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Identification, functional expression and kinetic analysis of two primary amine oxidases from *Rhodococcus opacus*

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ABSTRACT

Two native copper-containing amine oxidases (EC 1.4.3.21) have been isolated from *Rhodococcus opacus* and reveal phenotypic plasticity and catalytic activity with respect to structurally diverse natural and synthetic amines. Altering the amine growth substrate has enabled tailored and targeted oxidase upregulation, which with subsequent treatment by precipitation, ion exchange and gel filtration, achieved a 90–150 fold purification. MALDI-TOF mass spectrometric and genomic analysis has indicated multiple gene activation with complex biodegradation pathways and regulatory mechanisms. Additional post-purification characterisation has drawn on the use of carbonyl reagent and chelating agent inhibitors. Michaelis–Menten kinetics for common aliphatic and aromatic amine substrates and several structural analogues demonstrated a broad specificity and high affinity with Michaelis constants (K_M) ranging from 0.1 to 0.9 mM for C_1–C_5 aliphatic mono-amines and <0.2 mM for a range of aromatic amines. Potential exploitation of the enzymatic versatility of the two isolated oxidases in biosensing and bioprocessing is discussed.

1. Introduction

Amine oxidases (AOs) are an important class of enzyme in both industry and medicine but their widespread use has yet to be fully realised. Variable levels of expression, inconsistencies in enzyme nomenclature, as well as an inability to identify or find enzymes with appropriate levels of activity and specificity have all hindered the selection and isolation of suitable oxidases for a targeted end use. Nevertheless, applications in biocatalysis [1,2], biosensors [3–5] and therapeutics [6] have been proposed for this heterogeneous group of enzymes, which catalyse oxidative deamination generating the corresponding aldehyde, with ammonia and hydrogen peroxide as by-products [7,8].

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$

These enzymes have been the subject of a number of classical biochemical studies that have assessed substrate activity and redox cofactor (FAD or copper dependent topa quinone), classifying the oxidase based upon catalytic mechanism and reaction [9,10]. However, ambiguous and inconsistent nomenclature, coupled with the difficulties of inferring catalytic properties based on genomic information alone, has meant that it is often difficult to explicitly identify the enzyme that has actually been examined [11,12]. Consequently, there remains little correlation between sequence information and AO attributes, including catalytic behaviour, metabolic pathways and biotechnological relevance. This lack of correlation has limited practical implementation to a few well characterised oxidases, notably those from *Aspergillus sp.* [1–3], *Arthrobacter sp.* [13,14], pea seedling [3,15] and bovine serum [6,16]. As the number of fully characterised oxidases increase, the possibilities for commercial exploitation should escalate.

Genomic analysis has revealed the presence of several putative AOs in the gram positive soil bacterium *Rhodococcus opacus* [17,18], an organism that has been identified as particularly suited to industrial biocatalysis [19]. This genus has found application in fossil fuel biodesulphurisation, steroid biotransformation and acrylamide and acrylic acid production [20–22]. Such a level of utilisation results from a combination of facile growth with stability in chemical reaction systems [22]. Moreover, the associated metabolic versatility and biochemical diversity spans a range of structural groups, including halogenated aliphatics and aromatics, substituted benzenes and quinolines [23]. Despite current and potential commercial importance and progress in recent years [17], the *Rhodococcus* species remain poorly characterised in terms of enzymatic action. In this paper, we report the isolation, characterisation and identification of two novel copper AOs. We establish the catalytic action of these AOs in the oxidative deamination of

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a range of aliphatic and aromatic amines and identify potential biotechnological applications.

2. Materials and methods

2.1. Bacterial strain and culture conditions

R. opacus DSM 43250 was used throughout this investigation. The bacterium was cultured in M9 minimal media with glucose as the carbon source. For induction experiments, ammonium chloride was replaced in the medium with 10 mM of the appropriate amine. The bacteria were grown at 30 °C for 3 days under continuous shaking at 130 rpm. The cells were then centrifuged for 20 min at 4 °C and 2500*g*, the supernatant removed and the cell pellet frozen at −20° C.

2.2. Purification of amine oxidase

The frozen cell pellet was re-suspended in ice cold TES buffer (pH 7.5) at a concentration of 0.4 g/ml and centrifuged for 30 min at 4 °C and 20000*g*. On removal of the supernatant, the cells were resuspended in TES buffer (0.4 g/ml) and twice passed through a French press at a pressure of 138 MPa. In order to ensure complete lysis, cells were sonicated in 30 s intervals on ice for a further 10 min. The cell debris was removed by centrifugation (4° C and 60,000 *g*) for 1 h and passed through a 0.2 μm syringe filter. The filtered lysate was initially purified by a two cut ammonium sulphate precipitation, the first at 30% saturation with the second at 70%. The precipitated protein was re-suspended in TES buffer before purification by FPLC. The majority of the remaining protein was removed using an anion Hi-Prep 16 10 Q FF column (1.6 cm × 13 cm) with 50 mM Tris–hydrochloride (pH 7.5) and a linear 1 M sodium chloride gradient. Active fractions were pooled and diluted by a factor of 2 with running buffer to reduce the salt concentration. The protein was then run on a Mono Q 5/50 GL column (0.5 cm × 5 cm) using the same Tris–hydrochloride running buffer and a Tris–hydrochloride/sodium chloride elution buffer. Finally, selected active fractions were purified by gel filtration utilising a gel Superose 12 10/300 GL column (1 cm × 30 cm).

2.3. AO gel activity staining

AO activity staining was performed using a peroxidase-coupled system after electrophoresis on a native polyacrylamide gel, as previously described by Lee [24]. The native PAGE was equilibrated twice for 20 min in potassium phosphate buffer (pH 7.5). The gel was then transferred to the substrate solution containing 50 ml potassium phosphate buffer (pH 7.5), 10 mg 3-amino-9-ethylcarbazole and 5 mM amine substrate. After 5 min, 200 μl horseradish peroxidase (5 mg/ml) was added and the gel was gently shaken in the dark for 5–20 min, depending on the observed band intensity.

