Research Article

Received: 30 April 2021 Revised: 7 July 2021 Accepted article published: 12 July 2021 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jctb.6851

Oxidation of cadaverine by putrescine oxidase from *Rhodococcus erythropolis*

Valentine E Anyanwu,[a,b] Stephen J Hall,[a] Gill Stephens[a] and Anca Pordea[a*]

Abstract

BACKGROUND: Putrescine oxidase (EC 1.4.3.10) is of interest for the microbial production of unsubstituted platform nitrogen (*N*-)heterocycles, because it only requires inexpensive oxygen as co-substrate. Putrescine oxidase from *Rhodococcus erythropolis* (Re-PuO) was shown previously to catalyze the oxidation of cadaverine; however, there is little information in the literature about the robustness of this enzyme for biotechnological applications. The aim of this study was to investigate the suitability of Re-PuO for the bioproduction of 1-piperideine from cadaverine under different reaction conditions.

RESULTS: The formation of 1-piperideine catalyzed by Re-PuO was demonstrated using *o*-aminobenzaldehyde as a reagent to trap the cyclic imine and shift the equilibrium for cyclization. A direct assay of Re-PuO activity for cadaverine oxidation was then implemented, by monitoring oxygen consumption. Characterization of the reaction mixture by ^{1}H NMR and mass spectrometry confirmed the presence of piperideine dimers and trimers, yet the quantification of the reaction products could not be achieved. The optimum temperature and pH conditions for enzyme activity were determined as 55 °C and 8.5, respectively. At pH 7.5, the enzyme retained its activity after 65 h incubation at 25 °C, but lost 75% of its activity after 1 h incubation at 55 °C. The enzyme showed no substrate inhibition at concentrations as high as 100 mmol L^{-1} cadaverine. Complete biotransformation of cadaverine was observed in whole cells at physiological conditions.

CONCLUSIONS: These results successfully demonstrate the potential of putrescine oxidase for the bioproduction of *N*-heterocycles from cadaverine.

Supporting information may be found in the online version of this article.

Keywords: putrescine oxidase; cadaverine oxidation; whole-cell biotransformation; 1-piperideine

INTRODUCTION

Nitrogen (*N*-)heterocycles are valuable building blocks for the synthesis of high-value chemicals, with piperidine being one of the most frequently encountered N ring scaffolds in small molecule active pharmaceuticals.[1] One starting point for the synthesis of functionalized piperidine compounds is the unsaturated *N*-heterocycle, 1-piperideine (2,3,4,5-tetrahydropyridine or Δ^{1}-piperideine), which has been reported as an intermediate in the organic synthesis of structurally diverse heterocycles.[2-5] Owing to the chemical instability of 1-piperideine, this compound is generally formed *in situ* and characterized as a mixture with its dimeric and tetrameric forms.[3]

Current synthetic methods for the formation of 1-piperideine rely on piperidine oxidation with harsh chemicals, via *N*-chlorination and base-mediated HCl elimination.[3,4] However, the typical route in nature for the biosynthesis of this structure is derived from lysine metabolism, where lysine is decarboxylated to cadaverine, which in turn is cyclized upon oxidation.[6,7] As a result, 1-piperideine is a common intermediate of the biosynthesis of lysine-derived alkaloids, such as quinolizidine, indolizidine or lycopodium alkaloids.

The general vision of the current work was to study the enzymatic production of simple *N*-heterocycles. In nature, the oxidation of diamines, such as cadaverine or putrescine to the corresponding mono-aldehydes, followed by spontaneous cyclization to the imine heterocycle is catalyzed by transaminases or by amine oxidases.[8-11] Transaminases are enzymes that convert aldehydes and ketones into amines. They catalyze the transfer of an amino group from (di)amine donor molecules to carbonyl substrate acceptors, by using pyridoxal-5′-phosphate (PLP) as a cofactor.[12] However, flavin-dependent amine oxidases use molecular oxygen (O_2) to oxidatively deaminate the substrate.[13-15]

* *Correspondence to: A Pordea, Faculty of Engineering, University of Nottingham, University Park, NG7 2RD, Nottingham, UK. E-mail: anca.pordea@nottingham.ac.uk*

a *Sustainable Process Technologies, Faculty of Engineering, University of Nottingham, Nottingham, UK*

b *Molecular Therapeutics and Formulation, Schools of Pharmacy, University of Nottingham, Nottingham, UK*

Inspired by these alkaloid biosynthetic pathways, the biocatalytic synthesis of *N*-heterocycles using enzymatic cascades has attracted recent interest, and has involved transaminases or amine oxidases, either as isolated enzymes or in whole cells.[16-19] The requirement for O_2 as electron acceptor, instead of PLP and an additional carbonyl substrate makes the use of amine oxidases relatively inexpensive and better suited for applications in microbial production of heterocycles using engineered pathways compared with transaminases, because it affords better carbon efficiency and, thus, reduced energy demand and metabolic pressure on the cell. Therefore, we selected an amine oxidase for use in this study.

