Partial purification and characterisation of two actinomycete tyrosinases and their application in cross-linking reactions

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Note: Nucleotide sequence data reported are available in the GenBank database under the accession numbers KR030065, KR030066, KR030067 and KR030068.

Highlights:

- Partial purification of two extracellular tyrosinases from *Streptomyces* spp.
- Organic solvent resistance exhibited by tyrosinases
- Atypical characteristics, e.g. resistance to arbutin and SDS
- Catalysed cross-linking reactions of proteins, e.g. gelatine and casein

Abstract

Actinomycetes are a ubiquitous group of bacteria, and are hypothesised to produce tyrosinases for protection against the potential toxic effect of phenolic compounds and for the production of melanin. In this study, tyrosinase production by *Streptomyces pharetrae* CZA14^T (CZA14Tyr) and *Streptomyces polyantibioticus* SPR^T (SPRTyr) was optimised. The enzymes were partially

purified and biochemically characterised to determine their suitability for industrial applications. SPRTyr was stable up to 40°C and at pH 4.5-10.0, while CZA14Tyr was stable up to 40°C and at pH 6.5-10.0. The enzymes showed variable stability in the presence of water-miscible organic solvents and were able to oxidize L-DOPA in the presence of these solvents. A limited inhibitory effect was observed with arbutin, EDTA, sodium chloride and sodium dodecyl sulphate, while both enzymes were strongly inhibited by the reducing agents used in this study. Inhibition of enzyme activity was observed in the presence of 1 mM Cu^{2+} and 5 mM Co^{2+} for SPRTyr, and 5 mM Fe^{2+} and 5 mM Zn^{2+} for CZA14Tyr. When applied in various cross-linking reactions both tyrosinases were able to cross-link casein and gelatine in the absence of a phenolic compound, showing potential for application in the food industry and for the production of biomaterials.

Keywords:

biochemical characterisation, cross-linking, industrial application, *Streptomyces*, tyrosinase

1. Introduction

Tyrosinases (EC 1.14.18.1) are Type III copper proteins that utilise molecular oxygen to catalyse two types of reactions: monophenolase and diphenolase (catechol oxidase) type reactions [1,2]. In the monophenolase reaction, the *o*-hydroxylation of a monophenol occurs to produce an *o*-diphenol. The *o*-diphenol then serves as a substrate for the subsequent catechol oxidase reaction, resulting in the formation of *o*-quinones. In addition, tyrosinases have also been shown to catalyse the *o*-hydroxylation of aromatic amines and the oxidation of *o*-aminophenols to *o*-quinoneimines [3]. Tyrosinases, along with laccases (EC 1.10.3.2) and catechol oxidases (EC 1.10.3.1), are collectively known as phenol oxidases. However, it is only tyrosinases that are able to catalyse monophenolase type reactions, which is often used as a distinguishing feature in the biochemical description of phenol oxidases [1]. The Type III copper centre of tyrosinases consists of two copper atoms (Cu_A and Cu_B) that are co-ordinated by six highly conserved histidine residues (three residues per copper co-ordination) located in the active site [4]. The signature

sequence around the Cu_A and Cu_B co-ordination sites are characterised by the sequences H-X(n)-H-X(8)-H and H-X(3)-H-X(n)-H, respectively [1,5].

Tyrosinases are quite widespread in nature where they play various roles related to the health or pathogenicity of the producing organism [1]. In 1972, the first streptomycete tyrosinases were described: *Streptomyces glaucescens* [6] and *Streptomyces nigrifaciens* [7], but it is not clear whether the latter represents a true tyrosinase [2]. Very few streptomycete tyrosinases (less than twenty) have been isolated and described to date, which is quite surprising considering the fact that the genus currently consists of more than 600 validly published species (List of prokaryotic names with standing in nomenclature, 2015; http://www.bacterio.net/index.html). It has been proposed that there are three different types of tyrosinases in streptomycetes [8]: Type I or MelD (membrane bound, universal in streptomycetes, potential protective role against phenolics); Type II or MelC (secreted, sporadic occurrence in streptomycetes); and Type III (*o*-aminophenol oxidase, intracellular, rare, involved in secondary metabolite production). All three types of tyrosinases are encoded by bi-cistronic operons: one gene encodes for a chaperone or caddy protein and the downstream gene encodes for an inactive, apotyrosinase. For the streptomycete MelC extracellular tyrosinase, MelC1 is a copper chaperone involved in the binding of copper ions and the incorporation of copper into the apotyrosinase (MelC2). MelC1 is also responsible for the activation and secretion of the otherwise inactive apotyrosinase: MelC1 contains the twin arginine signal peptide sequence that allows for the export of the enzyme in its active form through the twin arginine translocation (TAT) secretion pathway [8]. In 2006, the crystal structure of the tyrosinase produced by *Streptomyces castaneoglobisporus* was elucidated, thereby providing a clearer understanding of the interaction of MelC1 with MelC2 during the formation of an active tyrosinase [9].

The ability of tyrosinases to oxidize mono- and diphenols has allowed for their applications in various biotechnological processes. The tyrosinase from *Agaricus bisporus* (common button mushroom) is most commonly utilized in research, largely because of its commercial availability

[10]. However, the limitations of this tyrosinase (e.g. low solvent and heat stability) have encouraged researchers to look towards alternative sources of tyrosinases, including bacteria due to ease of genetic manipulation and over-expression in expression hosts such as *Escherichia coli* [10]. The biotechnological relevance of bacterial tyrosinases spans their application in bioremediation (detoxification of phenol-contaminated wastewater and soil), the production of synthetic melanins, their application in biocatalysis reactions (production of L-3,4-dihydroxyphenylalanine or L-DOPA, and application in cross-linking reactions) and biosensor technology (detection of phenols and oxygen) [10,11]. In this study, tyrosinases from two novel streptomycetes, *Streptomyces polyantibioticus* SPR^T and *Streptomyces pharetrae* CZA14^T, were isolated and biochemically characterised (solvent stability, pH stability, thermostability, effect of inhibitors) to determine their suitability for application in industrial processes. The genome sequences of the two strains were analysed for the presence of bi-cistronic tyrosinase operons and the sequences subjected to bioinformatics analyses. Finally, both tyrosinases were applied in selected cross-linking reactions to determine their potential application in industries requiring covalent protein-protein cross-linking.

2. Experimental

2.1 Materials/Chemicals Used in this Study

All chemicals used in this study were obtained from Merck-Millipore (South Africa) and Sigma-Aldrich (South Africa), unless stated otherwise.

2.2 Tyrosinase-Producing Strains

S. polyantibioticus SPR^T (=DSM 44925^T =NRRL B-24448^T) was isolated from soil collected from the banks of the Umgeni River, KwaZulu Natal, South Africa [12] and *S. pharetrae* CZA14^T (=DSM 41856^T =JCM 13860^T =NRRL B-24333^T) was isolated from soil collected from the base of a giant quiver tree, Karoo Desert National Botanical Gardens, Worcester, South Africa [13]. The strains were maintained on International *Streptomyces* Project (ISP) medium number 2 (ISP2 or

Yeast extract-Malt Extract, YEME; g/L: 10.0 malt extract, 4.0 yeast extract, 4.0 glucose, 20.0 bacteriological agar, pH 7.3) [14] and as stock cultures in 20% (v/v) glycerol at -20°C.

2.3 Screening for Tyrosinase Activity: Solid Media

S. polyantibioticus SPR^T and *S. pharetrae* CZA14^T were streaked onto various solid media for the detection of melanin production (which served as an indicator of tyrosinase production). The following media were used: YEME (ISP2); ISP medium number 6 (ISP6; g/L: 15.0 peptone, 5.0 proteose peptone, 0.5 ferric ammonium citrate, 1.0 K_2HPO_4, 0.08 $Na_2S_2O_3$, 1.0 yeast extract, 15.0 bacteriological agar, pH 7.0) [14]; ISP medium number 7 (ISP7; g/L: 15.0 glycerol, 0.5 L-tyrosine, 1.0 L-asparagine monohydrate, 0.5 K_2HPO_4, 0.5 $MgSO_4.7H_2O$, 0.5 NaCl, 0.01 $FeSO_4.7H_2O$, 1.0 ml trace salts solution, 20.0 bacteriological agar, pH 7.2; Trace salts solution, g/100 ml: 0.1 $FeSO_4.7H_2O$, 0.1 $MnCl_2.4H_2O$, 0.1 $ZnSO_4.7H_2O$, filter sterilize) [14]; DSMZ medium 553 (GPHF-medium, g/L: 10.0 glucose, 5.0 peptone, 5.0 yeast extract, 5.0 beef extract, 0.74 $CaCl_2.2H_2O$, 15.0 bacteriological agar, pH 7.2); modified phenoxazinone production medium (MPPM; g/L: 10.0 glycerol, 10.0 glucose, 10.0 soya flour, 5.0 casamino acids, 5.0 yeast extract, 4.0 $CaCO_3$, 1ml trace salts solution, 15.0 bacteriological agar, pH 7.0; Trace salts solution, g/100 ml: 1.0 FeSO4; 0.9 ZnSO4, 0.2 $MnSO_4$) [15]; and Czapek solution agar (CZ; g/L: 30.0 sucrose, 2.0 $NaNO_3$, 1.0 K_2HPO_4, 0.5 KCl, 0.5 $MgSO_4.7H_2O$, 0.01 $FeSO_4.7H_2O$, 15.0 bacteriological agar, pH to 7.3 ± 0.2) [16]. *Streptomyces antibioticus* NRRL B-2770^T, a known tyrosinase producer, was included in all experiments as a positive control. Plates were incubated at 30°C for 7 days and monitored for the production of a dark brown to black pigment.