2.4. In-gel digestion of protein spots and protein identification

Protein identification was performed using native gels stained for AO activity, and purified enzyme on SDS–Tris–tricine gels. Gels were rinsed in Millipore filtered water (0.2 μm) three times for 5 min followed by staining with Coomassie Brilliant Blue for 1 h. Gels were de-stained using Millipore water and protein bands excised manually with a scalpel. The remaining stain was removed by incubating the gel slice at 37 °C twice with 0.2 ml 100 mM ammonium bicarbonate/50% acetonitrile for 45 min. The wash was removed and replaced with 100 μl 100% acetonitrile for 5 min and dried in Speed Vac for 15 min. Gel slices were rehydrated in 10 μl Trypsin Gold (Promega) (20 μg/ml) and 40 mM ammonium bicarbonate/10% acetonitrile at room temperature for 1 h. An additional 90 μl of 40 mM ammonium bicarbonate/10% acetonitrile was added to prevent complete evaporation before incubation overnight at 37 °C. The gel slices were further diluted with 100 μl Millipore water and mixed by vortexing for 10 min. The liquid was then transferred to a microcentrifuge tube and the gel slice digest extracted twice for 60 min by vortexing with 50% acetonitrile/5% trifluoroacetic acid. These extracts were pooled and dried in Speed Vac at room temperature for 2 h. Samples were reconstituted in 10 μl 0.1% trifluoroacetic acid, pipetted and expelled three times with ZipTips (Millipore) which had been preconditioned with 10 μl 100% acetonitrile followed by three washes with 0.1% trifluoroacetic acid. Contaminants were removed by washing ZipTips containing the bound protein a further three times with 0.1% trifluoroacetic acid before eluting 0.3 μl spots directly onto the Maldi plate using 1.5 μl matrix consisting of 70% acetonitrile/0.1% trifluoroacetic acid and 10 mg/ml α-cyano-4-hydroxycinnamic acid. Peptide spectra were generated using an Ettan Matrix-assisted laser desorption/ionization time of flight mass spectrometer (MALDI TOF MS). The identified peaks automatically selected by the peak seeker algorithm were searched against the NCBI database using MSFIT (http://prospector.ucsf.edu). Those with a high Mowse score, *i.e.* a weighted measure of how closely the spectrum matches a protein, were subsequently confirmed using the MASCOT programme (www.matrixscience.com), which generates an "expected" value, *i.e.* the number of (random) proteins that could achieve the same score for the given spectrum. Proteins, in the absence of post-translational modification, with an expectation score (*E*-value) of 0.05 and lower (95% confidence), a minimum sequence coverage of 10% and at least three independent peptides matches were taken as positive identifications.

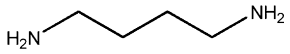
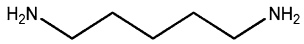
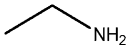
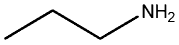
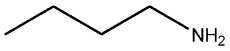
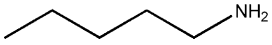
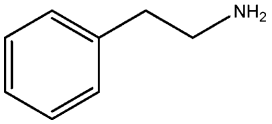
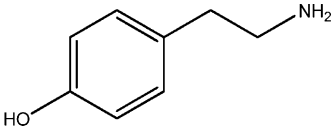
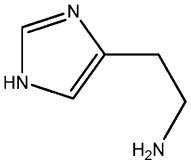
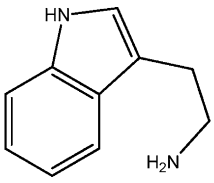
2.5. Enzyme assay

A colorimetric assay was performed in order to determine enzymatic activity. The assay is based on the formation of the hydrogen peroxide by-product, which in the presence of 4-aminoantipyrine, and 2,4,6-tribromo-3-hydroxybenzoic acid is converted in equimolar amounts by an added peroxidase to produce a quinoneimine dye [25]. The reaction was performed in 96 well plates containing 10 μl enzyme and 100 μl freshly prepared assay solution (200 mM potassium phosphate buffer pH 7.6, 1.5 mM 4-aminoantipyrine and 1 mM 2,4,6-tribromo-3-hydroxybenzoic acid). In addition, 20 μl amine substrate and 70 μl 1.4 mg/ml peroxidase from horseradish were added to give a final volume of 200 μl. Absorbance was measured at 510 nm and 30 °C using a plate reader ($VERSA_{max}$, Molecular Devices). All results were normalised against total protein using Bradford's reagent method [26]; readings were performed in triplicate and the results fell within a 5% relative standard deviation.

2.6. Enzyme inhibition

Enzyme inhibition was achieved using non-competitive inhibitors: semicarbazide; isoniazid; aminoguanidine; cuprizone; neocuprizone pargyline; clorgyline. The stock solutions contained 50 mM Tris–hydrochloride (pH 7.5). The purified oxidases were incubated on ice in triplicate with 0.1 mM of the inhibitor. After 30 min, the inhibitor-enzyme solution was measured for oxidase activity using the colorimetric assay with either 10 mM benzylamine (BEN) or phenylethylamine (PHE). The final concentration of inhibitor in the assay was 5 μM.

Table 1
Activity of lysed *Rhodococcus opacus* after inducing oxidase expression by limiting the nitrogen source to methylamine (MET), butylamine (BUT) and phenylethylamine (PHE): specific activity measured in μmole/min/g; oxidase activity relative to that recorded with ethylamine (ETH) as substrate is given in parentheses (as a percentage).

Substrate	Structure	Inorganic nitrogen (NH_4Cl) control	Activity of MET induced cells	Activity of BUT induced cells	Activity of PHE induced cells
Putrescine (PUT)		1.2	13.1 (546%)	5 (23%)	2 (15%)
Cadaverine (CAD)		0.2	0.7 (29%)	2 (9%)	3 (23%)
Methylamine (MET)		<0.2	2.6 (108%)	2 (9%)	1 (8%)
Ethylamine (ETH)		<0.2	2.4 (100%)	22 (100%)	13 (100%)
Propylamine (PRO)		<0.2	1.5 (63%)	47 (214%)	53 (408%)
Butylamine (BUT)		<0.2	2.4 (100%)	27 (123%)	62 (477%)
Amylamine (AMY)		<0.2	3 (125%)	17 (77%)	52 (400%)
Phenylethylamine (PHE)		<0.2	1.5 (63%)	16 (73%)	36 (277%)
Tyramine (TYR)		<0.2	0.8 (33%)	4 (18%)	9 (69%)
Histamine (HIS)		<0.2	1.5 (63%)	7 (32%)	22 (169%)
Tryptamine (TRY)		<0.2	0.7 (29%)	4 (18%)	11 (85%)

3. Results and discussion

3.1. Amine oxidase induction

As AOs are ubiquitously distributed in nature, oxidase specificity and activity within microorganisms are considered to vary substantially [27]. This diversity has classically been explored by limiting the nitrogen source to specific amines in order to induce an enzymatic response. Notable examples include the oxidase of the filamentous fungi *Aspergillus niger* and the yeast *Hansenula polymorpha* when grown on butylamine (BUT) and methylamine (MET) [28,29]. In bacteria, phenylethylamine (PHE) has been identified as an effective inducer of AO in *Escherichia coli* and *Arthrobacter globiformis* [30,31]. The latter has also been observed to possess inducible oxidases after culturing with either MET or histamine (HIS) [32]. These amines along with the structural analogues and non-physiological substrate benzylamine (BEN) [33–35] have demonstrated an ability to promote oxidase expression within a variety of microorganisms [36,37]. In this study, in order to optimise the detection of oxidase activity within *R. opacus*, MET, BUT and PHE were selected as amines to induce activity while prevalent natural substrates, that may arise primarily by decarboxylation or amination of simple aldehydes and ketones by higher plants [38], were utilised as substrates for oxidase screening. These substrates are identified in Table 1 and include aliphatic monoamines (C_1–C_5), diamines (putrescine (PUT) and cadaverine (CAD)), heterocylic amines (HIS and tryptamine (TRY)) and catecholamines (PHE and tyramine (TYR)). Oxidase expression in *R. opacus* grown under induction with MET displayed a substantial increase in substrate activity when compared with the control, *i.e.* ammonium chloride as the sole nitrogen source (see Table 1). The cells grown with inorganic nitrogen exhibited some aliphatic diamine activity, notably with respect to PUT, but no detectable catalytic response for the other substrates; detection limit = 0.1 μM/min. The MET induced cells displayed a ten-fold increase in PUT activity but a lesser activity for the longer chain CAD diamine. A comparable low activity level was recorded for the aliphatic monoamines but this was measurably greater than that observed for the aromatic or heterocyclic substrates. The enzymatic response exhibited by BUT induced cells was typically superior, with the exception of PUT and MET as substrates where the MET pre-treatment delivered higher activity. The expressed oxidases resulting from BUT treatment, principally acted on aliphatic monoamines, as shown in Table 1, with specific rates for the conversion of C_2–C_5 amines that were 5–30 times greater those recorded for MET induction. In addition, significant activity was also observed with respect to aromatic and heterocyclic

Table 2
Dry cell weight (mg) per ml of *Rhodococcus opacus* culture, grown with different nitrogen sources at 30 °C for 3 days.