Previously, Fraaije and co-workers discovered and characterized the putrescine oxidase (EC 1.4.3.10) from *Rhodococcus erythropolis* (Re-PuO).[20] This Flavin adenine dinucleotide (FAD)-dependent enzyme was shown to oxidize aliphatic diamines and to be relatively stable ($t_{1/2}$ = 2 h at 50 °C). The optimum temperature was found to be 30 °C, whilst the optimum pH for the oxidation of putrescine was reported to be pH 8, with no significant activity detected below pH 6.4. Putrescine was the best substrate, whilst the k_{cat} for cadaverine was >6.5-fold lower, and the K_M was more than two-fold greater than that for putrescine. The kinetic parameters and the temperature- and pH-dependence were determined using a coupled enzymatic assay, where horseradish peroxidase was used to determine the rate of hydrogen peroxide (H_2O_2) release from the reaction. The narrow substrate specificity was explained by the crystal structure, showing that the interaction between the nonoxidized amino group and a glutamate residue (Glu324) was responsible for determining the chain length of the aliphatic substrate that can fit into the active site.[14] The kinetic mechanism of Re-PuO also was studied by the same group by pre-steady state kinetic analysis, showing that, at high oxygen concentrations, an enzyme–product–O_2 complex was likely to form, whilst at low O_2 concentration the product was likely to be released before flavin re-oxidation by O_2.[13,16]

So far, only few biocatalytic approaches for the production of simple *N*-heterocycles have considered the use of putrescine oxidase.[19] Although Re-PuO was shown to catalyze the oxidation of cadaverine, product formation was not directly monitored and most catalytic assays relied on a coupled enzyme approach. Furthermore, there is little information in the literature about the robustness of this enzyme for biotechnological applications. Therefore, the aim of this work is to study the activity of putrescine oxidase from *Rhodococcus erythropolis* under conditions relevant to biotechnological applications, namely high substrate concentrations and a range of temperatures and pH values. The implementation of a direct enzymatic assay and the formation of 1-piperideine from cadaverine also was investigated. Our final goal was to demonstrate the oxidation of cadaverine in whole cells containing overexpressed putrescine oxidase.

MATERIALS AND METHODS

Bacterial strains and plasmids were purchased from Novagen (Merck Biosciences, Merck KGaA, Darmstadt, Germany). Materials for crude enzyme extraction, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), protein concentration and purification were purchased from Merck Millipore (Merck Biosciences, Merck KGaA, Darmstadt, Germany), GE Healthcare (now Cytiva, Marlborough, Massachusetts, US) and Bio-Rad (Hercules, California US). All other analytical grade chemicals used were purchased from Sigma-Aldrich, unless otherwise stated.

Expression of putrescine oxidase

The *R. erythropolis* gene encoding putrescine oxidase (GenBank accession no. EU240877), optimized for expression in *Escherichia coli*, was synthesized by Biomatik with the inclusion of *Nde*I and *Xho*I restriction sites to facilitate insertion into a pET-20b(+) plasmid, adapted for *C*-terminal histidine tagging. The plasmid construct was used to transform *E. coli* BL21 (DE3) and an empty plasmid was used as a negative control. Experimental cultures (in 50–250 mL Erlenmeyer flasks) were prepared from overnight precultures (in 50 mL Falcon tubes) grown at 37 °C and 250 rpm, which were derived by inoculation with single colonies grown overnight (16–18 h) on Luria–Bertani broth (LB) agar at 37 °C. All media were autoclaved at 121 °C for 15 min whereas antibiotics, isopropyl-β-D-1-thiogalactopyranoside (IPTG) and glucose solutions were filter-sterilized. The LB [10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 5 g L^{-1} sodium chloride (NaCl)] or Luria–Bertani agar (LB and 15 g L^{-1} bacto agar) used were supplemented with carbenicillin (50 μg mL^{-1}) and glucose (10 g L^{-1}). To prepare a standardized inoculum without toxic metabolites and spent medium, the precultures were collected aseptically by centrifugation (4032×*g*, 4 °C, 15 min), cell pellets were resuspended aseptically in 1 mL fresh sterile LB broth, and an appropriate aliquot was used for inoculation of expression cultures to an initial OD_{600} of 0.1. All expression experiments were conducted in 250- or 500-mL Erlenmeyer flasks containing 50 or 100 mL medium, respectively, at 25 °C and 250 rpm, because these conditions gave optimal expression of soluble protein (see Supplementary information, SI). Protein expression was induced by the addition of IPTG (0.4 mM) at an OD_{600} of approximately 0.6. Cells were harvested 5 h after induction by centrifugation at 4032×*g* and 4 °C for 15 min, resuspended in 100 mmol L^{-1} sodium–potassium (Na/K) phosphate buffer pH 7.5 and centrifuged. This washing process was repeated three times and the cell pellets were used for whole-cell biotransformation or for enzyme purification immediately or stored at −80 °C and used within 24 h.

