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Assessing the catalytic activity of three different sources of tyrosinase: A study of the oxidation of mono- and difluorinated monophenols

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ABSTRACT

Tyrosinase (tyr) purified from *Pseudomonas putida* F6, *Streptomyces antibioticus*, and *Agaricus bisporus* (mushroom) oxidises 3 regioisomers of fluorophenol as well as 3,4-difluorophenol (3,4FP). The catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$) of tyrosinase towards any one substrate is different for each enzyme source. Oftentimes a large difference in affinity (K_{m}), and turnover (k_{cat}) is observed for different enzyme sources with the same substrate. The best catalytic efficiency towards a fluorinated substrate was observed for *P. putida* F6 tyr with 4-fluorophenol (4FP). The presence of a second fluorine on the aromatic ring (3,4FP) resulted in a decrease in the catalytic efficiency of all three enzymes compared to values for 4FP. However, the K_{m} value for 3,4FP decreased for *P. putida* F6 tyr indicating a higher affinity for *P. putida* F6 tyr for 3,4FP compared to 4FP. Furthermore the k_{cat} value for 3,4FP increased for mushroom tyr in comparison to the value for 4FP indicating a higher maximum turnover of 3,4FP compared to 4FP for mushroom tyr. All three sources of tyr exhibited lower catalytic efficiencies for 3-fluorophenol (3FP) and 2-fluorophenol (2FP) compared to 4FP. However, the K_{m} value for 2FP was lower than that for 3FP for both *S. antibioticus* and mushroom tyr indicating a higher affinity for 2FP over 3FP.

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1. Introduction

Tyrosinase is a copper-containing enzyme that has been found widely distributed in microorganisms, plants and animals [1–3]. Tyrosinase (EC 1.14.18.1) catalyses the *o*-hydroxylation of monophenols into their corresponding *o*-diphenols (monophenolase or tyrosine hydroxylase (TH) activity), and the oxidation of *o*-diphenols to *o*-quinones (diphenolase or dopa oxidase activity), using molecular oxygen, which then polymerise to form brown or black pigments [4]. The synthesis of *o*-diphenols (catechols) is a potentially valuable catalytic ability and thus tyrosinase has attracted a lot of attention with respect to biotechnological applications [4,5]. The mechanism of action and the catalytic cycles of the TH and dopa oxidase activities of the enzyme have been studied with respect to the activity towards L-tyrosine and L-dopa [4]. The enzyme contains a dinuclear type-3 copper centre, in which

two copper ions are closely spaced and coordinated each by three histidines through the nitrogen atoms [6]. Cloning, sequencing and expression studies have been carried out on tyr from a number of bacterial sources including *Streptomyces antibioticus*, *Streptomyces lavendulae*, *Rhizobium etli* and *Pseudomonas maltophilia* generating information about the enzyme structure and function [7–10]. The enzyme has also been purified from many sources such as mouse melanoma, mushroom, plants, animals and bacteria [11–14].

The conversion of phenols to *o*-diphenols by tyrosinase is a potentially attractive catalytic ability and thus tyrosinase has attracted a lot of attention with respect to its biotechnological application as the catechol products are useful as drugs or drug synthons, e.g. L-dopa [1,15]. In order to assess the catalytic potential of tyrosinase we have taken different sources of the enzyme and directly compared their catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$) towards substituted phenols. The determination of the kinetic constants is the first step in the assessment of tyrosinase as a biocatalyst. We have focused on fluorinated phenols as fluorinated compounds possess interesting biological activity (e.g. enzyme inhibition, affect energy generation processes and cell to cell communication) and the vast majority of the research into the transformation of halogenated substrates has focused on chlorinated and brominated substrates [16]. In addition the small size of fluorine (similar to hydrogen) allows us to investigate the effects of substituent position on enzyme activity without an effect of substituent size.

Abbreviations: Tyr, tyrosinase; TH, tyrosine hydroxylase; 4FP, 4-fluorophenol; 3,4FP, 3,4-difluorophenol; 3FP, 3-fluorophenol; 2FP, 2-fluorophenol; 4EP, 4-ethylphenol; L-dopa, L-3,4-dihydroxyphenylalanine.