Nitrogen source	Dry cell weight (mg)
NH_4Cl	2.5
Methylamine (MET)	2.5
Butylamine (BUT)	2.4
Phenylethylamine (PHE)	2.1

amines. Oxidase expression for cells cultured with PHE was similar to that observed for BUT. There are, however, distinct differences in terms of the level of induction and preferred substrates, notably higher activity for the catechol, heterocyclic and C_4/C_5 aliphatic amines.

The observed variation in relative levels of activity is indicative of the presence of multiple enzymes within the cell with differing levels of expression. While limiting the nitrogen source to specific amines increased global oxidase expression within *R. opacus* as anticipated, the results suggest that there is a selective expression of AOs that is dependent on the growth substrate. For instance, the enhancement of activity with respect to aromatic amines after pre-treatment with PHE is a specific upregulation of a corresponding oxidase necessary for growth, which additionally is capable of acting upon other related substrates. However, such is the broad response to the three inducers that cellular stress is a likely contributing factor. Indeed, increased AO levels have been detected under conditions of limited carbon and nitrogen in other organisms [39,40]. The varying level of cellular response to MET, BUT and PHE treatment must be influenced by the efficiency and specificity of the oxidases (or other enzymes) to degrade these amines, along with transport across the cell membrane and possible toxic effects due to the substrate and resulting aldehyde [41]. Cellular influence on oxidase activity coupled with oxidase heterogeneity (in terms of specificity), constrains the explicit value of the information that can be gleaned from substrate specificity alone. However, a consideration of nitrogen limited conditions can lead to some insight into multiplicity and possible overlap of enzyme activities. For example, there are appreciable differences in AO activity with respect to the C_2–C_5 aliphatic monoamines (Table 1), which could be expected (as a first approximation) to share the same oxidase [42–44]. The observed variation of substrate preference within this amine grouping suggests that the recorded activity is a composite resulting from a contribution of more than one AO acting on the same substrates to different degrees. Conversely, a common proportionate difference in rate for either catechol or heterocyclic amines (activity for HIS is 1.8–2.0 times that of TRY, activity for PHE is 4 times greater than TYR for BUT and PHE induced cells), points to differing levels of induction. The latter response suggests the observed activity is primarily due to the action of a single oxidase or multiple enzymes with similar specificity.

The results presented in Table 1 indicate that the three sets of induction conditions generated three separate cellular responses. With MET as inducer, sub-optimal induction is achieved with relatively low levels of non-specific activity and measurably higher activity with respect to PUT. BUT also provides a broad cellular response but typically elevated activity, notably an order of magnitude increase in the case of C_2–C_4 aliphatic amines. The action of the aromatic PHE inducer also shows a degree of specificity, particularly for the longer chain monoamines and delivered the highest recorded rates. Given that *Rhodococcus* growth was largely unaffected by substrate, as shown in Table 2, the observed metabolic shift represents considerable flexibility to adapt as conditions dictate.

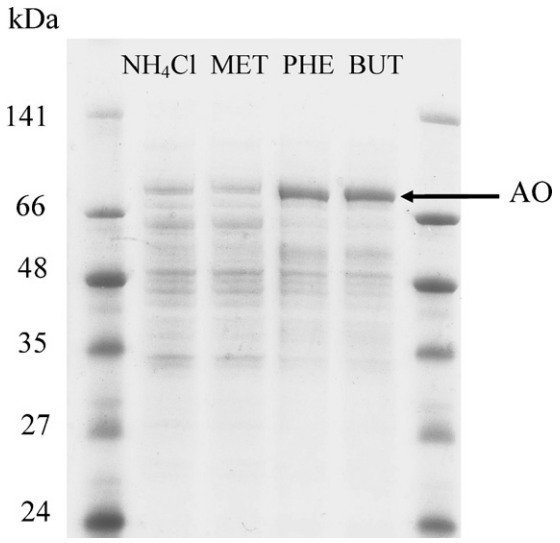


Fig. 1. SDS-PAGE of *Rhodococcus opacus* cell lysate from proteins from cells cultured with ammonium chloride, methylamine (MET), butylamine (BUT) or phenyethylamine (PHE) for 3 days at 30 °C.

3.2. Purification of oxidases post induction

Prior to any purification, a distinct protein band at a molecular weight of *ca.* 70 kDa was in evidence after culturing with either BUT or PHE, as shown in the SDS-PAGE presented in Fig. 1. The intensity of this band corresponded qualitatively with enzyme activity and was poorly defined after culturing with either MET or NH_4Cl. The associated molecular weight is close to the values calculated from the amino acid sequence of the genomically conserved copper oxidases (CuAO) found in highly similar *Rhodococcus* species (Table 3) [17,18,45]. The five other AOs, which utilise flavin as a redox co-factor and catalyse the same deamination reaction, have a predicted molecular weight in the range 49–55 kDa. The four possible CuAO exhibit similar characteristics, with molecular weights of the monomers prior to post-translational modification ±1.4 kDa and an isoelectric point estimated by the p*K* of the amino acid sequence ±0.3 pI [45]. Indeed, such is the sequence similarity that the oxidases may have occurred as paralogs with evolutionary selective pressures leading to divergent selectivity of the duplicated gene. Given the degree of structural commonality and overlapping substrate specificity, alternative oxidase induction conditions were required to facilitate enzyme separation and circumvent any inadvertent misidentification or erroneous characterisation. As MET provided low levels of oxidase induction for the given substrates and a similar substrate preference to that observed with BUT treated cells, the work was focused to consider the contrasting activities resulting from BUT and PHE induction. In order to differentiate between possible AOs, PHE was selected as both inducer and substrate due to the observed reaction specificity and high levels of activity with respect to aromatics. Taking the results presented in Table 1, BUT as substrate generated a wide range of activities for the induced cells, suggesting overlapping substrate specificity. Coupling BUT induced enzyme purification with BUT as test substrate would not facilitate explicit enzyme identification. Although the shorter chain aliphatic amines were not subject to such wide variation, they exhibited the drawback of relatively low activity. The non-physiological substrate benzylamine (BEN) was chosen as a suitable substrate as a high level of specific activity has been established for a number of AOs [28,35,40]. Additionally, the shorter alkyl chain (relative to PHE) linking the amino group to the benzene ring can hinder deamination for a number of oxidases. Murooka and co-workers [34] in analysing microbial

Table 3
Possible oxidases whose action directly liberates ammonia (oxidoreductases acting on the CH-NH_2 group of donors) that have been identified within the *Rhodococcus sp.* RHA1/*Rhodococcus opacus* B4 genome [17,18]. Homologues shown by Blast score with the corresponding molecular weight and pI of the monomer subunit as estimated by Compute pI/Mw [45].