Purification of putrescine oxidase

Cell pellets were resuspended in 1 mL buffer A (20 mmol L^{-1} Na phosphate buffer, 500 mmol L^{-1} NaCl, 30 mmol L^{-1} imidazole pH 7.4). Benzonase® nuclease (0.1 μL) and a half tablet of protease inhibitor cocktail were added to the resuspended cell pellet before lysing the cells by a pressure drop from 1724 bar using a Constant Systems cell disrupter (before the use of this system, all detachable components were placed on ice for 10–15 min). Cell debris was removed by centrifugation at 18 000×*g* and 4 °C for 10 min. The clear supernatant was purified by immobilized nickel ion affinity chromatography at 4°C, using an AKTA protein purification system (GE Healthcare). Crude extracts (5–10 mL) were applied to a HisTrap FF crude column (1 mL) using buffer A, and proteins were eluted on a linear gradient with buffer B (20 mmol L^{-1} Na phosphate buffer, 500 mmol L^{-1} NaCl, 500 mmol L^{-1} imidazole pH 7.4) at a flow rate of 1 mL min^{-1}. Elution was monitored by absorbance at 280 nm (see SI). Fractions (2 mL) were analyzed by SDS-PAGE (see analytical methods), and those showing bands equivalent to the molecular weight of Re-PuO (≈50 kDa) were combined (see SI). Purified protein was desalted by exchanging buffer B with 100 mmol L^{-1} Na/K phosphate buffer pH 7.5 by several concentration/dilution cycles using a Vivaspin® sample concentrator. The purified protein was quantified using the DC™ protein assay kit (Bio-Rad) with bovine serum albumin (BSA) as a standard.

o-Aminobenzaldehyde (*o*-ABA) coupled colorimetric assay

The reaction mixture contained 100 mmol L^{-1} Na/K phosphate buffer pH 7.5, putrescine or cadaverine substrate (1 mmol L^{-1}) and 1 mmol L^{-1} *o*-ABA. The phosphate buffer was saturated with

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

air by continuous bubbling of compressed air before use. Purified Re-PuO (0.1 mg mL^{-1} final concentration) was used to start the reaction. The change in absorbance was monitored at 30 °C for 5 min, by UV–visible spectrophotometry in 1-mL quartz cuvettes, at 435 nm when putrescine was used as substrate and at 465 nm when cadaverine was used. For the orange cadaverine adduct, an extinction coefficient of 2800 L mol^{-1} cm^{-1} at 465 nm was used.[21] For the yellow putrescine adduct, an extinction coefficient of 1860 L mol^{-1} cm^{-1} at 435 nm was used.[22]

Oxygen consumption assay

The rate of O_2 consumption was monitored using a Clark-type O_2 electrode (Oxygraph, Hansatech Instruments Ltd, Norfolk, UK). The cylindrical reaction vessel was fitted with a small magnetic stirrer and maintained at the relevant temperature using a heated circulating water-bath (Grant TC120). The assays contained 100 mmol L^{-1} Na/K phosphate buffer at the relevant pH (6.5; 7.5; 8.5 or 9.5), temperature (25; 30; 37; 45; 55; 65 or 75 °C) and putrescine or cadaverine substrate (1 mM or 0.8 mM), as described in the text. The phosphate buffer was saturated with air by continuous bubbling of compressed air before use. Purified Re-PuO (0.1 mg mL^{-1} final concentration) was used to start the reaction.

For the determination of Michaelis–Menten parameters, the assays contained 100 mmol L^{-1} Na/K phosphate buffer pH 7.5 and putrescine or cadaverine substrate (0.1–1 mmol L^{-1}). The phosphate buffer was saturated by continuous bubbling of compressed air (0.24 mmol L^{-1} O_2) before use. Purified Re-PuO (0.1 mg mL^{-1} final concentration in the cuvette) was used to start the reaction. Maximum velocities (V_{max}) and Michaelis constants (K_M) were estimated using the Michaelis–Menten model of PRISM v8.4.2 (GraphPad).

Whole-cell biotransformation

Reaction mixtures (10 mL) containing harvested cells (dry cell weight, 1.0 ± 0.1 mg mL^{-1}), cadaverine (5 mM) and 100 mmol L^{-1} Na/K phosphate buffer pH 7.5 were incubated in Erlenmeyer flasks (50 mL) at 25 °C and 250 rpm. Samples were collected aseptically at intervals and were centrifuged at 4032×*g*, 4 °C for 10 min. The supernatant was collected in a glass syringe, filtered using a 0.22-µm syringe filter and analyzed immediately or stored at −20 °C and analyzed within 24 h.

Analytical methods

Plasmid concentration was determined using a NanoDrop ND1000. The designed pET-20b(+)_ puo_{Rh} sequence was verified using Sanger sequencing services provided by Eurofins Genomics (UK Ltd). Spectrophotometric measurements were made using a Shimadzu UV-2600 UV–visible spectrophotometer coupled to heated circulating water-bath (Grant TC120) and a 1.0-cm light path was used for all measurements. Growth of recombinant cells were determined by measuring the optical density (OD; absorbance) at 600 nm (OD_{600}nm). Samples (1 mL) collected aseptically at hourly or stated intervals were transferred to cuvettes and the OD measured. Where the reading was outside of a range from 0.0–0.8, samples were 10-fold diluted (1 in 10) using LB medium to achieve OD_{600nm} within the specified range. Cell weight measurements were made in pre-weighed microcentrifuge tubes that had been dried by incubating in the presence of dry silica in a sealed container. Samples (1 mL) were harvested by centrifugation (18 000×*g*, 2 min) and the supernatant discarded. The centrifugation step was repeated, and any residual medium removed using a pipette. For the dry cell weights, the cell pellets were incubated at 70 °C to constant weight, then weighed again. The difference between the empty microcentrifuge tube and the cell pellet was used to calculate the cell weight per litre of culture.

