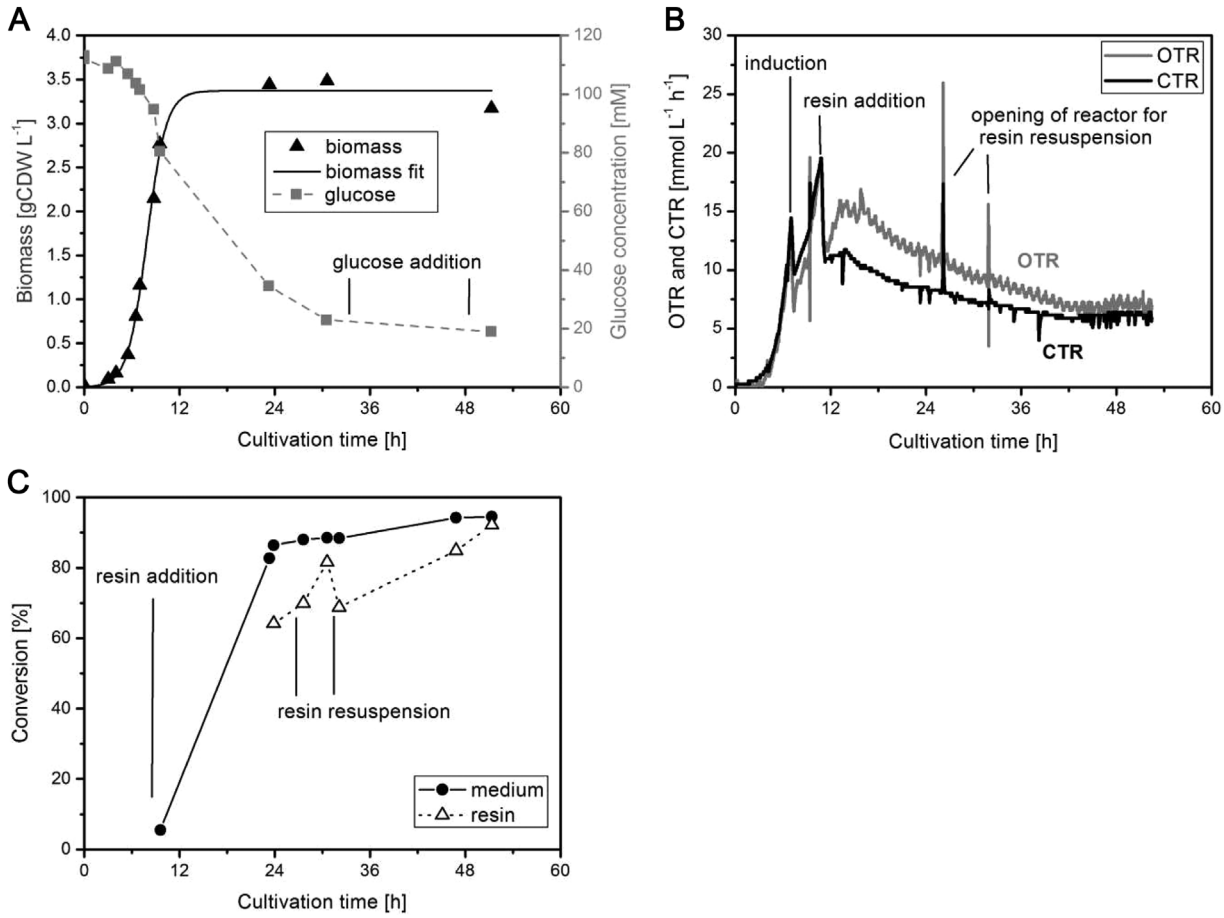

Figure 3. Biotransformation on a 4 L scale with ketone bound to Levatit® VP OC 1163 resin material. Modified M9 medium (3x $MgSO_4$ and 3x $(NH_4)_2SO_4$) with 20 g L^{-1} glucose, 1000 rpm agitation speed, 1 vvm specific aeration, $T_{initial} = 37°C$, $T_{expression} = 24°C$, induction with 0.2 mM IPTG at OD = 2.6. **(A)** Biomass formation and glucose concentration (immediate effect of glucose pulses on glucose concentration was not observed due to the sampling interval/constant consumption by *E. coli* cells). **(B)** Off-gas analysis (OTR and CTR), **(C)** Relative conversion of ketone to lactone in the liquid phase (by absolute quantification of using calibrated GC), and on the resin material (by relative quantification using GC) during the cultivation (see Materials and Methods for details).