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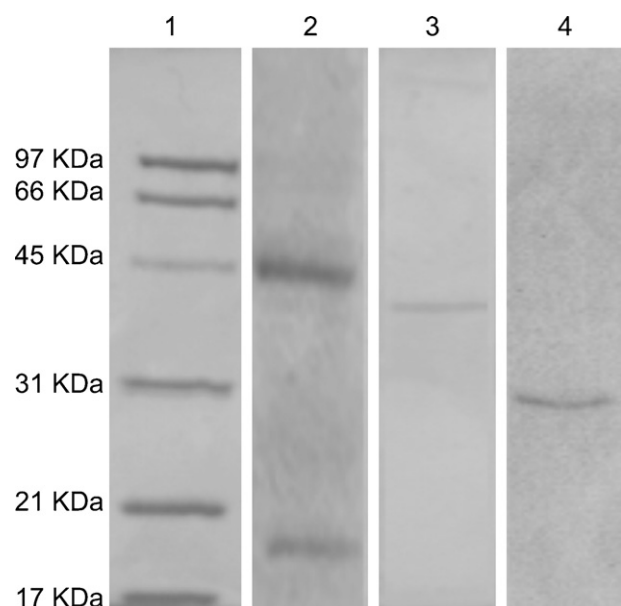


Fig. 1. SDS-PAGE of purified tyrosinases from three different sources: commercial *A. bisporus* (mushroom) (slice 2); *P. putida* F6 (slice 3); *S. antibioticus* (slice 4); molecular weight standards (slice 1). Proteins were stained with Coomassie Blue R250.

The kinetic characteristics of purified tyr from *Pseudomonas putida* F6 towards any fluorophenol has not been reported. While the activity of tyr from mushroom [17,18], *P. putida* F6 (crude cell extract) [19], and *S. antibioticus* [20] has been reported for 4FP no direct comparison of the purified enzyme activities has been undertaken. None of the tyr from the three sources used in this study have been tested for activity towards 3,4FP. Tyr from *S. antibioticus* has been reported to show activity towards 3FP but not 2FP [20]. To our knowledge mushroom tyr has not been tested for activity towards 2FP or 3FP.

In this study tyr from *P. putida* F6, *S. antibioticus* and *Agaricus bisporus* (commercial mushroom), all purified to homogeneity, were used to characterise the enzymes activity towards various substituted monophenols (Fig. 1) [14,21]. The substrates studied here include the three regioisomers of fluorophenol, 3,4FP and 4-ethylphenol (4EP). We wish to directly compare the activity of *A. bisporus*, *S. antibioticus* and *P. putida* F6 tyr towards a range of substrates and to gain a greater understanding of the effect of substitution on enzyme activity. Analysis of kinetic data for various sources of tyr towards mono- and di-substituted phenols will allow for better assessment of the biotechnological potential of the same catalytic activity from different sources.

2. Materials and methods

2.1. Reagents

4FP, 2FP, 3FP, 4EP, ferulic acid and mushroom tyr (3216 U/mg) were purchased from Fluka.

L-Tyrosine, copper (II) sulfate solution, DL-dithiothreitol and bicinchoninic acid solution were purchased from Sigma. 3,4FP was purchased from Acros Chemical Company. All other reagents were of analytical grade.

2.2. Protein molecular weight

The molecular weights of the 3 proteins used in this study are, *A. bisporus* is heterotetramer with two large subunits of 43 KDa and two small subunits of 17 KDa and overall molecular weight of 120 KDa [22], *P. putida* F6 39 KDa [14], and *S. antibioticus* 30 KDa [23]. Purity of the protein preparations was analysed by SDS-PAGE, according to standard protocols.

2.3. Preparation of *S. antibioticus* tyrosinase

S. antibioticus tyr was purified as previously described [24].

2.4. Preparation of commercial mushroom tyrosinase

Fluka (3216 U/mg) mushroom tyr was purified, by using anion exchange chromatography as previously described [21]. The column was equilibrated with 20 mM Tris-HCl pH 8.0. A stepwise gradient of increasing sodium chloride (NaCl, 0–500 mM) concentrations was applied (5 ml/min). Fractions (29–34) were collected, concentrated and used as the source of commercial enzyme as they exhibited the highest rate of tyr activity with tyrosine as the substrate.