For Re-PuO expression and purification analysis, SDS-PAGE was conducted with a Mini-Protean electrophoresis system using TGX precast gels. Cell samples (1 mL) were harvested by centrifugation (4032×*g*, 5 min, 4 °C) and extracts prepared using BugBuster® protein extraction reagent. Soluble protein (clear supernatant) was analyzed in comparison to insoluble (total) protein (resuspended cell sediments). Samples from protein purification process were used directly. All protein samples (20 µL) were mixed with 20 µL Laemmli sample buffer (contained 950 µL Laemmli buffer and 50 µL *β*-mercaptoethanol). Thereafter, the mixture was boiled at 105 °C for 5 min and stored on ice for 5–10 min. Subsequently, 10 µL of the prepared samples were loaded in each well and the electrophoresis was run in 1 × TGS (Tris-Glycine-SDS) running buffer at 150 V for 45–60 min. Thereafter, the gel was washed three times with distilled water and stained overnight with EZBlue Stain (Bio-Rad) or QC colloidal Coomassie protein stain. The solubility of the enzyme was estimated according to the fraction (soluble or insoluble) in which the protein band corresponding to the size of the enzyme was thickest by visual inspection. Pictures of the gels were obtained an uGenius Gel Documentation System (Syngene).

Cadaverine was analyzed by high-performance liquid chromatography (HPLC), after derivatization with dansyl chloride. The sample (1 mL) was mixed with saturated solution of Na_2CO_3 (0.5 mL), dansyl chloride solution (1 mL of 5 mg dansyl chloride in 1 mL acetone), vortexed for 1 min and then incubated for 1 h in the dark at 40 °C. After derivatization, 250 µL ammonia (NH_3) was added, the mixture vortexed for 1 min and incubated in the dark at room temperature for 15 min to remove excess dansyl chloride. Samples (10 µL) were analyzed using HPLC (Agilent Technologies 1200 series) on a Zorbax Eclipse XDB-C18 column (4.6 mm × 250 mm, particle size 5 µm) using a water/acetonitrile gradient at a flow rate of 0.8 mL min^{-1} starting with 65% of acetonitrile for 15 min, increasing to 80% acetonitrile after 15 min, to 100% after 20 min and held constant thereafter. Sample components were detected using a UV–visible detector at 254 nm.

RESULTS AND DISCUSSION

Enzymatic assays of Re-PuO and detection of oxidized products

The *R. erythropolis* gene encoding putrescine oxidase was optimized for expression in *E. coli* and cloned within the pET-20b(+) expression vector, which allowed the introduction of an *C*-terminal hexahistidine tag (see SI). The recombinant enzyme (Re-PuO hereafter) was expressed in the soluble form in *E. coli* BL21(DE3), after induction with 0.4 mmol L^{-1} IPTG at 25 °C. The fraction of soluble protein increased over time with increasing shaking speeds (see SI). Re-PuO was purified from the cell free extract by immobilized nickel affinity chromatography. As expected, the purified protein exhibited the characteristic UV–visible spectrum of a flavoprotein containing the oxidized FAD cofactor, with absorbance bands at 365 and 457 nm, which disappeared upon cofactor reduction by the addition of a saturating amount (30 mmol L^{-1}) of cadaverine substrate (see SI).[20]

Previous characterization of Re-PuO was performed using an indirect, coupled horseradish peroxidase (HRP) assay at pH 8, where the rate of H_2O_2 release was quantified by the HRP-catalyzed formation of a coloured product.[16,20] We characterized

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

the enzyme using a temperature of 30 °C and a Na/Kphosphate buffer at pH 7.5. To allow for direct measurement of heterocycle product formation, the characterization of Re-PuO was attempted initially by an established coupled colorimetric assay.[22,23] This assay relied on the formation of coloured adducts resulting from the rapid, noncatalyzed reaction of *o*-ABA with the oxidation products 1-piperideine and 1-pyrroline, formed during the PuO-catalyzed oxidation of cadaverine and putrescine, respectively [Fig. 1(a) and SI]. The formation of these adducts was monitored at 465 nm (for cadaverine) and 435 nm (for putrescine) to determine the enzymatic activity of purified Re-PuO [Fig. 1(b)]. With cadaverine, there was an initial lag, suggesting that the slow and/or incomplete formation of the orange adduct might be rate-limiting. The lag could be shortened partially when temperature, enzyme and *o*-ABA concentrations were increased (results not shown). Although this assay confirmed that cadaverine was converted to 1-piperideine, the lag hindered the reliable determination of the enzymatic activity for cadaverine oxidation using this method. Therefore, O_2 consumption was used as an alternative measure of Re-PuO activity and showed no lag phase with either substrate [Fig. 1(c)].[16] By contrast with data published previously, no protein precipitation was observed due to H_2O_2 accumulation under these conditions. The activity of the enzyme determined by direct O_2 consumption was two- to four-fold higher than the activity determined by the colorimetric reaction with *o*-ABA in the case of both substrates, further supporting the hypothesis that the kinetics of the coloured adduct formation had a limiting effect on the overall reaction and, therefore, hindered the accurate determination of the enzymatic activity. As expected, Re-PuO revealed a better activity for putrescine than for cadaverine, in agreement with reports published previously.[20]