We tested the residual biotransformation activity by adding ketone (5 mM) to a 10 mL sample of the culture broth: the biotransformation velocity was 0.29 mmol $gCDW^{-1}\,h^{-1}$, corresponding to 64% of the velocity measured for the pET system and glucose. The duration of the biotransformation could therefore have been prolonged, eventually even with a higher resin concentration. A total of 70 mM substrate in the biotransformation mixture (approx. 5 mM in the liquid phase) was approx. three times higher than a previously published value of a SFPR-based scale-up with a BVMO (Geitner et al., 2010).

In general, the efforts in up-scaling of the biotransformation successfully led to stable process parameters and a stable enzymatic activity over 52 h, and even beyond. We next focused on the development of an efficient product isolation and purification protocol.

Downstream Processing

We chose MTBE as the solvent for all extraction steps: it was suitable for the extraction of OOPN lactone (data not shown), and it was highly scored by the ACS GCI Pharmaceutical Roundtable Solvent Selection Guide. Ethyl acetate—another suitable solvent for OOPN lactone—was ranked lower with respect to waste production (GCIPR, 2011). We wanted to compare the possible ways of product recovery (step-wise vs. continuous extraction; separate extraction of the liquid; and solid phases) for their efficiency, and divided the culture into equal fractions. The cultivation broth and the resin were treated separately. The resin was collected by coarse filtration and then continuously extracted in a cellulose thimble using a Soxhlet apparatus. The first 20 extraction cycles yielded 39.0 g of crude product, another 40 cycles with fresh MTBE gave an additional 3.0 g. Pooled together, the two fractions had a lactone content of 75% w/w in the crude product (31.5 g, calculated value).

The supernatant of the centrifuged culture broth was divided in two equal fractions. One was manually extracted three times, yielding 0.9 g crude product (84% w/w lactone). The other was continuously extracted in a perforator, yielding 2.2 g with 93% w/w lactone content. These values clearly showed that a continuous extraction is mandatory, if the lactone should be efficiently

recovered from the medium (fourfold higher extraction efficiency for the lactone with equal solvent consumption). Overall, 93% of the total recovered material were collected from the resin (86% from the first 20 cycles of extraction, another 7% from additional 40 cycles). The medium only held 7% of the crude product mass (8% of final product mass by calculation).

All crude fractions were pooled, and a total lactone content of 74% was determined. The lactone was separated from the ketone by short-path distillation under vacuum, and subsequently purified to >99% (GC) by recrystallization. Overall, a high efficiency of 95% was reached in the DSP: 70% isolated yield from 74% content in the crude reaction product.

We were unable to avoid the incomplete conversion of ketone during the reaction, even though the walls and the lid were rinsed twice to wash the resin back into the culture. Not all of the material was re-submerged again during these washing steps. This problem could be tackled by specific reactor design: implementation of an appropriate macroporous containment for the resin beads would prevent their permanent separation from the aqueous phase, while still allowing equilibration of the adsorbed molecules with the solution. We calculated a volumetric yield of $8.2\,g\,L^{-1}$, based on isolated pure product from the 42 g ketone batch. Additionally, the extraction solvent MTBE was recycled to 84% with purity indistinguishable by NMR from the purchased material.

Conclusion

We successfully developed a non-hazardous process for the synthesis of a Nylon-9 precursor using BVMO catalysis, and translated the reaction from initial catalyst discovery stage to the multi-dozen gram scale under controlled conditions. The non-stereoselective enzyme CPDMO was successfully implemented as whole-cell biocatalyst in a minimal medium. Reaction engineering and bioprocess control enabled a biotransformation on the 42.5 g (281 mmol) scale in a 4 L bioreactor, converting the substrate in the SFPR–batch culture to 74% in 52 h. We showed that longer reaction times were possible, and that reactor design issues were the main impediment to a complete conversion of starting material. Continuous extraction of the polymer resin quickly recovered 85% of the total product; the organic solvent could also be recycled to a high extent. The product was then isolated from the crude in >99% purity using short-path distillation and recrystallization in 70% isolated yield. Overall, the optimized biotransformation conditions, together with the efficient downstream processing (95% mass efficiency from crude to pure product), resulted in a volumetric yield of 8 g pure lactone per liter culture, without chromatographic separation. Future BVMO-upscaling studies can benefit from the possibility to monitor the reaction via off-gas analysis and the stable biotransformation conditions described in this paper.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

The supporting information contains detailed descriptions of experimental methods and used materials, and additional data for biocatalyst screenings, effect of the applied dioxane concentration on *E. coli* growth, effect of OCHPN ketone on *E. coli* growth, and a loading study of ketone on resin material with concentration determination in the liquid phase.