2.5. Preparation of crude cell extract of *P. putida* F6 (CE)

P. putida F6 was grown in batch culture in E2 medium [25], with phenylacetic acid (5 mM) as the sole carbon and energy source and induced with ferulic acid (1 mM) for 2 h [26]. The cells were harvested, at an OD_{540 nm} of 0.9–1.0, by centrifugation at 15,000 × g for 10 min at 4 °C and washed twice in ice-cold 50 mM potassium phosphate buffer, pH 7.0. The cell pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.0 with 1 mM dithiothreitol and 10% (w/v) glycerol. Crude cell free extract (CE) was prepared by passing cells twice through a precooled French pressure cell (1000 psi), followed by centrifugation at 36,700 × g for 30 min. The resulting supernatant (CE) was collected and stored on ice.

2.6. *P. putida* F6 tyrosinase purification

P. putida F6 tyr was purified, using a DEAE sepharose column and gel filtration column as previously described [14].

2.7. Determination of protein by the Bicinchoninic method

This method is a modification of the Sigma Bicinchoninic acid protein assay method which itself is a modification of the method described by Smith et al. [27]. A 0.2 ml aliquot of bicinchoninic acid containing 2% (v/v) copper sulphate solution was added to duplicate wells in a 96-well microtitre plate containing 0.025 ml of the appropriate dilution of the test solution. The colour was allowed to develop for 30 min at 40 °C before reading the absorbance at 550 nm using a temperature controlled microplate reader SpectraMax 340 (Molecular Devices Corp., Sunnyvale, USA). Absorbance readings were converted to mg/ml protein using a standard curve of bovine serum albumin (BSA) 0.1 to 0.5 mg/ml.

2.8. Determination of molar extinction coefficient values for various substituted phenols

When tyr is supplied with substituted phenol substrates a dark colour is formed in the reaction medium. The relationship between monophenol substrate utilised and colour intensity of the product (molar extinction coefficient) was determined as previously described [28,29]. Mushroom tyr (0.5 units of tyrosinase activity) was added to 50 mM potassium phosphate buffer (pH 7.0) containing 0–0.5 mM substrate in a 1 ml spectrophotometric reaction carried out in triplicate. One unit of tyr activity is equal to 1 nmol of dopachrome produced per minute at 30 °C from L-tyrosine ($\epsilon = 3600 \text{ M}^{-1} \text{ cm}^{-1}$). All reactions were monitored in an ultraviolet–visible (UV) Helios β spectrophotometer (Unicam, Cambridge, UK). Temperature was controlled at 30 °C using a circulating water bath (Grant Instruments, Cambridge, UK). Reference cuvettes contained all reaction components except the substituted monophenols which, were replaced with 50 mM potassium phosphate buffer (pH 7.0).

The relationship between optical density at the maximum wavelength and concentration of phenol transformed was then determined by comparison of data from HPLC and spectrophotometry. The molar extinction coefficient (ϵ) of the reaction product was determined from the Beer–Lambert law.

2.9. High performance liquid chromatography

The HPLC apparatus included a C-18 Hypersil ODS 5 μ column (125 mm × 3 mm) (Hypersil, Runcorn, UK) and a Hewlett Packard HP1100 series instrument equipped with an Agilent 1100 series diode array detector. Metabolites were eluted under isocratic conditions using a mix of phosphoric acid (0.1%, v/v) and methanol as the mobile phase. The ratio of methanol to phosphoric acid (0.1%, v/v) varied according to substrate analysed (30:70 for 2FP, 3FP, 4FP and 3,4FP, 50:50 for 4EP). The flow rate was 0.5 ml/min and the injection volume of all samples was 20 μ l. 900 μ l of reaction samples were acidified with 100 μ l 1N HCl, centrifuged, filtered (0.2 μ m, 4 mm, nylon syringe filter) and substrate depletion analysed by HPLC.

2.10. Kinetic experiments

Kinetic studies on the oxidation of substituted monophenols were performed by spectrophotometric measurement of the corresponding o-quinone at 400 nm

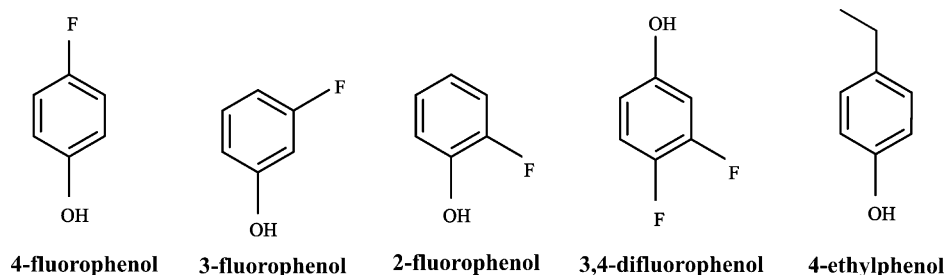


Fig. 2. Tyrosinase substrates: substituted monophenols used in this study.