Furthermore, the steady-state kinetic parameters of Re-PuO at pH 7.5, determined by the O_2 consumption assay, were $K_M = 0.24$ mmol L^{-1} and $V_{max} = 1.36$ nmol O_2 mL^{-1} s^{-1} for cadaverine and $K_M = 0.17$ mmol L^{-1} and $V_{max} = 7.36$ nmol O_2 mL^{-1} s^{-1} for putrescine (see SI). This result agrees with previous data that Re-PuO has a better catalytic efficiency (k_{cat}/K_M) for putrescine than for cadaverine.

Furthermore, the activity of Re-PuO increased to 10 mmol L^{-1} cadaverine and then remained constant at cadaverine concentrations ≤100 mmol L^{-1}; no inhibition was observed. To the best of our knowledge, this is the first report of cadaverine oxidation at substrate concentrations >10 mmol L^{-1}.

Attempts were made to characterize the reaction products from the enzymatic reactions. Under aqueous conditions, the unstable imine product from the enzymatic oxidation rapidly hydrolyses to aldehyde, which spontaneously cyclizes to 1-piperideine [Fig. 1 (a)]. This compound also is unstable and has been shown to form dimers and trimers, the speciation of which depends on both the pH and the temperature of the solution.[3] The formation of stable trimers was used previously as a way to displace the reaction equilibrium during ω-transaminase catalyzed reactions with cadaverine as a smart amine donor.[10] LC–MS analysis of the PuO-catalyzed oxidation of cadaverine showed the presence of dimers and trimers of 1-piperideine after 15 min (see SI). Direct analysis by ^{1}H nuclear magnetic resonance (NMR) of aqueous reaction samples after 24–40 h reaction at 25 °C and pH 7.5 confirmed the presence of trimers, indicated by characteristic peaks of isotripiperideine (≈2.5–2.6; 3.1 and 3.7 ppm) and potentially tripiperideine (2.7 and 3.1 ppm). However, a clear assignment of the peaks and the subsequent quantitative determination of the mixture composition was not possible due to the complexity of the