2.4 Enzyme Production

For the production of the tyrosinases, 10 ml pre-cultures of the two tyrosinase-producing strains were prepared in MPPM: 100 µl of the stock cultures was used to inoculate every 10 ml volume pre-culture media. *S. polyantibioticus* SPR^T was inoculated into MPPM, pH 5.5, while *S. pharetrae* CZA14^T was inoculated into MPPM, pH 6.5. The *S. polyantibioticus* SPR^T and *S. pharetrae* CZA14^T pre-cultures were incubated for 48 h at 22°C and 30°C, respectively, shaking

at 160 rpm. For both strains, a 5% pre-culture inoculum (20 ml) was used to inoculate a 400 ml volume of MPPM (in a 2 L shake flask), which was supplemented with 4 ml 100 mM filter sterilised $CuSO_4.5H_2O$ (final concentration of 1 mM). The 400 ml volume cultures were incubated as before, but only for 24 h. The cultures were centrifuged at 10 000 *g* for 5 min at 4°C and the culture supernatants used for the purification of the extracellular tyrosinases.

2.4.1 Partial Purification of the Tyrosinases: Ammonium Sulphate Precipitation, Dialysis and Anion Exchange

The culture supernatants from both strains were subjected to ammonium sulphate precipitation. The culture supernatant was initially brought to a 40% saturation level, stirred at 22°C for 1 h and centrifuged at 10 000 *g* for 10 min at 4°C. The resultant supernatant was brought to a 56% saturation level, stirred at 22°C for 1 h and centrifuged at 10 000 *g* for 10 min at 4°C. The pellets were resuspended in 50 mM potassium phosphate buffer (pH 6.5) and dialysed at 4°C for 24 h against ice-cold 50 mM potassium phosphate buffer, pH 6.5 (buffer replaced after the first 3 h and after 20 h). After 24 h, the dialysis buffer was replaced with a 20% (w/v) polyethylene glycol 8000 solution (prepared in 50 mM potassium phosphate buffer, pH 6.5) to concentrate the enzyme solution (incubated at 4°C for 24 h). The concentrate was redissolved in 50 mM potassium phosphate buffer, pH 6.5, and applied to a DEAE Sephadex™ A-50 (GE Healthcare) column. The enzymes were eluted with 50 mM potassium phosphate buffer, pH 6.5. The enzymes were designated SPRTyr for *S. polyantibioticus* SPR^T tyrosinase and CZA14Tyr for *S. pharetrae* $CZA14^T$ tyrosinase.

2.4.2 Enzyme Activity

Tyrosinase activity was determined using L-DOPA as a substrate: 10 mM L-DOPA was prepared in 50 mM potassium phosphate buffer (pH 6.5) [6] and its oxidation was monitored at 475 nm (ε = 3600 M^{-1} cm^{-1}) using an anthos Zenyth1100 microtiter plate reader. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of substrate per minute at 22 ±

2°C (ambient temperature). Tyrosinase from *S. antibioticus* NRRL B-2770^T was used as a positive control.

2.4.3 Protein Concentration Determination

The protein concentrations of the enzymes were determined using the Bradford's assay [17]. A standard curve was produced using a range of bovine serum albumin (BSA) concentrations. 20 µl of sample was added to 40 µl of Bradford's reagent and 140 µl of distilled water. Absorbance was measured at 595 nm.

2.4.4 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis was used to determine the molecular weight of the tyrosinases. A 5% stacking gel and 12.5% separating gel was used for the optimal separation of the proteins of interest. The gels were poured according to the manufacturer's instructions for the BIO-RAD PROTEAN mini-gel system. 20 µl of each tyrosinase sample (in duplicate) was mixed with 5 µl of 5x sample loading buffer containing dithiothreitol (DTT) [3.1 ml 1 M Tris-HCl (pH 6.8), 5 ml glycerol, 0.5 ml 1% (w/v) bromophenol blue, 5 mM DTT and 1.4 ml water]. The samples were boiled for 5 min prior to loading and 10 µl of the Spectra multicolour broad range protein ladder (Fermentas) was loaded onto the gel. The gel was run at 100 V in electrophoresis buffer [3.0 g Tris (25 mM), 14.4 g glycine (192 mM) and 1 g SDS in 1 litre water; pH 8.8]. The gel was stained with pre-made Thermo Scientific PageBlue protein staining solution overnight at 22°C on a rocking shaker. Excess stain was removed by washing the gels with distilled water for 10 min.

2.5 Characterisation of Partially Purified Tyrosinases

All characterisation studies were performed using the partially purified tyrosinases, SPRTyr and CZA14Tyr.

2.5.1 Optimum pH and Substrate Range

The substrate range of the two tyrosinases and the optimal pH for the oxidation of these substrates were determined. 50 mM sodium acetate buffers (pH 3-5.5 in 0.5 increments), 50 mM potassium phosphate buffers (pH 6-7.5 in 0.5 increments) and 50 mM Tris-HCl buffers (pH 8-10 in 0.5 increments) were used to determine the optimal pH for substrate oxidation. The following substrates were tested at a final concentration of 1 mM: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,4-dichlorophenol (2,4-DCP), 2,6-dimethoxyphenol (2,6-DMP), 3-hydroxyanthranilic acid (3-HAA), 4-methylcatechol, 4-*tert*-butylcatechol (4-TBC), caffeic acid, catechol, guaiacol, L-DOPA, L-tyrosine, *o*-aminophenol, *p*-cresol, phenol, pyrogallol, resorcinol and syringaldazine (SGZ). Oxidation of the monophenols L-tyrosine and *p*-cresol were also monitored in the presence of 1 µM L-DOPA. Supplementary Table S1 summarises the conditions under which the enzyme activity was determined.

2.5.2 Optimum Temperature

The optimum temperature for the oxidation of L-DOPA was determined using the colourimetric assay as described before. The oxidation of the substrate at different temperatures was monitored at 475 nm using a PerkinElmer UV/Vis spectrophotometer Lambda 25 with a PTP-6+6 Peltier system for temperature control. The assay was performed at 5-60°C in 5 degree increments.

2.5.3 Temperature Stability

The temperature stability of the two enzymes was determined through incubation of the enzymes in a Corning LSE™ digital dry bath at 30°C, 40°C, 50°C and 60°C. Triplicate samples were removed after 10 min, 20 min, 30 min and 60 min, and immediately cooled on ice. The untreated samples stored at 4°C were used as reference samples. All samples were tested for their ability to oxidise L-DOPA at ambient temperature (22 ± 2°C).

2.5.4 pH Stability

Equal amounts of each enzyme and 100 mM buffer solutions were mixed, and incubated at 4°C for 24 h. The buffers used were sodium acetate (pH 3-5.5 in 0.5 increments), potassium phosphate (pH 6-7.5 in 0.5 increments) and Tris-HCl (pH 8-10 in 0.5 increments). Activity was determined using the standard L-DOPA assay.

2.5.5 Effect of Inhibitors, Reducing Agents and Metals

The following compounds were tested to determine their effect on the ability of the two enzymes to oxidize L-DOPA: EDTA, L-ascorbic acid, L-cysteine, NaCl, *p*-arbutin, SDS and sodium metabisulphite (concentrations ranged from 0.01 to 50 mM). In addition, the effect of the following metals was also determined: Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} (concentrations ranged from 0.1 to 5 mM).

2.5.6 Effect of Organic Solvents on Enzyme Activity

The ability of the enzymes to oxidize L-DOPA in the presence of water-miscible organic solvents [2-propanol, acetone, acetonitrile, dimethylsulfoxide (DMSO), ethanol and methanol] was tested. Organic solvent was added to the L-DOPA/buffer mix immediately prior to performing the assay (final solvent concentrations ranged from 10 to 50%, v/v). The oxidation of L-DOPA in the absence of organic solvent was used as the reference control.

To determine the stability of the enzymes in the presence of organic solvents, the enzymes were incubated at 4°C for 20 h in the presence of different concentrations of water-miscible organic solvents (same as above). Activity was determined using the standard L-DOPA assay and tyrosinase samples not incubated in organic solvents used as reference controls.

2.5.7 Kinetics

The kinetic parameters, V_{max}, K_m and k_{cat}, were determined for the oxidation of L-DOPA and *p*-cresol. Varying concentrations of the substrates were used (1-10 mM) while maintaining a

standard concentration of the tyrosinases (1 U/ml). Assays were performed at 22°C using a PerkinElmer UV/Vis, temperature-controlled spectrophotometer Lambda 25.

2.6 Genomic DNA Isolation, Genome Sequencing and Annotation

Genomic DNA was isolated from both bacterial strains using the method described by Mandel and Marmur [18]. DNA concentration was determined using a Qubit® Fluorometer (Invitrogen). Sequencing libraries were prepared using the Roche 454 Titanium Rapid Library sequencing and Lib-L emulsion PCR kits. The integrity of the sequencing libraries was determined by Agilent Bioanalyser DNA 2100 chip. Libraries were sequenced on a Roche 454 GS Junior to a coverage of 20x. Both genomes were annotated using the online server, RAST version 4.0 (Rapid Annotation using Subsystem Technology) [19].

2.6.1 Tyrosinase Sequence Analyses

Tyrosinase sequences (MelC2) identified from the genome sequences were analysed using the program, DNAMAN version 4.13 (Lynnon Biosoft) for the prediction of amino acid content, MW and pI. The sequences were also analysed by protein BLAST [20] to identify related tyrosinase sequences. The MelC1 sequences were submitted to the TatP 1.0 server [21] to determine whether the signal peptides for export via the TAT pathway were present.

2.7 Application of the Tyrosinases: Cross-Linking Reactions

For the cross-linking experiments, the following reagents were prepared: 1.875 mg/ml target protein in sterile dH_2O; 10 mM L-tyrosine in 50 mM potassium phosphate buffer, pH 6.5; 10 mM L-DOPA in 50 mM potassium phosphate buffer, pH 6.5; and a mixture of 10 mM L-tyrosine and 10 mM L-DOPA (L-tyrosine/L-DOPA) in 50 mM potassium phosphate buffer, pH 6.5. The target proteins tested were: BSA, casein, gelatine, horse heart cytochrome *c* and horse myoglobin.