and recorded using a microplate reader SpectraMax 340 (Molecular Devices Corp., Sunnyvale, USA) on-line interfaced with a compatible PC and controlled with Softmax Pro 4.6 software. Temperature was controlled at 30 °C. Kinetic studies were performed in air saturated 50 mM phosphate buffer, pH 7.0, at 30 °C using a circulating water bath (Grant Instruments, Cambridge, UK). Reference wells contained all the components except the substrates, which were replaced with 50 mM potassium phosphate buffer (pH 7.0).

The three sources of tyr exhibited different rates of reaction with tyrosine (1 mM) as a substrate. In order to allow us to compare the activities of the enzyme towards various substrates it was imperative that the same units (8.8 units) of tyr activity were added to the assays (1 unit of tyr activity is equal to 1 nmol of dopachrome produced per minute at 30 °C from L-tyrosine ($\epsilon = 3600 \text{ M}^{-1} \text{ cm}^{-1}$)). Thus different concentrations of protein from the three sources were added into the assays, i.e. 0.006, 0.005, 0.06 mg/assay of protein from *P. putida* F6, *S. antibioticus* and mushroom, respectively. The assay medium (0.2 ml) at 30 °C contained 0.4 μM L-dopa, tyr and phenol substrate (added last to start the reaction). Substrate concentration was varied between 0.075 and 5.0 mM. The monophenolase activity of tyr shows a characteristic lag period before the maximum velocity of the hydroxylation step is reached [30]. The presence of catalytic amounts of diphenol such as L-dopa eliminates the lag period [31,32]. The low concentration of L-dopa (4 μM) used in this study did not contribute to the optical density when incubated with any source of tyr (data not shown).

2.11. Kinetic characterisation of tyrosinase

Initial rates were obtained by fitting linearly the change in absorbance ($\Delta A_{400 \text{ nm}}$) versus time plots. Kinetic parameters were obtained according to the Lineweaver and Burk (double reciprocal) method, which, allows for the determination of the Michaelis constant (K_m) and the maximum velocity (V_{max}) using a non-linear regression analysis software, Enzfitter for Windows 2.0.18.0 (Elsevier-Biosoft®, UK).

3. Results and discussion

Purified tyrosinases from three different sources were used in this study (Fig. 1). The addition of purified tyr from any of the three sources (*P. putida* F6, *S. antibioticus*, mushroom) to substituted monophenols (Fig. 2) resulted in the formation of a yellow/brown colour in the assay medium. This is indicative of the oxidation of the substrate by tyr activity, which results in the formation of coloured oxidised products. The intensity of the colour produced was seen to increase with increasing substrate concentration. The molar extinction coefficient of the coloured products was determined experimentally as no authentic standards were available commercially. A comparison of the data from the HPLC (substrate utilised) and spectrophotometer (colour formed) was used to determine the relationship between monophenol concentration utilised by tyr and the intensity of colour at λ_{max} (e.g. 4FP, Fig. 3). The value for the molar extinction coefficient (ϵ) was established for all of the substituted phenols in this study (Table 1). A linear relationship between colour and phenol concentration was observed up to 0.5 mM for 4EP and 2FP. However, a linear relationship only existed below 0.4 mM for 4FP, 3,4FP and 3FP. 4EP has the highest molar extinction coefficient value ($\epsilon_{400 \text{ nm}}$) while 4FP and 3FP display the lowest (Table 1). 4FP and 3FP are both oxidised by tyr to 4-fluorocatechol (data not shown) which is subsequently oxidised to a quinone which polymerises to form the coloured product and thus 4FP and 3FP have the same molar extinction coefficient value.