Rhodococcus sp. RHA1 GI number	*Rhodococcus opacus.* B4 GI number	Identities and Blast *E* value	Estimated molecular weight range (kDa)	Estimated pI range	Co-factor
111019599	226361748	98% *E* = 0	71.7–71.9	4.8	Copper
111022563	226243561	96% *E* = 0	73.0–73.1	4.9–5.0	Copper
111019825	226240457	96% *E* = 0	72.1–72.2	4.7–4.9	Copper
111025258	N/A	N/A	72.6	4.9	Copper
111022572	226365080	97% *E* = 0	49.3–49.5	4.9	Flavin
111019548	226361698	89% *E* = 0	48.9–49.0	5.7–5.8	Flavin
111018768	226360855	91% *E* = 0	48.9–49.1	5.4–5.5	Flavin
111022672	226365184	88%, *E* = 0	50.4–50.7	5.2–5.4	Flavin
111019880	226359955[a]	95%, *E* = 0	49.5–55.4	8.9	Flavin

[a] Initial 54 residues not observed in predicted protein; taking the full region from *Rhodococcus opacus* B4 genome identities = 87%.

Table 4
Sequential purification of amine oxidases (AOs) from *Rhodococcus opacus* on a series of FPLC columns. Analysis of the enzymes purified from (A) BUT induced culture (AO1) and (B) PHE induced culture (AO2).

(A) AO1 from BUT induced cells

	Total protein (mg)	Total volume (ml)	Protein (mg/ml)	Specific BEN activity (μmole/min/g)	Enzyme recovery (%)	Purification (fold)
Crude extract	67.01	400	0.168	39	–	–
$(NH_4)_2SO_4$ ppt	32.50	50	0.650	67	83.3	1.7
Hi Prep column	2.73	30	0.091	732	75.6	18.8
Mono Q column	0.16	1	0.160	2123	13.0	54.4
Superose column	0.04	1	0.040	6302	10.2	161.5

(B) AO2 from PHE induced cells

	Total protein (mg)	Total volume (ml)	Protein (mg/ml)	Specific PHE activity (μmole/min/g)	Enzyme recovery (%)	Purification (fold)
Crude extract	62.04	400	0.155	144	–	–
$(NH_4)_2SO_4$ ppt	31.03	50	0.621	204	70.8	1.4
Hi Prep column	4.51	50	0.090	1342	67.8	9.3
Mono Q column	0.23	2	0.115	5123	13.2	35.6
Superose column	0.07	1	0.070	13144	9.9	91.3

oxidase distribution found only a single organism capable of oxidative conversion of BEN, while enzymes from diverse bacterial genera readily catalysed deamination of PHE derivatives, including TYR. The results of the sequential purification steps are summarised in Table 4. The level of enzyme purity was monitored (after each purification) by gel electrophoresis, measurement of protein by Bradfords method [26] and enzyme activity with respect to BEN and PHE as substrates. Following ammonium sulphate precipitation the specific activity was increased by a factor of 1.4–1.7. The Hi-Prep ion exchange column separated the bulk of the protein from the AO component for both BUT and PHE induced cells with a 9–19 fold increase in specific activity relative to the crude extract. The active fractions (taking PHE induction as a representative case) displayed a normal distribution as shown in Fig. 2. The six fractions with the highest activity were pooled (denoted by the magnified area in Fig. 2), before separation using the Mono-Q column. The higher resolution obtained with the second ion exchange column provided a crucial separation of two AOs displaying differing specificity, as shown in Fig. 3a and b for the BUT and PHE induced cells, respectively. Several fractions showed activity towards BEN with no overlapping activity for PHE following BUT induction with the reverse response for the PHE induced cells. Although complete separation (to baseline) in terms of enzyme activity was not achieved, the level of purification was sufficient to isolate fractions associated with the two distinct activities, as denoted by the areas highlighted in Fig. 3. A possible third oxidase showing low PHE activity was also detected (Fig. 3b). The oxidase (denoted as AO1) that displayed activity towards BEN was eluted prior to the oxidase (AO2) with activity towards PHE. As a fraction of total enzyme activity, the oxidase that eluted first (AO1) was expressed in greater quantities following BUT induction. In the case of purification of PHE induced cells (Fig. 3b), the oxidase showing activity with respect to PHE was dominant with a far lesser fraction exhibiting active towards BEN. A single fraction that displayed no detectable activity overlap (Fig. 3) was taken in each case, resulting in a low enzyme recovery during the final purification by gel filtration on the Sephadex column (Table 4). Despite losing a signification proportion of the total starting enzyme, at the final stage of purification the relative activity was 162 and 91 times greater than that of the crude extract post BUT and PHE induction, respectively.

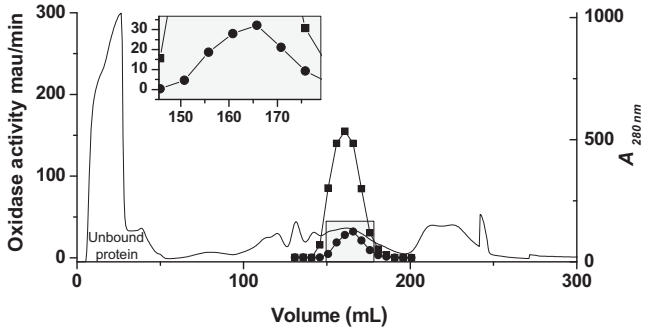


Fig. 2. PHE (■) and BEN (●) oxidase activity purification profile for PHE induced cells after purification on the Hi-Prep ion exchange column. Selected fractions for further purification are indicated by the magnified area.

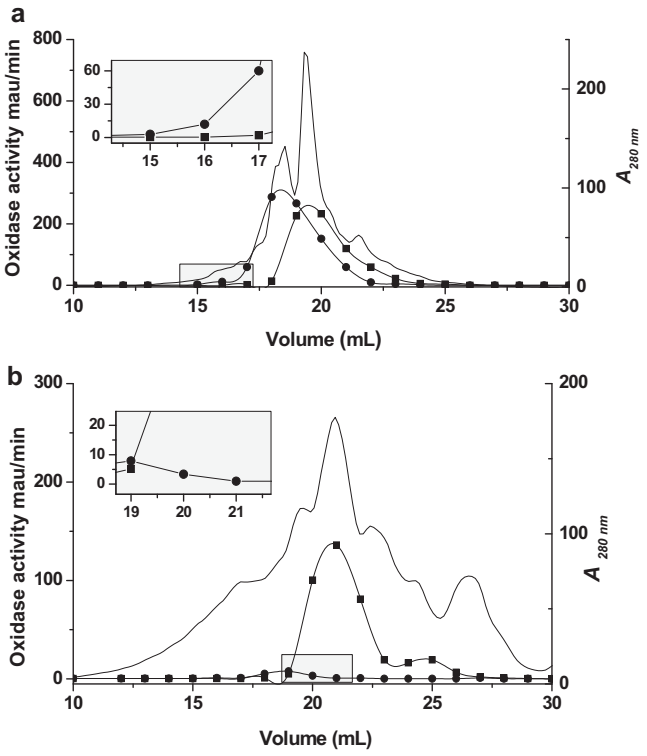


Fig. 3. PHE (■) and BEN (●) oxidase activity purification profile for (a) BUT and (b) PHE induced cells after purification on the Mono Q column. Magnified area shows fractions with no overlapping activity.