The following reactions were prepared for each target protein with each tyrosinase: 1) target protein only; 2) target protein + enzyme; 3) target protein + enzyme + L-tyrosine; 4) target protein

+ enzyme + L-DOPA; and 5) target protein + enzyme + L-tyrosine/L-DOPA. Final reaction volumes were 500 µl (made up with buffer) and the final concentrations in the reaction vessels were: 0.75 mg/ml target protein, 1 mM L-tyrosine, 1 mM L-DOPA and 1 U/ml enzyme.

The reactions were incubated at room temperature (22 ± 2°C) for 24 h on a Labnet LabRoller II, at 20 rpm. 20 µl of each reaction was mixed with 5 µl 5x sample loading buffer containing 1 mM DTT. The samples (except for the horse heart cytochrome *c* and horse myoglobin samples) were boiled for 10 min and centrifuged prior to loading onto an SDS-polyacrylamide gel (5% stacking gel, 12.5% separating gel). A pre-stained protein ladder was included for comparative purposes. The gels were stained with the pre-made Thermo Scientific PageBlue protein staining solution overnight and washed with distilled water to visualise the bands. The gels with the horse heart cytochrome *c* and horse myoglobin cross-linking experiment samples were not stained with the PageBlue Staining solution, but were stained with an *o*-dianisidine solution. Directly after electrophoresis, the gels were rinsed for 10 min with distilled water followed by a 10 min wash with a 10% (v/v) trichloroacetic acid solution. The gels were rinsed with distilled water for two 10 min washes and finally stained with an *o*-dianisidine solution [50 mM sodium citrate buffer, pH 4.4, 0.7% (v/v) hydrogen peroxide and 1 mg/ml *o*-dianisidine] [2].

3. Results

3.1 Screening for Tyrosinase Activity: Solid Media

Melanin production was clearly visible for the two test strains and the control strain. For all three strains, the most extensive melanin production occurred on DSMZ medium #553 and the MPPM agar media (Fig. 1). Both strains were also positive for melanin production on ISP6 and ISP7 agar plates (greater production on ISP6 agar).

3.2 Partial Purification of the Tyrosinases

A yield of 11.5% (purification fold of 11.84) and 24.6% (purification fold of 5.16) was obtained for the SPRTyr and CZA14Tyr, respectively (Table 1). The production of SPRTyr was variable, with a

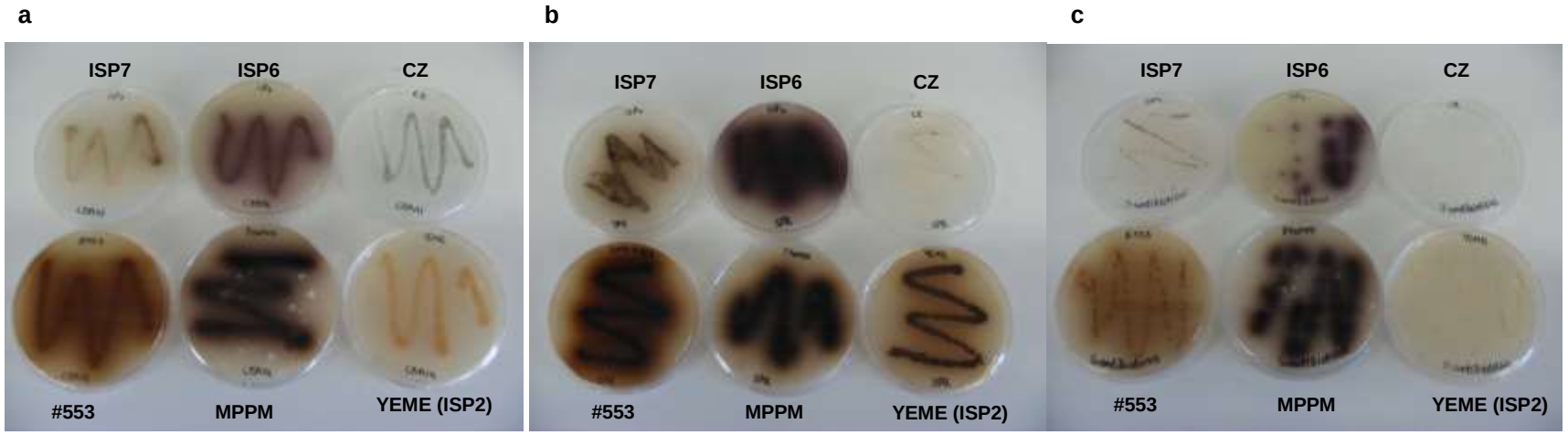

Fig. 1: Agar plate screening for melanin production on various agar media (Top row: ISP7, ISP6, CZ; Bottom row: DSMZ medium #553, MPPM, YEME). **a**: *S. pharetrae* CZA14^{T}; **b**: *S. polyantibioticus* SPR^{T}; and **c**: *S. antibioticus* (positive control).

Table 1: Purification of SPRTyr and CZA14Tyr: ammonium sulphate precipitation and anion exchange chromatography. Enzyme activity was determined using the standard L-DOPA assay and protein concentration was determined using the Bradford's assay.

Sample	**Total activity (U)**	**Total protein (mg)**	**Specific activity (U/mg)**	**Purification fold**	**Yield (%)**
S. polyantibioticus SPR^{I} culture filtrate	1256	624	2.01	1	100
After 40% ammonium sulphate precipitation	801	523.8	1.53	0.76	63.8
After anion exchange	144	4.26	23.80	11.84	11.5
S. pharetrae CZA14^{I} culture filtrate	1246	426.41	2.92	1	100
After 40% ammonium sulphate precipitation	1535	368.57	4.17	1.43	123.2
After anion exchange	306.88	20.36	15.07	5.16	24.6

shift in the optimum growth temperature observed during the course of this study (optimal growth and production was initially at 30°C, but later changed to 22°C). The yield was consistently higher and reproducible for the production of CZA14Tyr. A maximum level of 5.32 mg/L and 16.97 mg/L tyrosinase was purified from the culture filtrates for *S. polyantibioticus* SPR^{T} and *S. pharetrae* CZA14^{T}, respectively.

3.3 SDS-PAGE Analysis

Using the protein ladder, a standard curve was generated to determine the size of the two tyrosinases (data not shown). Both samples had two major bands, one at 43.7 kDa, and a second band at 30.5 kDa and 32.6 kDa for *S. polyantibioticus* SPR^{T} and *S. pharetrae* CZA14^{T}, respectively (Fig. 2).

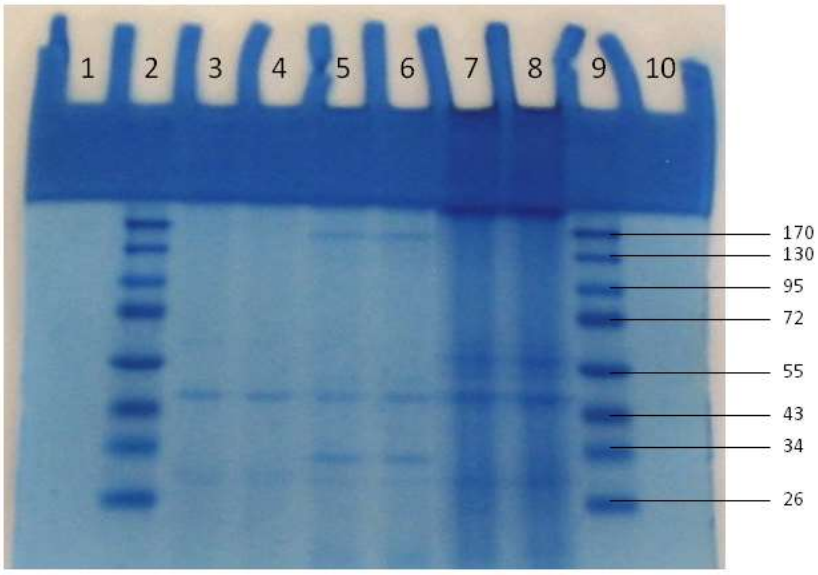

Fig. 2: SDS-PAGE analysis of the tyrosinases used in this study. Lanes 2 and 9: Protein ladder; Lanes 3 and 4: SPRTyr; Lanes 5 and 6: CZA14Tyr (partially purified); and Lanes 7 and 8: Crude extract from *S. antibioticus* (positive control used in this study).

3.4 Characterisation of Partially Purified Tyrosinases

3.4.1 Optimum pH and Substrate Range

Typical tyrosinase substrates were oxidized by both tyrosinases, whereas typical peroxidase substrates (2,4-DCP, phenol; in the presence and absence of H_2O_2) and laccase substrates (2,6-DMP, ABTS, guaiacol, resorcinol, syringaldazine) were not oxidized (Table 2). A slight difference in specific activity and optimal pH for oxidation of the substrates was observed between the two tyrosinases. For the oxidation of the monophenols, L-tyrosine and *p*-cresol, the effect of the addition of 10 µM L-DOPA was determined: for the oxidation of *p*-cresol, the specific activity doubled, whereas for the oxidation of L-tyrosine, little variation in the specific activity was observed. In addition, the monophenolase:diphenolase ratio was determined for both monophenol/diphenol combinations: the L-tyrosine:L-DOPA ratio was 0.059 (0.058 when 10 µM L-DOPA was added to the L-tyrosine) for the SPRTyr and 0.049 (0.051 with 10 µM L-DOPA) for CZA14Tyr; and the *p*-cresol: L-DOPA ratio was 0.072 (0.143 with 10 µM L-DOPA) for SPRTyr and

Table 2: The specific activity determined for SPRTyr and CZA14Tyr using different substrates and the optimal pH for their oxidation; no activity was detected using 2,4-DCP, 2,6-DMP, ABTS, guaiacol, phenol, resorcinol and syringaldazine (mean±SEM; n=3).