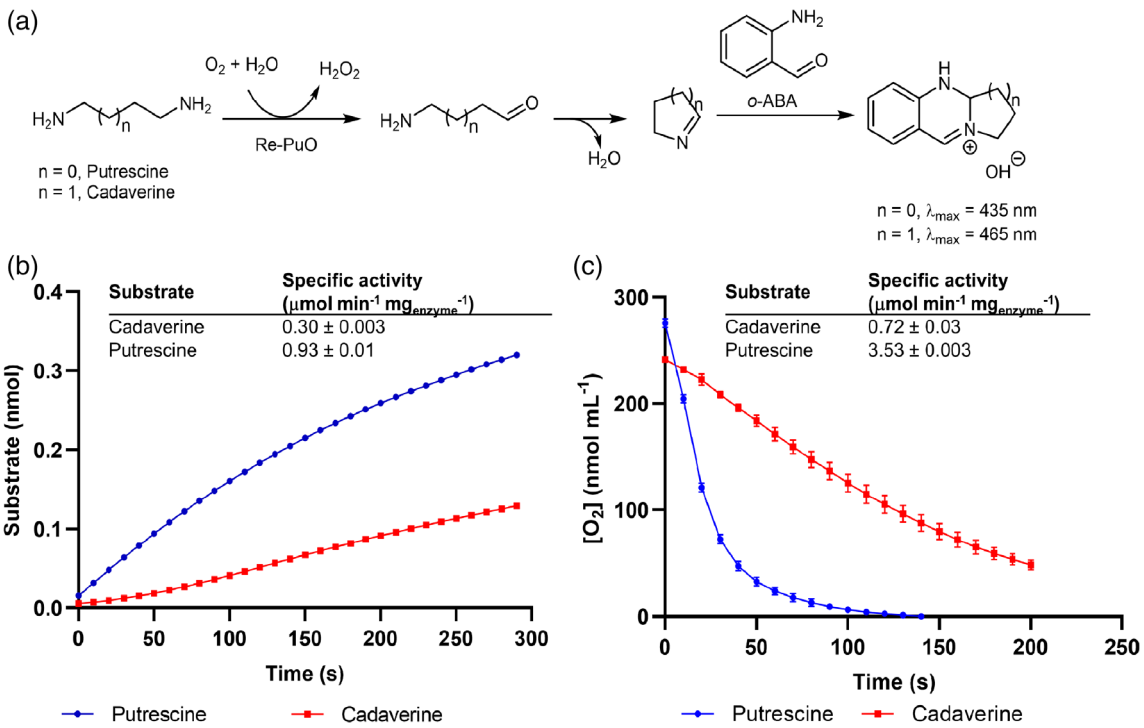

Figure 1. Enzymatic assays of putrescine oxidase from *R. erythropolis*. (a) Enzymatic oxidation of putrescine and cadaverine followed by cyclization to form 1-pyrroline and 1-piperideine and subsequent reaction with *o*-ABA to give coloured dihydroquinazolinium adducts; (b) *o*-ABA coupled assay; (c) O_2 consumption assay. Assays were performed with purified Re-PuO (0.1 mg mL^{-1}) in 100 mmol L^{-1} Na/K phosphate buffer (pH 7.5) at 30 °C, with a substrate concentration of 1 mmol L^{-1}. before to the assay.

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

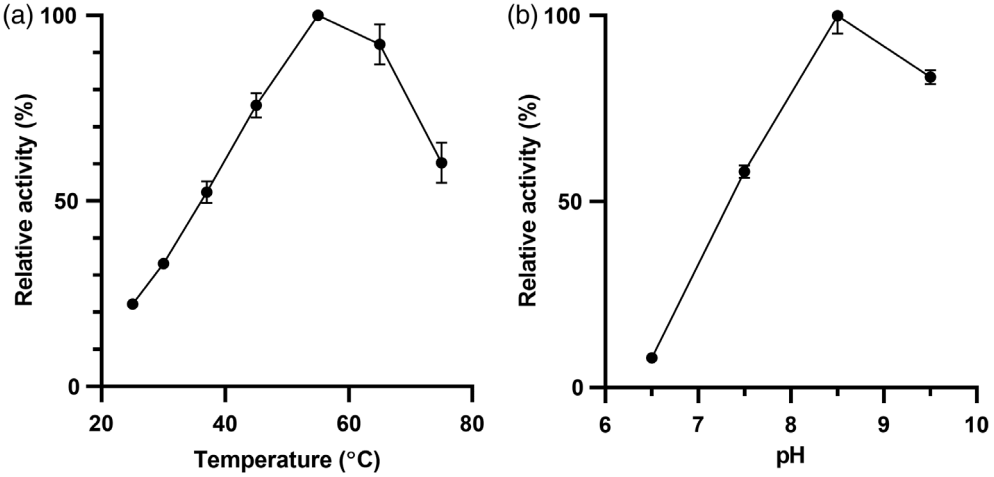

Figure 2. Effect of (a) temperature and (b) pH on the activity of Re-PuO. The oxidation of cadaverine (0.8 mmol L^{-1}) by Re-PuO (0.1 mg mL^{-1}) was measured in 100 mmol L^{-1} Na/K phosphate buffer by monitoring the rate of O_2 consumption at different temperatures and pH values. For the variation of temperature, a pH of 7.5 was maintained. For the variation of pH, a temperature of 30 °C was maintained. In each case, relative activity was calculated as a percentage of the highest activity: for (a) at 55 °C, specific activity 9.05 ± 0.5 μmol O_2 min^{-1} $mg_{enzyme}{}^{-1}$; and for (b) at pH 8.5, specific activity 20.7 ± 0.70 μmol O_2 min^{-1} $mg_{enzyme}{}^{-1}$.

mixture (see SI). The quantitative ^{1}H NMR analysis also confirmed >85% consumption of cadaverine after 40 h reaction at 25 °C and pH 7.5, with 27 mmol L^{-1} initial cadaverine concentration and 1 mg mL^{-1} Re-PuO.

Temperature- and pH-dependence of Re-PuO activity

The activity of Re-PuO was determined at different temperatures and pH values, in order to identify the optimum reaction parameters (Fig. 2). At pH 7.5, the O_2 consumption activity with cadaverine as substrate increased with temperature up to 55 °C, and the enzyme continued to be active up to 75 °C [Fig. 2(a)]. This contrasts with previous reports that 30 °C is the optimum temperature for enzyme activity; however, this result was determined using HRP-coupled assay.[20] The optimal activity of HRP is 30 °C, and HRP denatures at temperatures >42 °C,[24] so this might explain the difference between our results and the temperature optimum reported previously. As expected, a decrease in enzyme activity was observed with increased acidity; the optimum pH was 8.5 at all of the temperatures studied [Fig. 2(b)]. Furthermore, the enzyme activity remained unaffected for ≤65 h when stored at 25 °C and at a pH range of 7.5–9.5. However, stability decreased with increase in temperature, and a sharp decrease of enzyme activity was observed after incubation at the optimal temperature of 55 °C. At this temperature, ≈75% of activity was lost after 1 h incubation at pH 7.5, whilst at pH 9.5, 95% activity was lost after 1 h and complete inactivation was observed after 2.5 h. In conclusion, the optimal conditions for Re-PuO activity decrease the enzyme's stability, which may hinder the use and re-use of the isolated enzyme. Thus, the use of harvested whole cells containing overexpressed Re-PuO offers an alternative approach.