3.3. Enzyme characterisation

AO1 isolated from BUT induced cells with a distinct specificity towards BEN (relative to PHE), was found to act on a broad range of aliphatic monoamines (C_1–C_5). AO2 isolated from PHE treated cells displayed activity towards PHE as well as TYR and heterocyclic amines but negligible conversion of BEN and short chain aliphatic amines. Neither AO1 nor AO2 exhibited any measurable activity when using diamines (PUT or CAD) as substrate. These observations also apply to cells induced with BEN and TYR (data not shown), where enzymes AO1 and AO2 were again isolated. Both oxidases were further characterised by native gel electrophoresis (Fig. 4), staining the dimers (*ca.* 140 kDa) directly on the gel with BUT as substrate (see Section 2.3), where two distinct oxidation bands are evident at slightly different migration points. The overlap of the bands is consistent with overlap in terms of oxidase activity, as shown in Fig. 3a.

As AOs are subject to inhibition by an array of compounds, this enables some differentiation and, with substrate specificity, can serve as a basis for enzyme classification [46]. AOs that possess a copper co-factor are inhibited by reactive carbonyl

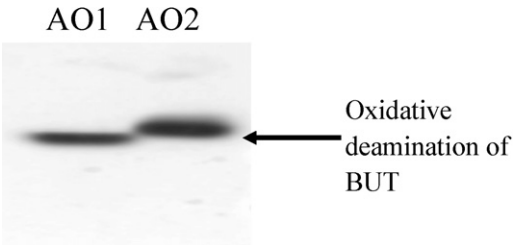


Fig. 4. Native-PAGE oxidase activity gel of AO1 and AO2 indicating difference in molecular weight and overlapping specificity towards BUT as substrate.

Table 5
Inhibition of purified oxidases from BUT (AO1) and PHE (AO2) induced cells. Activity of the cells incubated (30 min on ice) with selected inhibitors is presented as a percentage of that obtained for the untreated cells.

Inhibitor	BUT induced cells (AO1) % BEN activity	PHE induced cells (AO2) % PHE activity
Pargyline	100	100
Clorgyline	99	100
Semicarbazide	1	2
Isonazid	14	16
Aminoguanidine	18	17
Cuprizone	2	1
Neocuproine	98	99

reagents such as semicarbazide, isoniazid and aminoguanidine. As the redox role of copper during the oxidative catalytic cycle remains somewhat controversial [47], AO1 and AO2 were also treated with chelating agents (cuprizone and neocuproine). Flavin dependent monoamine oxidases are not sensitive to these compounds at low concentrations but are inhibited by parglyline and clorgyline, which have no effect on AOs with a copper co-factor [48]. Neither enzyme displayed any detectable inhibition with either parglyline or clorgyline (Table 5). Both enzymes were, however, inhibited by the carbonyl reagents and cuprizone, a response that is consistent with CuAO. All detectable activity was irreversibly inhibited by semicarbazide. Treatment with isoniazid and aminoguanidine, which are known to preferentially (but not selectively) inhibit diamine oxidases [49], also acted to significantly (but not totally) inhibit both AO1 and AO2. Application of cuprizone (cupric chelating agent) served to suppress catalytic action whereas neocuproine (cuprous inhibitor) had no significant inhibitory effect. As both AOs displayed activity towards monoamines and possess a copper co-factor, they can both be classified in terms of the IUBMB (International Union of Biochemistry and Molecular Biology) nomenclature as primary amine oxidases (EC 1.4.3.21) [46] (see supplement 14, http://www.chem.qmul.ac.uk/iubmb/enzyme/supplements/sup2008/. Based on our results, we can discount classification as diamine oxidase EC 1.4.3.22 (due to substrate preference), monoamine oxidase EC 1.4.3.4 and polyamine oxidase EC 1.5.3.11 (due to co-factor).

3.4. Proteomic identification and genomic analysis

The enzyme bands were extracted from the native activity gel and identified by MALDI-TOF analysis. The results were matched to the proteins associated with *Rhodococcus* RHA1 and *Rhodococcus opacus* B4, which are genomically similar and display synteny conservation [17,18,50]. Matching at least seven tryptic digest fragments (with greatest confidence to the proteins of *Rhodococcus* RHA1), analysis confirmed the identification of two oxidases based on an *E*-value (expectation value, see Section 2.4) $\leq$0.05. Additional induction conditions that resulted in similar phenotypes validated further the matching identification of the oxidases; the results are summarised in Table 6. AO1 was matched to GI 111019599 (*E*-value in the range $9.1e^{-06}$–$1.2e^{-05}$) and AO2 identification was consistent with GI 111019825 (*E*-value in the range 0.009–0.021) from two separate induction conditions. These highly significant expectation values were calculated from the entire NCBI database, which was not limited by organism, PI or size. As shown by the representative MALDI-TOF spectrum (for AO1) in Fig. 5, over twenty distinct peaks in the m/z range 500–3000 were observed, only three of which were not matched to this oxidase. One of these represents a low molecular weight (m/z = 569) and a second (m/z = 2386) exhibited low intensity. The high percentage of matching peptide peaks, coupled with high sequence coverage (Table 6) provides a high confidence

Table 6
Amine oxidases expressed by *Rhodococcus opacus* after inducing the cells with four different amines. All proteins were identified by MALDI-TOF MS analysis and matched to the *Rhodococcus* RHA1 using both MSFIT and Mascot.

Inducer	GI number	M_r (Da)	*PI*	% Sequence coverage/peptides	Mowse Score MSFIT	*E*-value Mascot
BUT	111019599	71695	4.6	29/11	$1.5e^{+8}$	$9.1e^{-06}$
BEN	111019599	71695	4.6	23/9	$3.8e^{+7}$	$1.2e^{-05}$
TYR	111019825	72051	4.9	23/12	15758	0.009
PHE	111019825	72051	4.9	18/11	70024	0.021

for positive identification. With the exception of the near identical oxidases from *R. opacus* B4 (Table 3), the protein with greatest homology (by BLAST) to AO1 is AO2 (63% "identities", 75% "positives"). The majority of the sequence variation between AO1 and AO2 occurs on domains that lie on the periphery of the protein sequences. The enzyme component that forms the active site, and therefore has a considerable influence on specificity, exhibited 72% "identities" and 83% "positives". The closest characterised enzyme to both AOs occurs in another gram-positive soil bacterium (*A. globiformis*) that shares 57 and 64% "identities" with AO1 and AO2, respectively. It should be noted that *Arthrobacter sp.* possesses two CuAO with high homology and specificity towards PHE or HIS [32].