Tyrosinase	**Substrate**	**Optimal pH for oxidation**	**Specific activity (U/mg)**
SPRTyr	3-HAA	8.5	0.37 ± 0.04
CZA14Tyr		9.5	0.18 ± 0.02
SPRTyr	4-methylcatechol	5.5	20.00 ± 4.45
CZA14Tyr		5.5	16.80 ± 1.35
SPRTyr	4-TBC (400 nm)	7.5	29.73 ± 2.73
CZA14Tyr		7.0	27.02 ± 0.92
SPRTyr	4-TBC (475 nm)	8.0	0.82 ± 0.05
CZA14Tyr		8.0	0.68 ± 0.06
SPRTyr	Caffeic acid	5.5	6.67 ± 0.90
CZA14Tyr		5.5	6.15 ± 0.73
SPRTyr	Catechol	7.0	19.27 ± 0.25
CZA14Tyr		7.0	24.37 ± 0.39
SPRTyr	L-DOPA	5.5	14.09 ± 0.10
CZA14Tyr		5.5	16.00 ± 0.54
SPRTyr	L-tyrosine	8.0	0.83 ± 0.04
CZA14Tyr		8.0	0.78 ± 0.01
SPRTyr	L-tyrosine + 10 µM L-DOPA	9.0	0.81 ± 0.03
CZA14Tyr		9.0	0.62 ± 0.02
SPRTyr	*o*-aminophenol	9.0	0.42 ± 0.01
CZA14Tyr		9.0	0.25 ± 0.01
SPRTyr	*p*-cresol	8.0	1.01 ± 0.02
CZA14Tyr		8.0	0.77 ± 0.03
SPRTyr	*p*-cresol + 10 µM L-DOPA	8.0	2.02 ± 0.15
CZA14Tyr		8.0	1.57 ± 0.06
SPRTyr	Pyrogallol	7.0	1.30 ± 0.15
CZA14Tyr		7.0	1.35 ± 0.02

0.048 (0.098 with 10 µM L-DOPA) for CZA14Tyr. For the oxidation of the monophenols, a lag-period of more than 10 min was observed for both enzymes before the *o*-hydroxylation reactions occurred.

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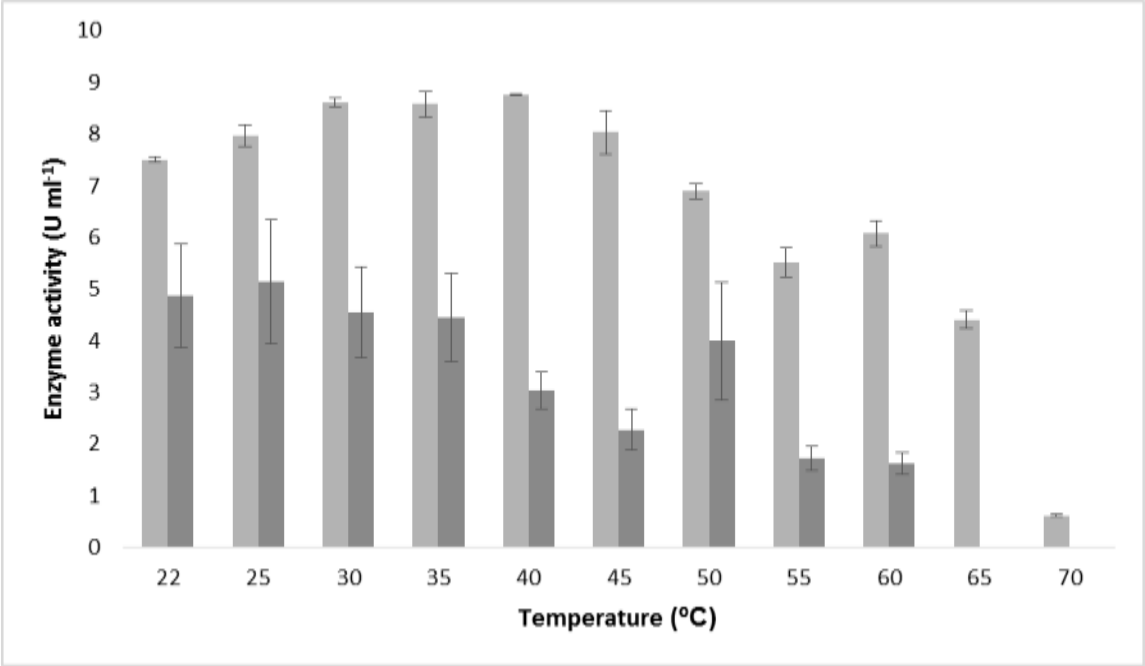


b

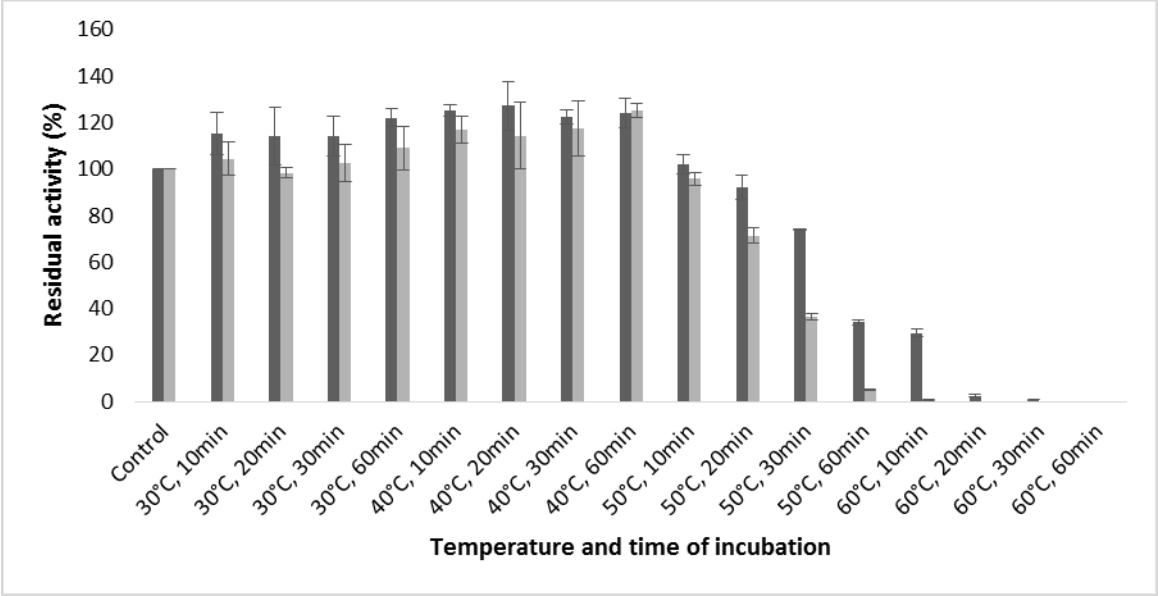


Fig. 3: a The effect of temperature on the oxidation of 10 mM L-DOPA for CZA14Tyr ( ) and SPRTyr ( ). **b** Thermostability of CZA14Tyr ( ) and SPRTyr ( ) when incubated at different temperatures for 10, 20, 30 and 60 min. Activity was measured using the standard L-DOPA assay.

3.4.2 Optimum Temperature and Temperature Stability

For the oxidation of L-DOPA (10 mM), CZA14Tyr was able to oxidise the substrate optimally across a range of temperatures: 30-40°C with a second peak at 60°C (Fig. 3a). In contrast, SPRTyr oxidised L-DOPA optimally around 22-35°C, with a second peak at 50°C. The tyrosinases were stable for 1 h up to 40°C, but a decline in activity was observed for both enzymes when incubated at 50°C (Fig. 3b). CZA14Tyr retained more activity than SPRTyr, with 33.96 ± 1.05% residual activity compared to 4.90 ± 0.26% residual activity for SPRTyr after 1 h of incubation at 50°C. CZA14Tyr showed a residual activity of 29.50 ± 1.72% (0.53 ± 0.14% for SPRTyr) after 10 min incubation at 60°C, but rapidly lost activity after 20 min of incubation at 60°C (2.46 ± 0.40% residual activity).

3.4.3 pH Stability

SPRTyr retained 100% of its activity after 24 h of incubation in the presence of buffers ranging from pH 5-10. Below pH 5.0, the residual enzyme activity declined from 70.75 ± 5.51% at pH 4.5 to 13.90 ± 1.93% at pH 4.0 and 1.54 ± 0.74% at pH 3.5. Total loss of activity occurred at pH 3.0. CZA14Tyr was more sensitive than SPRTyr to lower pH levels, with 100% residual activity detected in the presence of buffers ranging from pH 6.5–10 and a total loss of activity observed for samples incubated in buffers below pH 5.0 (pH 6.0: 96.98 ± 1.67%; pH 5.5: 93.29 ± 3.61%; and pH 5.0: 29.01 ± 5.16%).

3.4.4 Effect of Reducing Agents, Inhibitors and Metals

Both tyrosinases were strongly inhibited by the reducing agents tested in this study (L-ascorbic acid, L-cysteine and sodium metabisulphite; Table 3). Total inhibition of the CZA14Tyr occurred in the presence of 0.5 mM L-cysteine, 0.2 mM sodium metabisulphite and 1 mM L-ascorbic acid, while the SPRTyr was totally inhibited by 1 mM L-cysteine, 0.5 mM sodium metabisulphite and 2 mM L-ascorbic acid. Minimal effect was observed with NaCl (> 80% residual activity for both enzymes at 50 mM), as well as SDS and *p*-arbutin (> 90% residual activity for both enzymes at

Table 3: Effect of inhibitors, reducing agents and metals on the activity of the SPR and CZA14 tyrosinases.