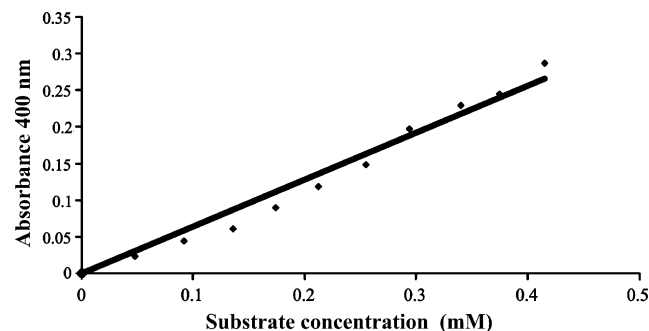


Fig. 3. The relationship between absorbance and substrate concentration (4-fluorophenol) utilised.

Table 1

Molar extinction coefficients of tyrosinase assay products formed from various substituted monophenols

Substrate	$\epsilon \text{ (M}^{-1} \text{ cm}^{-1})^a$
4-Fluorophenol	641
3-Fluorophenol	641
2-Fluorophenol	903
3,4-Difluorophenol	860
4-Ethylphenol	1123

^a All values are determined from the slope of the line from standard curves of triplicate results.

Kinetic measurements of tyr activity were carried out as a function of substrate concentration. A study by Orenes-Pinero et al. [33] on the kinetic characterisation of dopa oxidase activity of *S. antibioticus* showed a lag period was also seen for monophenolase activity whose duration was dependant on substrate concentration. To remove the lag period in the present study 4 μM L-dopa was added to the kinetic assays [34].

A value for the kinetic parameters K_m and k_{cat} were calculated for each substrate using the double reciprocal plots of the inverse reaction rates of tyr as a function of the inverse substrate concentration (4FP) in the presence of various enzyme sources. By way of example the plots for 4FP are shown in Fig. 4. The K_m value of tyr from *P. putida* F6 for 4FP was 1.9- and 2.5-fold lower than that observed for *S. antibioticus* and mushroom, respectively. Previous studies using *S. antibioticus* have shown a 5.7-fold higher K_m value for 4FP than that observed in this study [20]. The k_{cat} (number of molecules of the substrate turned over per second per mole of enzyme) was higher for mushroom tyr compared to the other two enzyme sources. However, *P. putida* F6 Tyr still exhibited the highest catalytic efficiency (k_{cat}/K_m) towards 4FP (Table 2).

Fluorine is a small substituent similar in size to hydrogen, but with a much stronger electron withdrawing effect on the benzene ring. The supply of a difluorinated monophenol (3,4-difluorophenol) allows us to investigate the electronic effects of