The catalytic response recorded in this study provides some insight into possible implications for the surrounding genes, given the small evolutionary distance between the species [51] and conservation of the implicated regions (>95% identities by Blast) within both *Rhodococcus* RHA1 and *R. opacus* B4. AO1 is clustered among genes that are predicted to be involved in the metabolism of a range of xenobiotics [17]. A gene encoding an aldehyde dehydrogenase (GI 111019601) is separated from AO1 by a single amino acid permease and, as such, is likely to act on the resulting generated aldehyde. The possible involvement of this enzyme is significant in that while numerous microorganisms are capable of oxidising BEN, few can utilise the resulting benzaldehyde due to its high toxicity [35]. To date, only *Pseudomonas putida*, *Paracoccus denitrificans* IFO 12442 and *Mycobacterium sp.* JC1 DSM3803 have shown significant growth with BEN as the sole substrate [35] *Mycobacterium* is the only example where this action is mediated by a copper amine oxidase [35]. In our investigation, *R. opacus* was also found to grow on BEN as the sole energy source. We can propose a possible contribution due to nearby *padR* (GI 111019609), the gene encoding a known repressor in the transcriptional control of catabolic pathways of aromatic compounds [52]. AO2 is found in close proximity to a group of previously studied PAA genes [53]. Phenylacetaldehyde generated from PHE oxidation is further degraded *via* a phenylacetic acid pathway, as demonstrated by knockout mutagenesis studies on *Rhodococcus* RHA1 [53]. When either PAAN, a putative ring-opening enzyme, or PAAF phenylacetyl-CoA ligase were mutated, no growth was observed with PHE [53]. This observation suggests that PHE is the main substrate for AO2 and phenylacetaldehyde is catabolised by nearby genes as part of the phenylalanine degradation pathway.

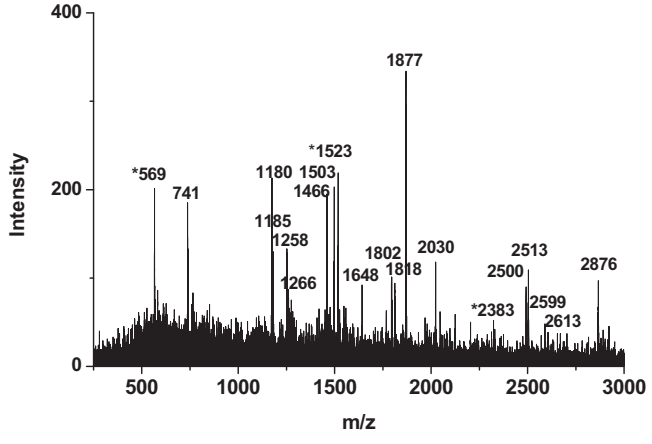


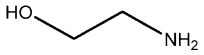
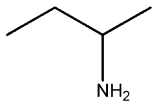
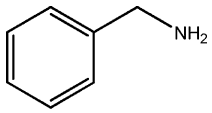
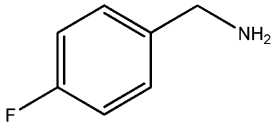
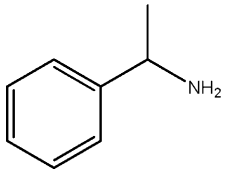
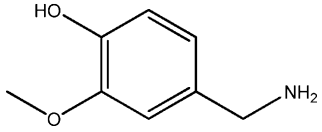
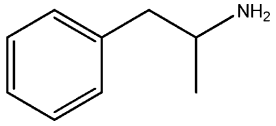
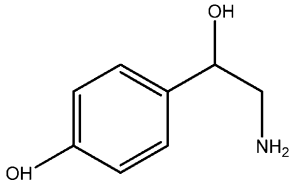
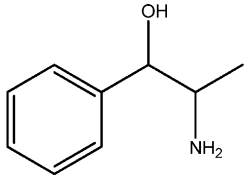
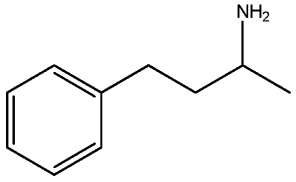
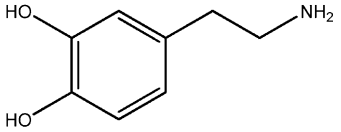
Fig. 5. MALDI-TOF Mass spectrum of AO1 digested with trypsin. Detected peaks using the peakseeker algorithm are displayed, with corresponding matches within 1 Da to the predicted enzyme (at m/z = 741.2, 1180.4[P], 1185.5, 1258.4, 1266.3[P], 1466.4, 1503.4[D], 1648.4, 1802.4, 1818.3, 1877.3, 2030.4[m], 2500.4, 2513.1, 2599.2, 2613.1, 2876.9). Unmatched peaks (at m/z = 569.2, 1523.5, 2383.9) denoted by *. Possible post-translational modifications: [P]Phosphorylation; [D]Dihydroxylation; [m]Dimethylation.

3.5. Kinetic analysis

Kinetic analysis was undertaken using a standard Michaelis–Menten approach over an extended concentration range in order to quantify the catalytic action of the purified AOs. Crucial for adaptation and survival, the range of substrates was extended beyond those presented in Table 1 to include structural analogues that are synthetic or less naturally abundant; these are identified in Table 7. As molecular surface topology and charge considerably influence catalytic efficiency of AOs [54], their action is determined by substrate chain length, steric hindrance and charge distribution. Therefore, taking AO1, additional aliphatic mono-amines and BEN derivatives were also tested (Table 7). These included ethanolamine (ETO) possessing a polar functionality, apolar branched *sec*-butylamine (*sec*-BUT), aromatic methylbenzylamine (MBEN) and halogen (*4*-fluorobenzylamine (FBEN)) and methoxy (vanillylamine (VAN)) congeners. The catalytic response for AO2 was probed by considering substituted phenethylamines as substrates, which included amphetamine (AMP), *1*-methyl-*3*-phenylpropylamine (MPP) and encompassed hydroxyl subsituent(s) on the benzene ring (octopamine (OCT) and dopamine (DOP)) and the carbon chain linking the amino-group to the ring (phenylpropanolamine (PPA)).

Representative activity/substrate concentration plots for oxidative deamination promoted using AO1 and AO2 are presented in Fig. 6a and b, respectively. An increase in activity was observed with increasing concentration to attain activity maxima in the range 0.1–1 mM, with typically a subsequent decrease at higher concentrations, suggesting substrate inhibition. Substrate inhibition was not observed for the shorter chain amines where catalytic efficiency was lower in AO1. This extended to the conversion of AMY and HIS by AO2, where relative catalytic efficiency and substrate affinity were poor relative to the preferential substrates. Similar observations have been reported for *Porcine* AO, where a decrease in activity at higher concentrations was attributed to the binding of more than one substrate molecule to the active site [55]. In the case of *A. globiformis*, it was reasoned that two amines attach during the reductive cycle of the reaction where the second amine binds near the active site or interacts with the post-translated amino acid residue (TPQ) that is required for electron transfer [56]. In common with those studies, inhibition of AO1 and AO2 was consistent with a simple one-site binding model, where $V = V_{max}[S]/(K_M + [S] + [S^2]/K_I$, as shown in Fig. 6.