Inhibitor, reducing agent or metal	**Concentration tested (mM)**	**Residual activity (%) - SPRTyr**	**Residual activity (%) - CZA14Tyr**
Arbutin	50	96.47 ± 1.72	96.08 ± 0.59
EDTA	1	78.71 ± 14.36	81.16 ± 0.46
	2	79.89 ± 5.30	80.57 ± 1.97
	5	77.03 ± 13.32	79.14 ± 0.37
	10	82.03 ± 7.91	78.08 ± 1.76
	25	89.38 ± 8.68	69.67 ± 1.02
	50	82.56 ± 7.77	48.50 ± 4.12
L-ascorbic acid	0.2	98.01 ± 3.28	96.98 ± 3.53
	0.5	60.59 ± 10.35	53.10 ± 2.24
	1	19.54 ± 4.19	0
	2	0	0
L-cysteine	0.05	91.66 ± 2.14	95.59 ± 1.30
	0.1	88.33 ± 5.10	88.71 ± 0.55
	0.2	71.96 ± 9.90	55.86 ± 1.57
	0.5	23.71 ± 2.49	0
	1	0	0
NaCl	25	100.42 ± 6.45	85.54 ± 3.83
	50	89.38 ± 7.20	81.18 ± 0.87
SDS	50	97.67 ± 2.60	117.0 ± 3.41
Sodium metabisulphite	0.02	87.42 ± 6.78	95.58 ± 1.60
	0.05	76.08 ± 13.81	95.17 ± 0.90
	0.1	84.43 ± 5.63	64.52 ± 1.02
	0.2	63.14 ± 5.63	0
	0.5	0	0
Ca^{2+}	0.1 - 5	101.45 ± 3.63	99.48 ± 8.00
Co^{2+}	0.1 - 2	111.02 ± 1.71	118.01 ± 3.21
	5	78.37 ± 0.49	100.56 ± 1.69
Cu^{2+}	2	70.95 ± 4.48	52.88 ± 22.33
	5	36.23 ± 2.98	33.27 ± 1.86
Fe^{2+}	0.1 - 2	106.40 ± 5.23	126.36 ± 12.82
	5	100.27 ± 5.39	0
Mg^{2+}	0.1 - 5	113.63 ± 1.69	109.19 ± 5.56
Mn^{2+}	0.1 - 5	106.17 ± 1.70	98.60 ± 3.91
Zn^{2+}	0.1 - 2	104.77 ± 1.92	102.74 ± 3.36
	5	110.13 ± 4.47	29.79 ± 1.55

50 mM). EDTA had a variable effect on the activity of SPRTyr, but inhibited CZA14Tyr, with a residual activity of 48.50 ± 4.12% at 50 mM.

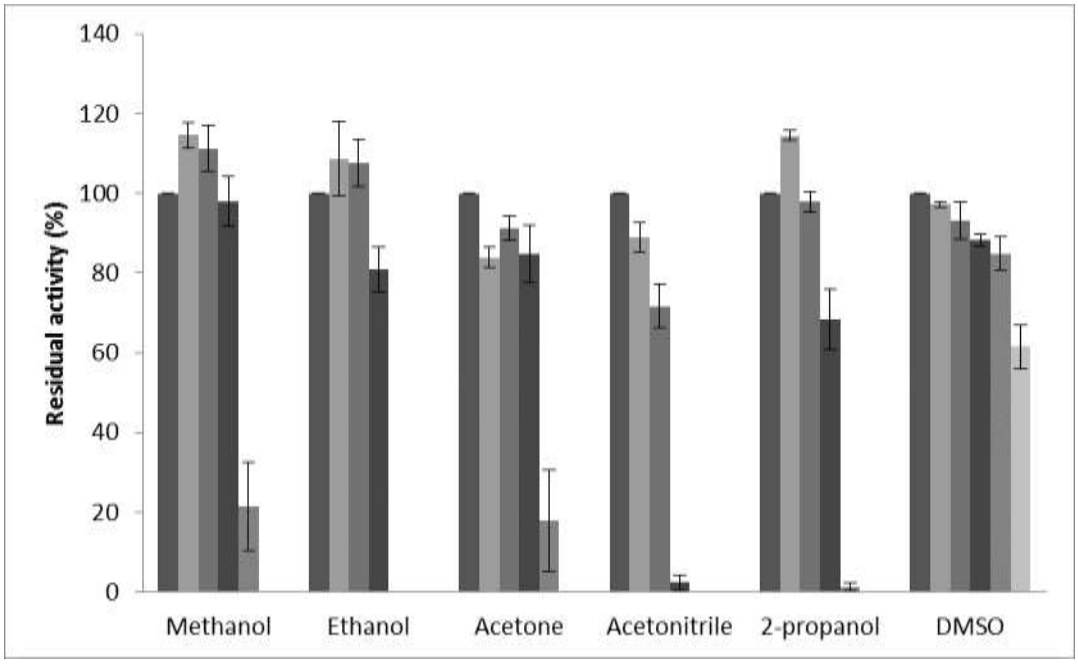
Tyrosinase activity was enhanced by Ca^{2+}, Mg^{2+} and Mn^{2+}. CZA14Tyr was unaffected at 5 mM Co^{2+}, while SPRTyr had a residual activity of 78.37 ± 0.49%. Increasing Cu^{2+} concentrations increased inhibition of both tyrosinases (< 40% residual activity for both enzymes at 5 mM Cu^{2+}; Table 3). SPRTyr was unaffected by 5 mM Fe^{2+}, while CZA14Tyr was completely inhibited. Similarly, SPRTyr was unaffected at 5 mM Zn^{2+}, while only 29.79 ± 1.55% residual activity was observed for CZA14Tyr.

3.4.5 Effect of Organic Solvents on Enzyme Stability and Activity

The stability of the two tyrosinases in the presence of water-miscible organic solvents was determined through incubation of the enzymes with varying concentrations of solvent for 20 h. Both enzymes were strongly inhibited by 30% (v/v) acetonitrile with < 15% residual activity detected (10.73 ± 1.31% for CZA14Tyr and 2.40 ± 1.99% for SPRTyr) (Fig. 4). At lower acetonitrile concentrations (20%, v/v), CZA14Tyr was more strongly inhibited than SPRTyr (47.20 ± 5.35% residual activity for CZA14Tyr and 71.71 ± 5.57% residual activity for SPRTyr). Ethanol, acetone and 2-propanol showed strong inhibitory effects only at concentrations above 40% (v/v). The SPRTyr was strongly inhibited in the presence of 40% (v/v) methanol (21.37 ± 11.12% residual activity), while the CZA14Tyr exhibited 74.66 ± 1.92% residual activity at 40% (v/v) methanol and 17.64 ± 3.79% at 50% (v/v) methanol (SPRTyr completely inhibited at 50%, v/v). Both enzymes were highly stable in the presence of 50% (v/v) DMSO: 61.61 ± 5.46% residual activity for SPRTyr and 60.80 ± 4.40% residual activity for CZA14Tyr. At 10% (v/v) of ethanol, methanol and 2-propanol, and 20% (v/v) ethanol and methanol, an enhancing effect was observed for the SPRTyr.

Both enzymes were able to oxidise L-DOPA in the presence of the organic solvents tested (Fig. 5). More than 50% residual activity was observed for SPRTyr in the presence of 40% (v/v)

a



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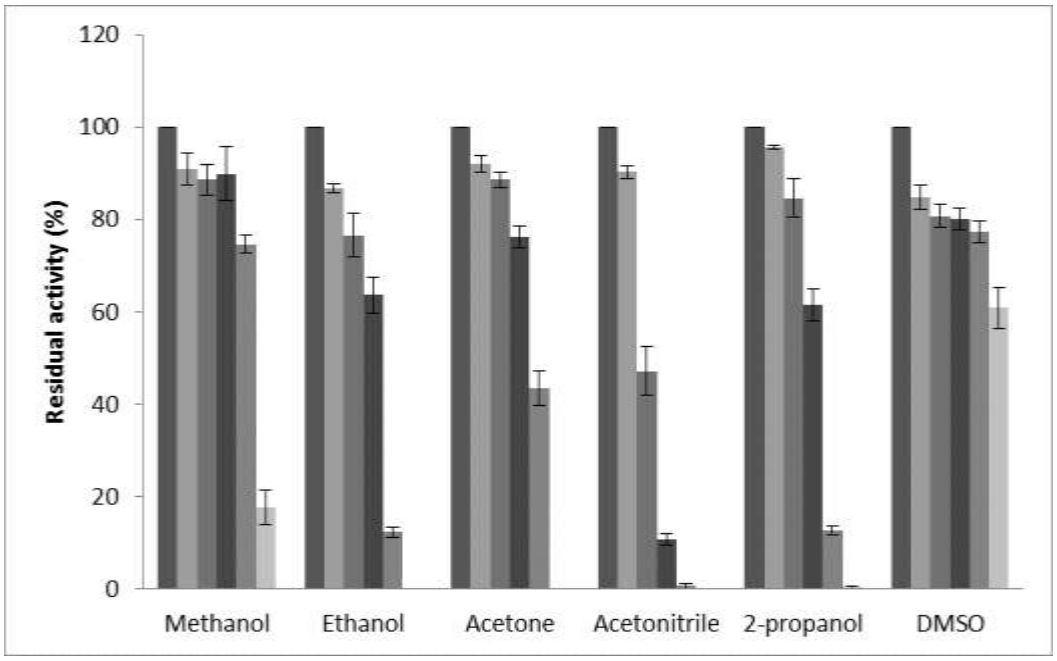
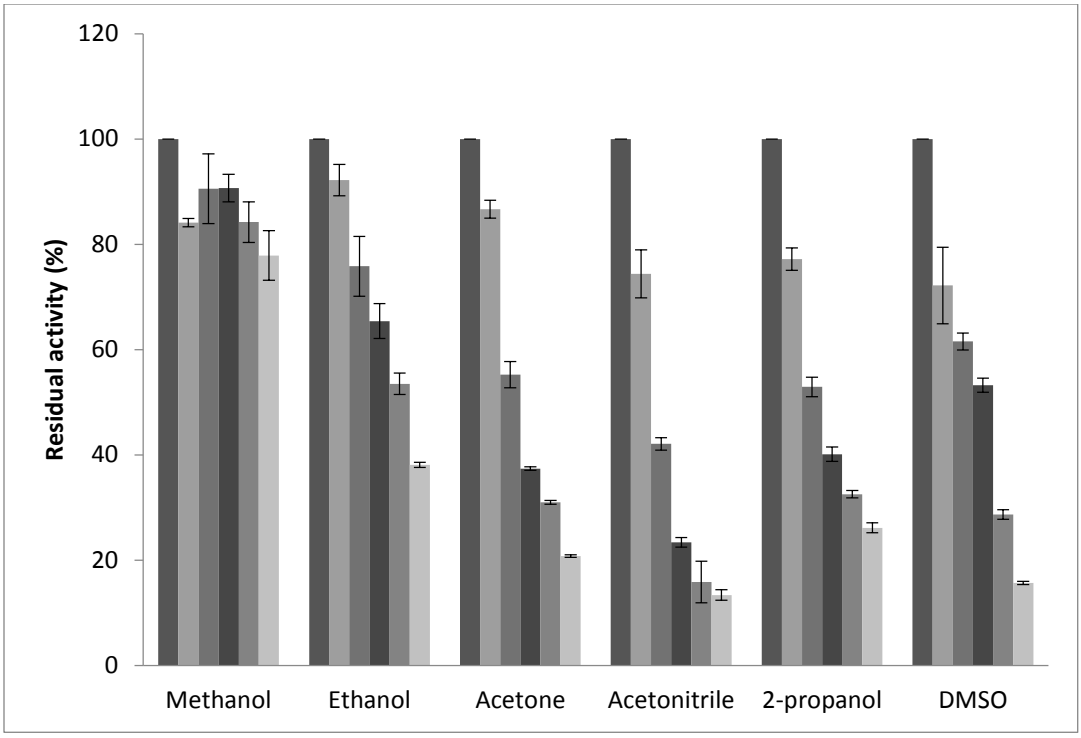