Oxidation of cadaverine by harvested cells

The aim of this study was to demonstrate the biocatalytic synthesis of the unsaturated *N*-heterocycle 1-piperideine, which is a starting point for the synthesis of functionalized piperidine compounds. Therefore, biocatalysis in whole cells was performed using cadaverine as a substrate for oxidation. The cadaverine oxidation activity of Re-PuO was investigated with harvested, recombinant whole cells expressing Re-PuO, by monitoring cadaverine consumption

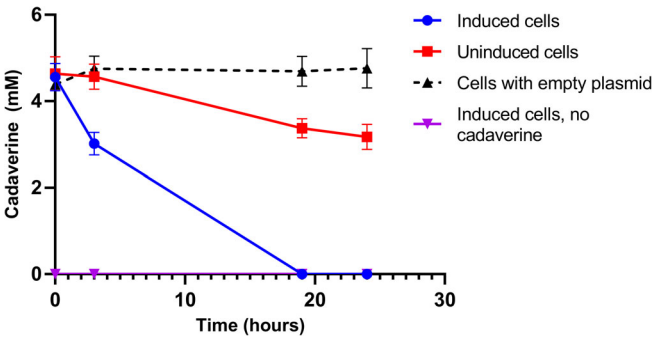

Figure 3. Cadaverine consumption by whole cells containing Re-PuO. Reaction mixtures contained washed cell suspensions harvested 5 h after induction (dry cell weight 1 ± 0.1 mg mL^{-1}), and cadaverine (5 mmol L^{-1}) in 100 mmol L^{-1} Na/K phosphate buffer pH?. Controls contained cells where expression had not been induced with IPTG, cells containing an empty plasmid, or induced cells in the absence of cadaverine.

by HPLC. Complete biotransformation of 5 mmol L^{-1} cadaverine was observed after 19 h of incubation at pH 7.5 and 30 °C with harvested cells containing Re-PuO (Fig. 3). This suggests that *E. coli* has a transport system for cadaverine, possibly via the putative cadaverine transport protein CadB.[25] Control experiments with uninduced cells showed slow biotransformation of some of the cadaverine, which was attributed to leaky expression of the enzyme, because cells containing empty plasmids lacking the Re-PuO gene did not consume any cadaverine. The latter observation indicated that the biotransformation was exclusively due to the expression of Re-PuO and not to other endogenous enzymes that can transform cadaverine. No product was formed in the absence of cadaverine, indicating that cadaverine was not produced by *E. coli*, and that the presence of Re-PuO does not induce its production.

CONCLUSIONS

We successfully expressed putrescine oxidase from *R. erythropolis* (Re-PuO) in *E. coli* and characterized the enzyme by directly

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

monitoring its O_2 consumption during cadaverine oxidation. A colorimetric assay using *o*-ABA also was used and indicated that 1-piperideine was the product of the reaction. Whilst this assay could be used as a rapid activity screen, the slow kinetics of *o*-ABA condensation with the 5-aminopentanal product of cadaverine condensation hindered the reliable determination of enzymatic parameters. Preliminary characterization of the reaction mixture by ^{1}H NMR and MS confirmed the presence of piperideine dimers and trimers; however, the quantification of the reaction products could not be achieved. The spontaneous formation of these products will hinder the future exploitation of the enzyme unless coupled to a cascade to capture the piperideine and convert it to the final target product.[16] We are presently investigating this approach. The isolated enzyme displayed optimal activity at pH 8.5 and 55 °C, although it was unstable at this high temperature. The use of harvested whole cells containing overexpressed Re-PuO showed complete cadaverine consumption. Thus, Re-PuO offers significant promise for the bioproduction of *N*-heterocyles, providing that challenges associated with the instability of the reaction products can be addressed.

ACKNOWLEDGEMENTS

This research was supported by a PhD Scholarship from the Faculty of Engineering University of Nottingham, UK to VEA.

CONFLICTS OF INTEREST

Authors declare no conflicts of interest.