Table 7
Additional amines selected for a Michaelis–Menten kinetic analysis of the catalytic action of AO1 and AO2.

Substrate	Structure
Ethanolamine (ETO)	
sec-Butylamine (*sec*-BUT)	
Benzylamine (BEN)	
4- Fluorobenzylamine (FBEN)	
Methylbenzylamine (MBEN)	
Vanillylamine (VAN)	
Amphetamine (AMP)	
Octopamine (OCT)	
Phenylpropanolamine (PPA)	
1-Methyl-*3*-phenylpropylamine (MPP)	
Dopamine (DOP)	

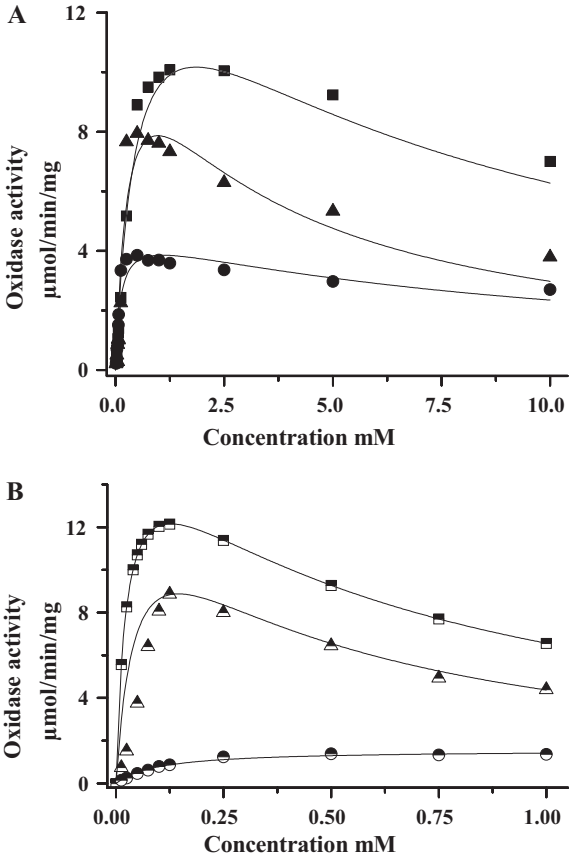


Fig. 6. Initial reaction rate as a function of substrate (PRO (■), BUT (▲), AMY (●), PHE (⬒), TYR (◭) and DOP (◓)) concentration for oxidative deamination promoted using (**A**) AO1 and (**B**) AO2. Note: curves generated by fitting the data to the expression $V = V_{max}\,[S]/(K_M + [S] + [S^2]/K_I)$.

3.5.1. Analysis of AO1 kinetics

From a consideration of the Michaelis (K_M) and rate (k_{cat}) constants given in Table 8, it is clear that the oxidase catalytic action is strongly dependent on the nature of the substrate. Simple aliphatic amines are characterised by K_M values that, with the exception of MET, were inversely related to chain length. This can be attributed to an increased stabilization and substrate positioning afforded by an enhanced interaction with the enzyme. In the case of *bovine* serum AO, substrates were proposed to bind to two different regions, depending on structure and charge distribution of the substrate [57]. The lower K_M observed for MET as well as the aromatic substrates (an order of magnitude lower for BEN and VAN) suggest different interaction/docking at the AO1 active site that is sensitive to amine structure and aromaticity. Amine recognition within this class of enzyme is partially achieved by a "substrate channel" that governs access to the active site within the protein structure. This channel is composed of amino acid side chains that facilitate the catalytic reaction by directing and positioning the amino group of the substrate [58]. In the case of ETO, the polar hydroxyl moiety, as opposed to any steric influence, is the likely source of the considerably higher K_M and lower associated k_{cat}. It must be noted that there was no detectable conversion of *sec*-BUT as substrate where steric hindrance, arising from branched C-NH_2, must inhibit the oxidation reaction; this response extended to MBEN. The highest k_{cat} values were obtained with PRO and BUT, which may reflect their abundance in the natural environment, as both these amines are associated with the degradation of vegetation and animal waste [38,59]. The highest catalytic efficiency, in terms of low K_M and high k_{cat} was achieved with the aromatic BEN substrate

Table 8
Kinetic parameters obtained from a Michealis–Menten treatment of oxidative deamination promoted by AO1 and AO2; "ND" denotes where no inhibition was detected.

AO1

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})	K_I (mM)
Methylamine (MET)	0.18	0.4	2	ND
Ethylamine (ETH)	0.86	7.2	8	ND
Propylamine (PRO)	0.49	18.9	39	9.9
Butylamine (BUT)	0.32	15.3	48	3.1
Amylamine (AMY)	0.11	5.9	54	10.2
Ethanolamine (ETO)	2.05	3.3	2	ND
Benzylamine (BEN)	0.04	12.1	303	4.1
Vanillylamine (VAN)	0.02	1.0	50	2.9
4-Fluorobenzylamine (FBEN)	0.13	17.3	133	0.8

AO2

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})	K_I (mM)
Phenyethylamine (PHE)	0.02	20.9	1045	0.6
Tyramine (TYR)	0.05	16.1	322	0.4
Octopamine (OCT)	0.03	3.5	116	0.6
Dopamine (DOP)	0.10	1.9	19	1.4
Histamine (HIS)	0.28	13.2	47	ND
Butylamine (BUT)	0.32	4.3	13	ND
Amylamine (AMY)	0.16	12.7	79	ND

(k_{cat}/K_M = 303 mM^{-1} s^{-1}, see Table 8). The presence of fluorine as a ring substituent in the *para*-position (FBEN) served to increase both K_M and k_{cat} relative to BEN. Similar observations have been reported for both mouse and human SSAO where k_{cat} increased from 0.18 s^{-1} (BEN) to 0.23 s^{-1} (FBEN) and 0.45 s^{-1} to 0.71 s^{-1}, respectively [60]. It was proposed that the smaller BEN substrate was more readily accommodated in the active site but hindered release of the aldehyde product to a greater extent than the bulkier FBEN [60]. To the best of our knowledge, this is the first instance of FBEN oxidative deamination using a micro-organism. The presence of hydoxyl and methoxy groups on the benzene ring (in VAN) did not significantly alter K_M, but resulted in an appreciably lower k_{cat}.