Fig. 4: The stability of the two tyrosinases, SPRTyr (**a**) and CZA14Tyr (**b**) incubated in the presence of 0 10 20 30 40 and 50% (v/v) water-miscible organic solvents (incubated for 20 h at 4°C). Residual activity was calculated compared to a sample incubated without any organic solvent present. Activity was determined using the standard L-DOPA assay.

a



b

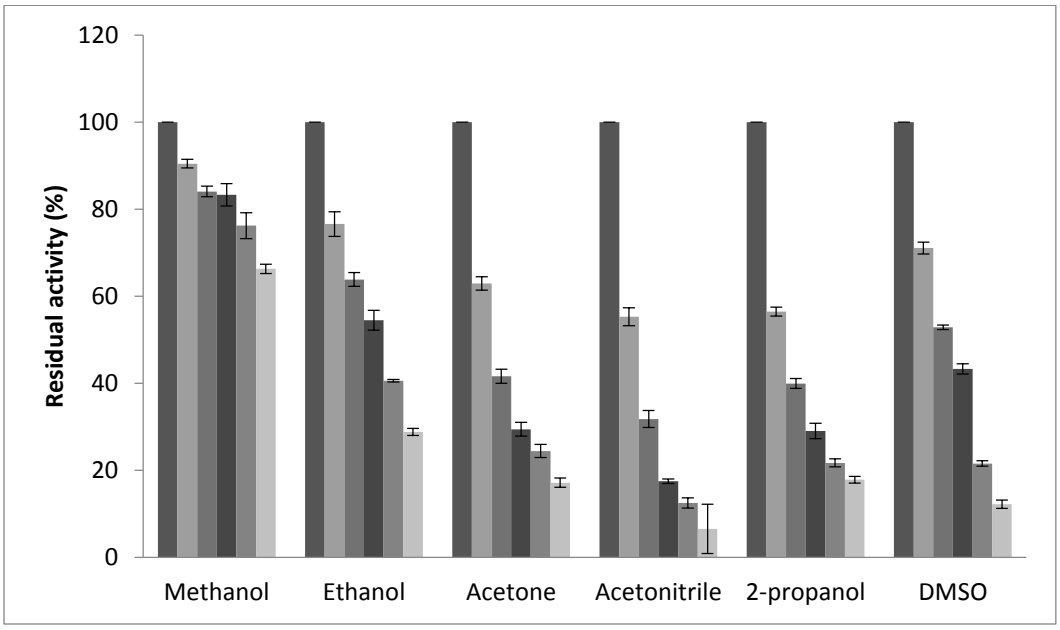


Fig. 5: The effect of organic solvents on the ability of the two tyrosinases, SPRTyr (**a**) and CZA14Tyr (**b**), to oxidise L-DOPA. Oxidation of L-DOPA was performed in the presence of 0 10 20 30 40 and 50% (v/v) water-miscible organic solvents Residual activity was calculated compared to the oxidation of L-DOPA under standard conditions.

ethanol, 20% (v/v) acetone, 30% (v/v) DMSO and 20% (v/v) 2-propanol, and > 70% residual activity in the presence of 10% (v/v) acetonitrile and 50% (v/v) methanol. For CZA14Tyr, > 50% residual activity was observed in the presence of 30% (v/v) ethanol, 10% (v/v) acetone, 10% (v/v) acetonitrile, 20% (v/v) DMSO and 20% (v/v) 2-propanol, while there was > 60% residual activity detected in the presence of 50% (v/v) methanol.

3.5 Kinetics

The kinetic parameters for the oxidation of *p*-cresol and L-DOPA were determined for both enzymes. CZA14Tyr has a higher K_m value for *p*-cresol (K_m = 1.54 ± 0.54 mM; V_{max} = 0.73 ± 0.06 μM/min) and a lower K_m value for L-DOPA (K_m = 1.79 ± 0.86 mM; V_{max} = 163.67 ± 24.01 μM/min) than SPRTyr (*p*-cresol: K_m = 0.66 ± 0.20 mM, V_{max} = 0.71 ± 0.01 μM/min; and L-DOPA: K_m = 3.13 ± 0.28 mM, V_{max} = 92.87 ± 2.73 μM/min). Calculation of k_{cat}/K_m showed that SPRTyr has a greater specificity for *p*-cresol (85.25 s^{-1} mM^{-1}; k_{cat} = 56.27 s^{-1}) than CZA14Tyr (15.01 s^{-1} mM^{-1}; k_{cat} = 23.11 s^{-1}). In contrast, CZA14Tyr has a greater specificity for L-DOPA with a k_{cat}/K_m of 2894.43 s^{-1} mM^{-1} (k_{cat} = 5181.03 s^{-1}) compared to SPRTyr (2351.40 s^{-1} mM^{-1}; k_{cat} = 7359.84 s^{-1}).

3.6 Sequence Analyses

Genome sequence analysis revealed the presence of MelC1-MelC2 tyrosinase operons for both strains. BLAST analysis revealed that these operons are related to the extracellular streptomycete MelC1-MelC2 tyrosinases typically described in literature. The MelC2 sequence from *S. polyantibioticus* SPR^T (GenBank accession number: KR030067) showed 63% sequence similarity to the tyrosinase from *Streptomyces venezuelae* ATCC10712 (YP_006880197), while the MelC2 from *S. pharetrae* CZA14^T (GenBank accession number: KR030068) showed 81% sequence similarity to the tyrosinase from *Streptomyces ascidiscabies* 84-104 (WP_010358344). Multiple sequence alignment of selected tyrosinase sequences allowed for the confirmation of the presence of the histidine residues involved in the co-ordination of CuA and CuB (Supplementary Fig. S1). Analysis of the MelC2 sequence from *S. polyantibioticus* SPR^T revealed the following: CuA signature sequence of H_{39}-X(15)-H_{55}-X(8)-H_{64} and a CuB signature sequence of H_{203}-X(3)-

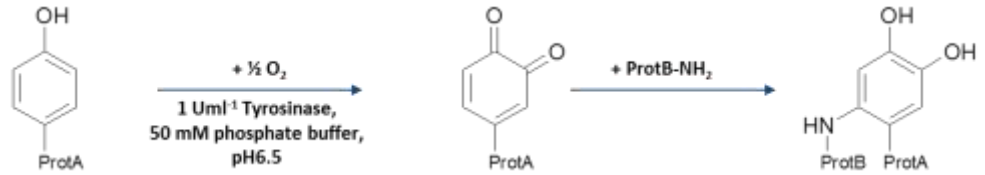
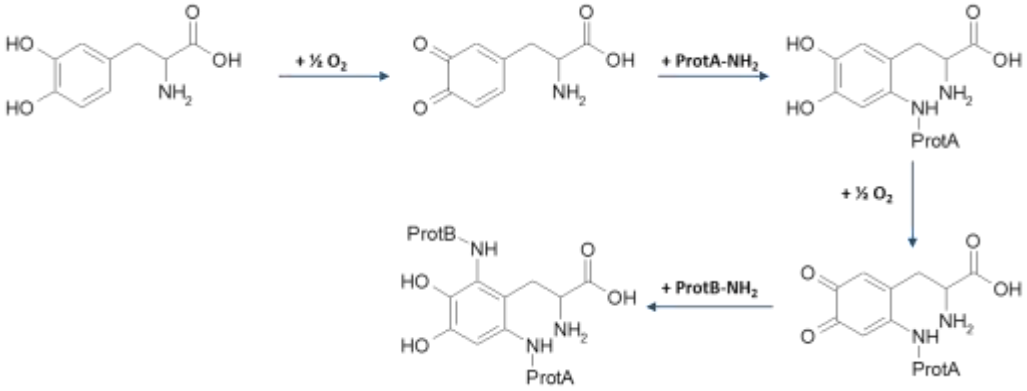
H_{207}-X(35)-H_{243}. The CuA signature sequence for the MelC2 sequence from *S. pharetrae* CZA14^T is the same as for *S. polyantibioticus* SPR^T, with the exception that the CuB signature sequence was found to be H_{200}-X(3)-H_{204}-X(35)-H_{240}. In addition, an analysis of the MelC1 sequences (GenBank accession numbers: KR030065 and KR030066, for strains SPR^T and CZA14^T, respectively) from both strains using the online server, TatP 1.0, revealed the presence of the signal peptide sequence for the export of proteins via the TAT pathway (Supplementary Fig. S2).