ASSOCIATED CONTENT

Supporting information. The Electronic Supporting Information is available free of charge.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Vitaku E, Smith DT and Njardarson JT, Analysis of the structural diversity, substitution patterns, and frequency of nitrogen heterocycles among U.S. FDA approved pharmaceuticals. *J Med Chem* **57**: 10257–10274 (2014).
2 Gravel E, Poupon E and Hocquemiller R, Biomimetic investigations from reactive lysine-derived C_5 units: one step synthesis of complex polycyclic alkaloids from the *Nitraria* genus. *Tetrahedron* **62**:5248–5253 (2006)).
3 Rouchaud A and Braekman J-C, Synthesis of new analogues of the tetraponerines. *Eur J Org Chem* **2009**:2666–2674 (2009)).
4 Monaco MR, Renzi P, Scarpino Schietroma DM and Bella M, Biomimetic organocatalytic asymmetric synthesis of 2-substituted piperidine-type alkaloids and their analogues. *Org Lett* **13**:4546–4549 (2011)).
5 Unsworth WP, Coulthard G, Kitsiou C and Taylor RJK, Direct imine acylation for molecular diversity in heterocyclic synthesis. *J Org Chem* **79**:1368–1376 (2014).
6 Bunsupa S, Yamazaki M and Saito K, Quinolizidine alkaloid biosynthesis: recent advances and future prospects. *Front Plant Sci* **3**:239 (2012).
7 Bunsupa S, Yamazaki M and Saito K, Lysine-derived alkaloids: overview and update on biosynthesis and medicinal applications with emphasis on quinolizidine alkaloids. *Mini Rev Med Chem* **17**:1002–1012 (2017)).
8 Sun J, Morita H, Chen G, Noguchi H and Abe I, Molecular cloning and characterization of copper amine oxidase from *Huperzia serrata*. *Bioorg Med Chem Lett* **22**:5784–5790 (2012)).
9 O'Reilly E, Iglesias C, Ghislieri D, Hopwood J, Galman JL, Lloyd RC *et al*., A regio- and stereoselective ω-transaminase/monoamine oxidase cascade for the synthesis of chiral 2,5-disubstituted pyrrolidines. *Angew Chem Int Ed* **53**:2447–2450 (2014)).
10 Gomm A, Lewis W, Green AP and O'Reilly E, A new generation of smart amine donors for transaminase-mediated biotransformations. *Chem A Eur J* **22**:12692–12695 (2016)).
11 Slabu I, Galman JL, Weise NJ, Lloyd RC and Turner NJ, Putrescine transaminases for the synthesis of saturated nitrogen heterocycles from polyamines. *ChemCatChem* **8**:1038–1042 (2016)).
12 Cha HJ, Jeong J-H, Rojviriya C and Kim Y-G, Structure of putrescine aminotransferase from *Escherichia coli* provides insights into the substrate specificity among class III aminotransferases. *PLoS One* **9**: e113212 (2014)).
13 Kopacz MM, Heuts DPHM and Fraaije MW, Kinetic mechanism of putrescine oxidase from *Rhodococcus erythropolis*. *FEBS J* **281**: 4384–4393 (2014)).
14 Kopacz MM, Rovida S, van Duijn E, Fraaije MW and Mattevi A, Structure-based redesign of cofactor binding in putrescine oxidase. *Biochemistry* **50**:4209–4217 (2011)).
15 Dijkman WP, de Gonzalo G, Mattevi A and Fraaije MW, Flavoprotein oxidases: classification and applications. *Appl Microbiol Biotechnol* **97**:5177–5188 (2013)).
16 Al-Shameri A, Borlinghaus N, Weinmann L, Scheller PN, Nestl BM and Lauterbach L, Synthesis of *N*-heterocycles from diamines *via* H_2-driven NADPH recycling in the presence of O_2. *Green Chem* **21**: 1396–1400 (2019)).
17 France SP, Hussain S, Hill AM, Hepworth LJ, Howard RM, Mulholland KR *et al*., One-pot cascade synthesis of mono- and disubstituted piperidines and pyrrolidines using carboxylic acid reductase (CAR), ω-transaminase (ω-TA), and imine reductase (IRED) biocatalysts. *ACS Catal* **6**:3753–3759 (2016)).
18 Galman JL, Slabu I, Weise NJ, Iglesias C, Parmeggiani F, Lloyd RC *et al*., Biocatalytic transamination with near-stoichiometric inexpensive amine donors mediated by bifunctional mono- and di-amine transaminases. *Green Chem* **19**:361–366 (2017)).
19 Ping Y, Li X, Xu B, Wei W, Wei W, Kai G *et al*., Building microbial hosts for heterologous production of *N*-methylpyrrolinium. *ACS Synth Biol* **8**: 257–263 (2019)).
20 van Hellemond EW, van Dijk M, Heuts DP, Janssen DB and Fraaije MW, Discovery and characterization of a putrescine oxidase from *Rhodococcus erythropolis* NCIMB 11540. *Appl Microbiol Biotechnol* **78**: 455–463 (2008)).
21 Fothergill JC and Guest JR, Catabolism of L-lysine by *Pseudomonas aeruginosa*. *J Gen Microbiol* **99**:139–155 (1977)).
22 Bachrach U and Oser IS, Enzymatic assay for spermidine. *J Biol Chem* **238**:2098–2101 (1963)).
23 Frydman RB, Ruiz O, Kreisel M and Bachrach U, Oxidation of *N*-alkyl and *C*-alkylputrescines by diamine oxidases. *FEBS Lett* **219**:380–386 (1987)).
24 Lavery CB, MacInnis MC, MacDonald MJ, Williams JB, Spencer CA, Burke AA *et al*., Purification of peroxidase from horseradish (*Armoracia rusticana*) roots. *J Agric Food Chem* **58**:8471–8476 (2010)).
25 Soksawatmaekhin W, Kuraishi A, Sakata K, Kashiwagi K and Igarashi K, Excretion and uptake of cadaverine by CadB and its physiological functions in *Escherichia coli*. *Mol Microbiol* **51**:1401–1412 (2004)).