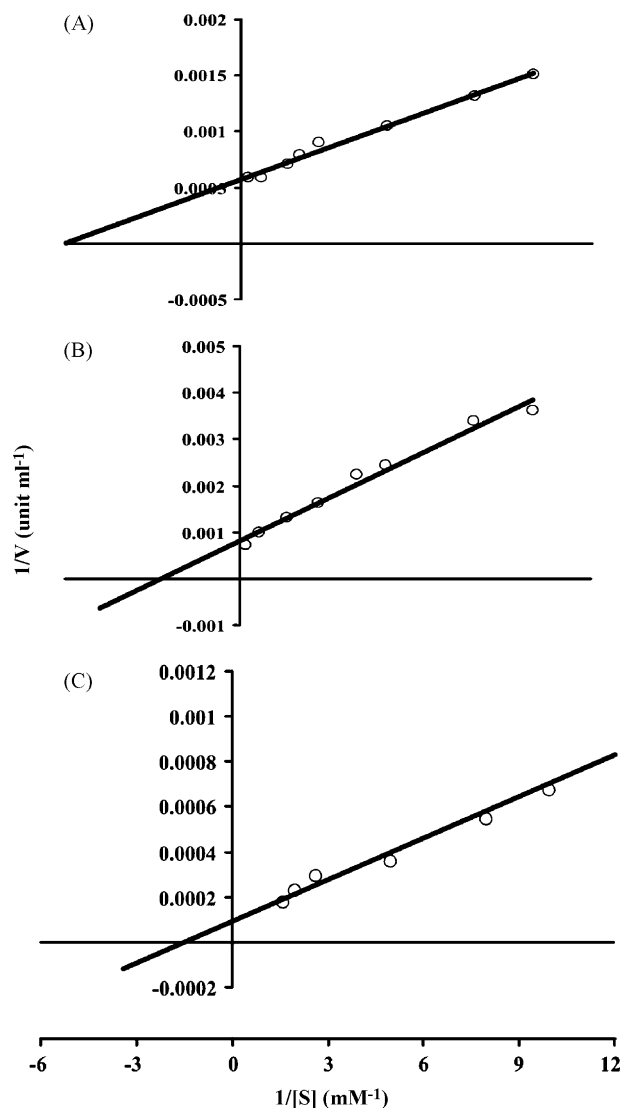


Fig. 4. Double reciprocal plots of the kinetics of tyrosinase catalysed oxidation of 4-fluorophenol with various purified tyrosinase sources: (A) *P. putida* F6, (B) *S. antibioticus* and (C) commercial mushroom. The experimental conditions were 50 mM phosphate buffer pH 7.0, temperature 30 °C. The protein concentrations varied at 0.006 mg/assay for *P. putida* F6 (A), 0.005 mg/assay for *S. antibioticus* (B) and 0.06 mg/assay for mushroom (C). The substrate concentrations ranged between 0.1 and 5.0 mM.

fluorine on tyr enzyme activity without an effect of substituent size.

While all three sources of tyr exhibited a lower catalytic efficiency value for 3,4FP compared to 4FP the K_m and k_{cat} values for

the three enzyme sources differed (Table 2). Indeed the affinity of *P. putida* F6 tyr was greater for 3,4FP compared to 4FP. However, a 17.9-fold lower k_{cat} value for 3,4FP compared to 4FP indicates that 3,4FP is a poor substrate for *P. putida* F6 tyr (Table 2). In contrast to *P. putida* F6 both mushroom and *S. antibioticus* tyr exhibited a lower affinity for 3,4FP compared to 4FP (Table 2). While mushroom tyr exhibits the highest K_m of the three enzymes and also showed the largest difference in K_m values (3.4-fold) between 4FP and 3,4FP the k_{cat}/K_m value for mushroom was higher than *S. antibioticus* or *Pseudomonas* tyr. Indeed mushroom tyr exhibits a 1.3-fold higher k_{cat} value for 3,4FP (42.7 s^{-1}) compared to 4FP (33.0 s^{-1}). The higher k_{cat} value for 3,4FP is surprising as the presence of an extra electron withdrawing group such as fluorine should reduce the reactivity of the enzyme towards that substrate. Thus one would have expected a reduction in the k_{cat} value compared to 4FP as a substrate. The presence of electron donating groups has been shown to assist in the hydroxylation of monophenols by tyr [35–37]. In keeping with this prediction the substitution of the fluorine moiety of 4FP with an ethyl moiety (4EP) results in higher catalytic efficiencies of all three sources of tyr. The increase in catalytic efficiency for *P. putida* F6 is due to both a lower K_m and higher k_{cat} value (Table 2). However, the k_{cat} value for 4EP is lower than that of 4FP for both *S. antibioticus* and mushroom tyr and thus the increased affinity and not turnover for 4EP is responsible for the increased catalytic efficiency towards this substrate (Table 2). While the k_{cat}/K_m value agrees with the predicted reactivity of 3,4FP and 4EP the increase in the k_{cat} value for the difluorinated substrate and the decrease in k_{cat} for 4EP are currently inexplicable.

The effect of substituent position (regiochemistry) was also investigated with 2 and 3FP as substrates. The K_m value for 3FP for *P. putida* F6 and *S. antibioticus* tyr increased 2.3- and 3.2-fold compared to 4FP. However, mushroom tyr exhibited a 1.2-fold lower K_m value for 3FP compared to 4FP. Moreover the k_{cat} for all three enzyme sources decreased and thus the catalytic efficiency of the enzymes towards 3FP also decreased (Table 2). In a previous study of the oxidation of 4FP and 3FP by *S. antibioticus* tyr a similar trend for both K_m and k_{cat} values was observed [20].