Analysis of the MelC2 sequences, allowed for the prediction of the MW, length and pI of the tyrosinases. The extracellular MelC2 of *S. polyantibioticus* SPR^T and *S. pharetrae* CZA14^T had similar molecular weights and characteristics: 292 aa, 33.76 kDa, pI of 9.92 for the MelC2 from *S. polyantibioticus* SPR^T; and 288 aa, 33.618 kDa, pI of 10.03 for the MelC2 from *S. pharetrae* CZA14^T. The predicted MW and the observed MW from SDS-PAGE analysis (30.5 kDa and 32.6 kDa for *S. polyantibioticus* SPR^T and *S. pharetrae* CZA14^T, respectively) was within a similar range.

3.7 Application of the Tyrosinases: Cross-Linking Reactions

SPRTyr and CZA14Tyr were both able to cross-link the target proteins used in this study to various extents (Table 4). Cross-linking was observed by formation of a distinctive precipitate n the reaction vessels (Supplementary Fig. S3a), which when viewed under a light microscope, was visible as an extensively cross-linked matrix (Supplementary Fig. S3b). This was confirmed by SDS-PAGE analyses where cross-linked proteins were visualised as high molecular weight bands. Both enzymes were able to cross-link gelatine (Supplementary Fig. S4a) and casein (Supplementary Fig. S4b) in the presence and absence of a phenolic compound. BSA was only partially cross-linked and required a 48 h incubation period; the cross-linking of this protein was only clearly visible in the sample containing both L-tyrosine and L-DOPA (Supplementary Fig. S4c). Partial cross-linking of horse heart cytochrome *c* (Supplementary Fig. S5a) and horse myoglobin (Supplementary Fig. S5b) occurred in the absence of a phenolic compound. However,

Table 4: A summary of the cross-linking ability of SPRTyr and CZA14Tyr when selected target proteins were used. The various target proteins, the reaction conditions and the reaction mechanisms are indicated. Phenolic compounds used in this study were L-DOPA and L-Tyrosine.

Target proteins	**Optimal reaction conditions for cross-linking**	**Reaction mechanism**
Casein Gelatine See Supplementary Fig S4a and b	Total cross-linking within 24 h. Cross-linking takes place in the absence of phenolic compounds.	
Bovine serum albumin See Supplementary Fig S4c	Partial cross-linking only after a 48 h incubation. Cross-linking only takes place in the presence of both phenolic compounds.	
Horse heart cytochrome *c* Horse myoglobin See Supplementary Fig S5a and b	Partial cross-linking after 24 h in the absence and presence of phenolic compounds. Optimal cross-linking in the presence of both phenolic compounds	

cross-linking of these two proteins was more prominent in the presence of L-DOPA and L-tyrosine. CZA14Tyr was more effective at cross-linking horse heart cytochrome *c* than SPRTyr.

4. Discussion

The true biological role of tyrosinases in streptomycetes is still unclear, but it is proposed that they are involved in melanin production, and have putative roles in lignin degradation and self-defence against toxic phenolics produced and released by plants [8]. Melanin production in solid media occurs readily in the presence of an inducing compound such as trace elements and amino acids (e.g. ISP6 and ISP7 agar media used in actinomycete systematics as an indicator of melanin production) [14]. A recent study by Guo et al. [22] defined a medium for optimal melanin production by *Streptomyces kathirae.* The optimal medium contained a high concentration of yeast extract (37 g L^{-1}), salts (NaCl and $CaCl_2$), amylodextrin as a carbon source, and $CuSO_4$. It is therefore not surprising, that melanin production and tyrosinase induction has been demonstrated in various studies. Held and Kutzner [23] showed that tyrosinase production in *S. glaucescens* can be induced by various amino acids: L-methionine, L-leucine, L-phenylalanine, but not L-tyrosine. Similarly, Ikeda et al. [24] showed that L-methionine induces production of tyrosinase in *S. antibioticus*, *S. glaucescens* and *S. castaneoglobisporus*, but not in *Streptomyces michiganensis*, where Cu^{2+} was found to induce tyrosinase production [23]. The addition of Cu^{2+} and glucose to the *S. antibioticus* culture medium has also been found to enhance the production of tyrosinase [25]. In this study, *S. polyantibioticus* SPR^T and *S. pharetrae* $CZA14^T$ were cultured on various solid media to determine their ability to produce melanin (Fig. 1), a possible indicator for tyrosinase production [1]. These media were also used as liquid media screens for tyrosinase production. Optimal production of tyrosinase was found to occur in the complex medium, MPPM, a medium typically used in the production of bioactive secondary metabolites [15]. In addition, similar to other streptomycete strains [23], the addition of $CuSO_4.5H_2O$ to the medium resulted in the induction of tyrosinase production in both strains, while the addition of L-methionine had a limited effect (data not shown).

The production of CZA14Tyr was quite consistent, while difficulties were experienced with the production of the tyrosinase from *S. polyantibioticus* SPR^T. After an adjustment of the production temperature from 30°C to 22°C, the production of SPRTyr was more consistent and reproducible. As indicated in the review by Faccio et al. [26], bacterial tyrosinases are typically produced at very low levels, especially since most have been produced from the native strains. The production levels observed for SPRTyr (5.32 mg L^{-1}) and CZA14Tyr (16.97 mg L^{-1}) were higher than that previously observed for *Streptomyces albus* (0.2 mg L^{-1}) [27]. Higher production levels for streptomycete tyrosinases have been reported, but were only achieved when the operon encoding for the tyrosinase was overexpressed either in the native strain (e.g. *S. antibioticus* at 20 mg L^{-1} and *S. kathirae*) [28,29] or in an expression host (e.g. the tyrosinase from *Streptomyces* sp. REN-21 in *E. coli* at 54 mg L^{-1}) [3]. SDS-PAGE analysis (Fig. 2) showed that the enzymes fall within the expected size range for streptomycete tyrosinases (29-35 kDa) [1] with SPRTyr at 30.5 kDa and CZA14Tyr at 32.6 kDa (MelC2 only). The MelC1-MelC2 complex is clearly visible for both enzymes (Fig. 2; 50-52 kDa).

Over the past decade, there has been a global move towards the implementation of a bio-based economy. This has led to an increased effort in the identification of biocatalysts that can be applied in bio-processes. In this study, the biochemical characterisation of the two partially purified tyrosinases revealed that both enzymes exhibit properties ideal for industrial application. Both enzymes are stable under alkaline conditions and are able to oxidize a range of substrates (Table 2), including 3-hydroxyanthranilic acid and *o*-aminophenol, which can potentially be used as substrates for the production of phenoxazinone-type antibiotics. Even though other proteins were visible in the SDS-polyacrylamide gel (Fig. 2), these proteins represented a small percentage of the total protein content, while in commercial tyrosinases, additional proteins may represent up to 97%, depending on the source of the commercial enzyme [30]. Laccases and peroxidases are often considered to be the most likely enzymes to interfere with tyrosinase activity. No laccase activity or peroxidase activity was detected for the partially purified tyrosinases produced in this study. As expected, very low monophenolase:diphenolase ratios

were obtained and both tyrosinases exhibited a much higher affinity for diphenols as compared to monophenols, a phenomenon reported for most bacterial tyrosinases [31]. Optimal oxidation of L-DOPA occurred at different temperatures for the two enzymes (Fig. 3a), with optimal oxidation of L-DOPA occurring under ambient conditions for SPRTyr (22–25°C). A bioprocess, such as the production of synthetic melanins, based on the application of SPRTyr would therefore not require any additional energy requirements. Furthermore, incubation of the enzymes at 40°C had an enhancing effect on both enzymes: more than 100% activity was detected after 1 h of incubation (Fig. 3b), making the enzymes suitable for application in processes requiring higher temperatures of up to 40°C.

Many phenolic substrates used in biocatalysis reactions are insoluble in water and require enzymes that are resistant to the organic solvent used for solubilisation. The OSRT produced by *Streptomyces* sp. REN-21 [3,32] for example, retained > 80% activity in up to 30% (v/v) ethanol, 20% (v/v) acetone, and 20% (v/v) DMSO after incubation for 20 h. In this study, SPRTyr was found to be more stable than OSRT and CZA14Tyr in the presence of organic solvents (Fig. 4). Furthermore, both SPRTyr and CZA14Tyr retained more activity in the presence of 50% methanol than the known organic solvent resistant OSRT [3]. As with OSRT, rapid loss of activity occurred in the presence of higher concentrations of acetone, acetonitrile, 2-propanol and DMSO.

SPRTyr had a k_{cat}/K_m value of 85.25 s^{-1} mM^{-1} for the oxidation of *p*-cresol, which is higher than that reported for *S. glaucescens* (48 s^{-1} mM^{-1}) (calculated from values reported in [6]) and that determined for CZA14Tyr (15.01 s^{-1} mM^{-1}). In addition, both SPRTyr and CZA14Tyr exhibited much higher k_{cat}/K_m values for the oxidation of L-DOPA than previously reported at 2351.40 s^{-1} mM^{-1} and 2894.43 s^{-1} mM^{-1}, respectively. These values are between 3 to a 100 fold greater than those reported in literature: 23.5 s^{-1} mM^{-1} for the *Streptomyces* sp. KY-453 tyrosinase [33]; 250 s^{-1} mM^{-1} for the *S. glaucescens* tyrosinase (calculated from values reported in [6]); 271 s^{-1} mM^{-1} for the *Streptomyces* sp. REN-21 OSRT [32]; 742.9 s^{-1} mM^{-1} for the *Streptomyces albus* tyrosinase [27]; and 880 s^{-1} mM^{-1} for the *S. castaneoglobisporus* tyrosinase [34]. The high affinity for L-

DOPA, make SPRTyr and CZA14Tyr excellent candidates for application in the production of synthetic melanins and for application as biosensors for the detection of L-DOPA [10]. The fact that both enzymes retained > 80% residual activity in the presence of 25 mM *p*-arbutin, EDTA, NaCl and SDS, as well as their ability to retain 100% activity in the presence of 5 mM Ca^{2+}, Mg^{2+} and Mn^{2+}, further supports their potential for application in biosensors. The strong inhibition by the reducing agents, L-ascorbic acid, L-cysteine and sodium metabisulphite, was to be expected, and probably results from the disruption of disulphide bridges in the protein structure. The ability of SPRTyr to retain activity in the presence of 1 mM L-ascorbic acid, however, will allow for the use of this reducing agent in controlled biocatalysis reactions.