The heterogeneity that characterises AOs makes comparison with published work difficult as the studies that have considered variations in both specificity and activity have been limited with respect to the range of substrates that were considered. There are several instances where oxidases with low K_M have been recorded for individual substrates but the response does not appear to apply to a broad spectrum of amines. For instance, K_M in the range 0.2–2 mM for C_1–C_4 amines was achieved with the methylamine oxidase of *Arthrobacter P1* [42] and can be compared with 0.24–1.6 mM for C_3–C_6 amines using *A. niger* [43] and 0.23–1.5 mM for C_1–C_5 amines with *Candida boidinii* [36]. When these AOs were tested for aromatic activity, *C. boidinii* did not display any activity with respect to BEN [36], the oxidase of *Arthrobacter P1* exhibited poor substrate affinity (K_M = 3.76 mM) while BEN readily underwent oxidative conversion by *A. niger* due to two separate AOs; K_M = 0.24 mM, k_{cat} of 37 s^{-1} for the copper oxidase and K_M = 0.75 mM, k_{cat} of 100 s^{-1} for FAD dependent oxidase [42,43]. The observed deviation in K_M presumably provides an innate advantage for a variety of environments and must be partially responsible for the isolation of *Rhodococcus* in diverse conditions [51]. The enzymatic and cellular characteristics provide opportunities for biotechnological implementation of AOs as medical sensors and in bioprocessing. AO1 can potentially be utilised in the detection of phospholipids if coupled with phospholipase D and an assay has been developed to detect phosphatidylethanolamine, a prominent phospholipid in mammals that is linked to apoptosis, cell signalling and coagulation [61]. The *Arthrobacter* oxidase utilised to detect phosphatidylethanolamine displayed a K_M = 15 mM and k_{cat} = 1.2 s^{-1} with respect to ETH. The sensitivity can be improved with AO1, which exhibited a significantly lower K_M and higher k_{cat}, as shown in Table 8. An alternative application is in vanillin production, a commercially important flavouring agent in foods/beverages and in pharmaceutical processes [62]. Currently, the vast majority of vanillin is chemically synthesised, as natural forms are expensive and limited by plant supply. A demand, however, remains for natural vanillin, providing an opportunity for microbial conversion as demonstrated by the successful but still relatively expensive approach that utilises ferulic acid [63]. Peppers and capsicums are low cost sources of VAN, which can be formed *via* cleavage of the secondary metabolite capsaicin by proteases. Application of AO from *Escherichia coli* and *A. niger* has been considered where immobilisation of the more activate *A. niger* oxidase resulted in a significant drop (by *ca.* 80% from k_{cat} = 1.0 s^{-1}) in enzyme activity, possibly due to structural changes resulting from enzyme binding to the cellulose support [1]. AO1 exhibited an equivalent VAN conversion rate in its unbound form but shares a relatively low sequence homology with the *Aspergillus* oxidase. Although further study is required to assess AO1 in an immobilised form, the results generated in this study suggest that it is a viable alternative, especially given the presence of eugenol oxidase in *Rhodococcus*, a second enzyme that can oxidise VAN, providing an additional vanillin bioconversion pathway [64,65].

3.5.2. Analysis of AO2 kinetics

As was the case with AO1, AO2 did not exhibit any activity with branched amine substrates, including *sec*-BUT, PPA, MPP or AMP. It should be noted that the CuAO of *Klebsiella oxytoca* has been reported to promote a low but measurable oxidation of AMP [66]. The highest k_{cat}/K_M ratio was delivered by PHE, supporting the genomic analysis that this is the preferred substrate. The presence of a *para*-positioned hydroxyl substituent on the ring (TYR) lowered catalytic efficiency, with an increase in K_M and lower k_{cat}, which we ascribe to steric hindrance and polar effects. This was further compounded in the case of DOP that bears a second hydroxyl substituent. The presence of a hydroxyl substituent on the side chain (in OCT) did not significantly affect K_M but resulted in a significantly lower k_{cat}. The importance of the phenyl ring to influence substrate positioning and enhance substrate affinity in AO2 is illustrated in the case of HIS where the associated k_{cat}/K_M was lower by a factor of 22 relative to PHE. A similarly low catalytic efficiency was recorded for the aliphatic amines. Lower activity with respect to heterocyclic HIS relative to PHE and decreased affinity for aliphatic amines that was inversely proportional to chain length has been noted in the literature [43,44,61] The AOs of *A. niger*, *K. oxytoca* and *Arthrobacter sp.* have all been reported to act on aromatic, aliphatic and heterocyclic amines [43,61,66]. However, this capacity is not as prevalent in bacteria where the work of Murooka *et al.* is notable in that 16 strains were found to show catecholamine activity but only one was active with respect to HIS [34]. The oxidase from *A. niger* exhibited K_M = 0.12–0.6 mM for aromatic amines [43], appreciably higher than that measured for AO2 but the k_{cat}/K_M (=1142 s^{-1} mM^{-1}) for PHE obtained with *A. niger* is close that recorded for AO2 (Table 8). In contrast, the oxidase of *Arthrobacter glomerulus* exhibited a lower K_M for PHE (0.003 mM) and TYR (0.017 mM) and comparable k_{cat} values 8.9 s^{-1} and 25.2 s^{-1}, respectively, but the highest substrate concentration tested (0.033 M) due to substrate inhibition was much lower than that used in this study (Fig. 6) [61]. The oxidases of *K. oxytoca*, on the other hand, possessed lower K_M (0.029–0.003 mM) but similar K_I (0.7–2.6 mM) values [66].

In terms of potential applications, AO2 specificity towards pathophysiological amines that are associated with food spoilage can be exploited to control amine levels in food [67]. In an

investigation of biogenic amine degradation from 166 food strains, complete degradation of 0.54 mM HIS and 0.58 mM TYR after 24 h from a starter culture was only achieved with *Rhodococcus sp.* [67]. Other oxidases with similar specificity have found use in biosensing to determine quality and freshness of fish [4,5] and other foodstuffs [13] where there is still considerable scope for improvement in terms of specificity and activity. As amines are also environmental pollutants, the ability of *R. opacus* to act on a range of aromatic amines as presented here, in addition to a number of other harmful compounds such as naphthalene, herbicides and PCBs [51] serves to highlight the potential of this organism not only in biotechnological applications but also in bioremediation of contaminated waters and soils.

4. Conclusions

Our study demonstrates that *R. opacus* is a catabolically rich strain, with the capacity to act on an array of amine substrates. In the approach that we have taken by limiting the nitrogen source to inorganic nitrogen (NH_4Cl), MET, BUT or PHE, the contrasting phenotypic response in terms of AO expression provided the means of resolving enzymatic multiplicity and facilitating isolation. Coupling of enzyme purification with use of BEN and PHE as test substrates and the action of inhibitors has enabled the isolation and identification of two copper oxidases (EC 1.4.3.21) that we label AO1 and AO2 and which share 63% homology with overlapping specificity. Despite the similarities, the two oxidases appear to participate in alternative degradation pathways as indicated by gene clustering. The prevalence of *R. opacus* in multiple environments can, in part, be attributed to its large genome and considerable catabolic potential. Functional expression, broad substrate affinity and turnover of multiple AOs with their subsequent catabolic pathways exemplify genomic capacity, enabling adaptation and survival. A Michaelis–Menten analysis of AO1 has revealed that this enzyme preferentially deaminates aliphatic amines and aromatic amines where the amino group is attached to the benzene ring *via* a short alkyl chain (e.g. BEN). The AO2 oxidase can also act on aliphatic amines but with a preference for aromatic amines that bear a longer alkyl chain linkage (e.g. PHE or TYR). As both enzymes possess appreciable substrate plasticity (including synthetic amines), coupled with high catalytic efficiency, we believe there is considerable scope for exploitation in bioprocessing and biosensing. Additional oxidases within this organism remain to be characterised. Future work will focus on the identification of these enzymes and their relevance for *R. opacus* as part of both the carbon and nitrogen cycle.

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