Mushroom tyr was the only source of enzyme capable of the oxidation of 2FP under the standard set of conditions described in this study (data not shown). The inability of *P. putida* F6 and *S. antibioticus* to oxidise 2FP may be due to lower protein concentrations (0.006 and 0.005 mg/assay, respectively) in the assay medium compared to mushroom tyr (0.06 mg/assay). In order to detect activity towards 2FP the concentration of protein in the *S. antibioticus* and *P. putida* F6 tyr preparations was increased to 0.011 mg/assay for both enzyme sources (Table 2). *P. putida* F6 displayed the highest K_m values showing a poor affinity of the enzyme for this substrate, however, a poorer catalytic efficiency was seen for *S. antibioticus* tyr (Table 2). While the catalytic efficiency of the bacterial sources towards 2FP is between 6.9- and 7.7-fold lower in comparison to 3FP and 4FP mushroom tyr displayed a

Table 2

Kinetic constants characterising the reaction of tyrosinase from different sources with various substrates

Substrate	<i>Pseudomonas putida</i> F6			<i>S. antibioticus</i>			Purified commercial mushroom		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1}\text{mM}^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1}\text{mM}^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1}\text{mM}^{-1}$)
4-Fluorophenol	0.27 ± 0.01	23.15 ± 0.94	85.74 ± 0.94	0.51 ± 0.09	13.99 ± 0.77	27.45 ± 0.78	0.67 ± 0.29	33.0 ± 7.0	49.25 ± 7.0
3,4-Difluorophenol	0.14 ± 0.01	1.29 ± 0.02	9.21 ± 0.04	0.89 ± 0.15	4.41 ± 0.26	4.96 ± 0.30	2.29 ± 0.53	42.7 ± 5.0	18.65 ± 5.0
3-Fluorophenol	0.62 ± 0.15	2.58 ± 0.001	4.16 ± 0.25	1.67 ± 0.3	3.60 ± 0.28	2.15 ± 0.41	0.55 ± 0.08	7.90 ± 0.4	14.33 ± 0.17
2-Fluorophenol ^a	2.05 ± 0.99	1.23 ± 0.4	0.60 ± 0.07	0.86 ± 0.61	0.24 ± 0.06	0.28 ± 0.61	0.32 ± 0.04	3.00 ± 0.04	9.3 ± 0.057
4-Ethylphenol	0.05 ± 0.01	35.37 ± 1.24	786 ± 1.24	0.06 ± 0.01	8.70 ± 0.26	153 ± 26	0.03 ± 0.006	4.8 ± 0.1	160 ± 0.1

The experimental conditions were 50 mM phosphate buffer pH 7.0, temperature 30 °C. The protein concentrations varied at 0.006 mg/assay for *P. putida* F6, 0.005 mg/assay for *S. antibioticus* and 0.06 mg/assay for purified commercial mushroom. The substrate concentrations varied between 0.1 and 5.0 mM.

^a Protein concentration altered to 0.011 mg/assay for assays with 2-fluorophenol due to a lack of activity for *P. putida* F6 and *S. antibioticus* tyrosinase at lower protein concentrations with this substrate.

catalytic efficiency only 1.5-fold lower for 2FP compared to 3FP (Table 2).

The activity of tyr from all three sources towards the three mono-substituted regioisomers followed a predictable trend, i.e. the catalytic efficiency of any one enzyme was higher for 4FP compared to 3FP and 2FP. Furthermore the presence of an extra electron withdrawing moiety on the aromatic ring (3,4FP) did result in a dramatic decrease in the catalytic efficiency of all three enzyme sources compared to 4FP. However, a large variation in the K_m , k_{cat} and catalytic efficiency was observed with different sources of tyr for any one substrate which suggests that while these enzymes follow similar trends the catalytic ability of the enzyme is dependent upon its biological source. Indeed the oxidation of 4FP is most efficiently catalysed by tyr from *P. putida* F6 however purified commercial mushroom tyr shows greatest catalytic efficiency towards 34FP.

The activity of the three sources of tyr towards tyrosine and L-dopa follow a similar trend to that observed in this study in that the catalytic ability of the enzyme is dependent upon its biological source. *P. putida* F6 tyr has a 1.5-fold higher affinity for L-tyrosine (K_m 0.33) compared to L-dopa but the rate of reaction was 2.3-fold higher for the latter substrate (0.9 $\mu\text{mol}/\text{min}$) [14]. *S. antibioticus* has a 27-fold higher K_m value for L-dopa compared to *P. putida* F6. Many studies have been carried out using mushroom tyr on various aromatic phenols including L-dopa and L-tyrosine however the commercial sources differ as does the method of purification for the enzyme leading to difficulty in comparing the kinetic parameters [22,38–40]. Thus the application of tyrosinase for the biotransformation of phenols to catechols will require a matching of catalyst to substrate as some sources of tyrosinase will oxidise specific phenols more efficiently.

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