Scattered, non-standardised biochemical information and the general lack of sequence information on well-described tyrosinases precluded a direct comparison between SPRTyr, CZA14Tyr and their closest phylogenetic neighbours. The varied biochemical information available is most probably due to the fact that past studies were more focused a specific application (e.g. organic solvent tolerance or melanin production), rather than gauging the full biotechnological potential of the enzymes. The lack of sequence information, coupled to a general lack of access to the producing strains, have also made it difficult to perform comparative studies and to ensure the inclusion of suitable positive controls (e.g. many bacterial-based studies make use of mushroom tyrosinase as a positive control simply because of its commercial availability). Furthermore, the past two decades have seen a dramatic increase in the number of genomes being sequenced. Although these genomes serve as useful resources they do not provide any information on the potential biochemical properties or applications of the enzymes. It is therefore not surprising that the sequences with the highest similarity to SPRTyr and CZA14Tyr were tyrosinase sequences derived from genome sequencing events. The analysis of the MelC2 sequences, which relate to the tyrosinases described in this paper, allowed for the detection of the conserved histidine residues involved in the co-ordination of CuA and CuB (Supplementary Fig. S1), confirming the sequence-based classification of these enzymes as Type I tyrosinases (according to the classification proposed by [10]). Furthermore, the predicted pI for the MelC2

sequences (9.92 and 10.03, for SPRTyr and CZA14Tyr, respectively) were similar to those reported for *S. michiganensis* (pI = 9.0) [35] and *Streptomyces* sp. KY-453 (pI = 9.9) [33]. Future studies should consider sequence information, ensure that the producing organism is readily available (e.g. from international culture collections), and should include biochemical information that would determine the suitability of the enzyme for its application in bio-based processes as defined by the bioeconomy approach (e.g. ability to catalyse non-standard reactions, ability to function in the presence of organic solvents or other media, etc.).

In nature, a wide range of protein cross-linking processes occur [36]. Some of the industries that have explored this phenomenon include the food processing industry, leather and textile fabrication industry, and tissue engineering field [36]. Enzymes are considered to be viable alternatives to chemical cross-linkers such as gluteraldehyde and formaldehyde, which have related toxicity and expenses. Enzymes that have been explored as potential cross-linking agents include transglutaminases, peptidases, tyrosinases, laccases, peroxidases, and lysyl oxidases/amine oxidases [36]. For tyrosinases, tyrosine side chains on proteins act as a substrate and are converted to *o*-quinones which react spontaneously with lysine, tyrosine, histidine and cysteine residues, resulting in covalent protein-protein cross-linking [36]. Unfolded proteins therefore serve as the best substrates for tyrosinases, with limited cross-linking occurring when globular proteins are used. Cross-linking can also be induced through the addition of small phenolic compounds, which act as cross-linking mediators, thereby widening the range of proteins that can be cross-linked by tyrosinases [36]. In this study, limited cross-linking occurred when BSA, horse heart cytochrome *c*, and horse myoglobin were used as substrates (Table 4; Supplementary Fig. S4c and Supplementary Fig. S5) probably due to the lack of free tyrosyl side chains and the globular structure of the substrate proteins. However, enhanced cross-linking was observed when L-DOPA and L-tyrosine were added as phenolic mediators. In contrast, both tyrosinases were able to cross-link casein and gelatine without the addition of a phenolic compound (Table 4; Supplementary Fig. S4a and b). The cross-linking of casein in milk is believed to mitigate its allergenic properties; SPRTyr and CZA14Tyr therefore have potential for

application in the dairy industry. In addition, biomimetic materials such as hydrogels, are often made from collagen or gelatine [36] and the ability of both enzymes to cross-link gelatine in the absence of a phenolic compound shows potential application in the production of biological scaffolds for tissue engineering and will be explored in future studies.

5. Conclusions

The unique properties of the tyrosinases produced by *S. polyantibioticus* SPR^T and *S. pharetrae* CZA14^T, such as their organic solvent stability, activity in the presence of organic solvents, broad pH stability, and activity in the presence of inhibitory compounds, make these enzymes ideal for application in biocatalysis, bioremediation and biosensor technology. In addition, their ability to cross-link certain target proteins allows for potential application in the production of novel biomaterials. These two novel tyrosinases are therefore useful additions to the arsenal of biotechnologically relevant enzymes with potential for application in bio-based processes as defined by the needs of a global bioeconomy.

Acknowledgements

This work was supported by the National Research Foundation (NRF) of South Africa for project funding [Grant No. 73691; New cross-linking enzymes to S.B.]; and the Cape Peninsula University of Technology (CPUT) University Research Funding awarded to M.LR-H. J.R. held an NRF Innovation Postdoctoral Fellowship during the course of this study and Z.P. an NRF Honours Scholarship. Any opinion, findings and conclusions or recommendations expressed in this material are those of the author(s) and therefore the NRF does not accept any liability in regard thereto.

Conflict of Interest

The authors declare that they have no conflict of interest. This research did not involve human or animal studies. Good laboratory practice was followed at all times during the course of this study.

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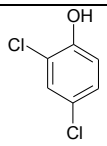
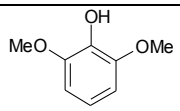
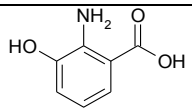
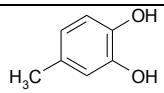
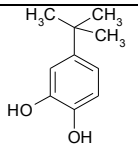
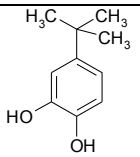
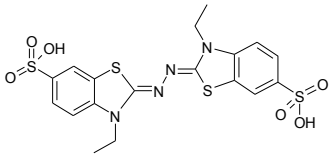
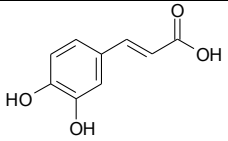
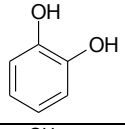
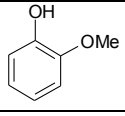
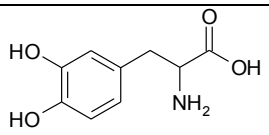
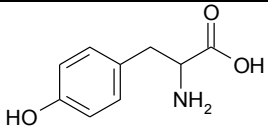
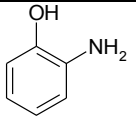
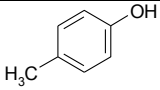
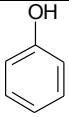
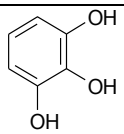
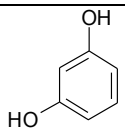
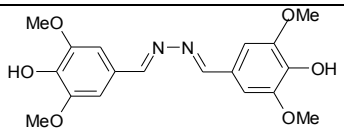
Supplementary Table

Table S1: Substrates used in this study – structure, extinction co-efficient and the wavelength at which oxidation of the substrate was monitored.

Substrate	Structure	Extinction co-efficient (M^{-1} cm^{-1})	Wavelength to monitor oxidation	Reference
2,4-DCP		21 674	510 nm	[1]
2,6-DMP		14 800	468 nm	[2]
3-HAA		18 000	452 nm	[3]
4-methylcatechol		1 433	400 nm	[4]
4-TBC		3 300	475 nm	[5]
4-TBC		1 150	400 nm	[5]
ABTS		36 000	420 nm	[3]
Caffeic acid		1 900	480 nm	[6]
Catechol		740	410 nm	[7]
Guaiacol		26 600	470 nm	[8]

L-DOPA		3 600	475 nm	[9]
L-tyrosine		3 600	475 nm	[10]
o-aminophenol		9 600	433 nm	[11]
p-cresol		1 433	400 nm	[12]
Phenol		7 100	510 nm	[13]
Pyrogallol		4 400	450 nm	[14]
Resorcinol		32 800	500 nm	[14]
Syringaldazine		65 000	530 nm	[15]

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Supplementary Figures

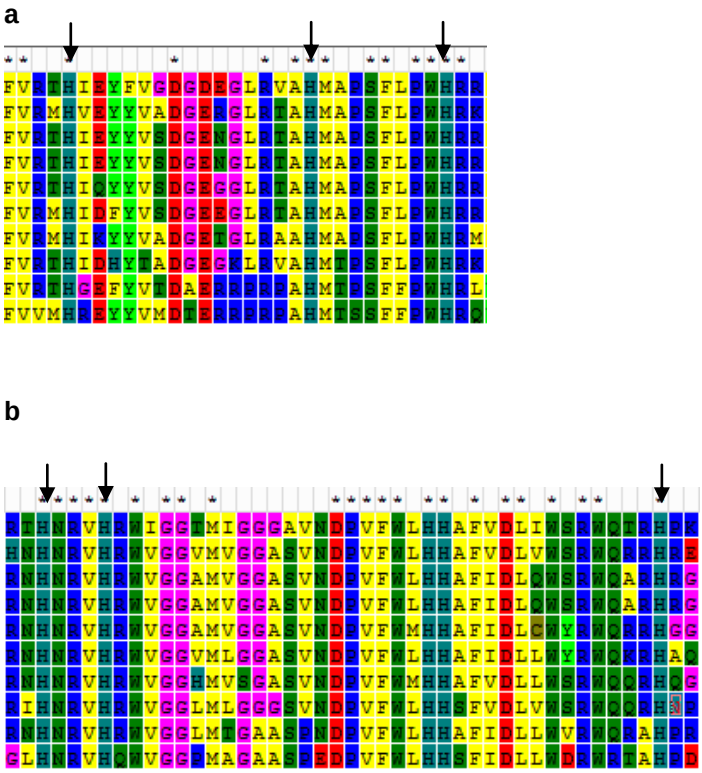
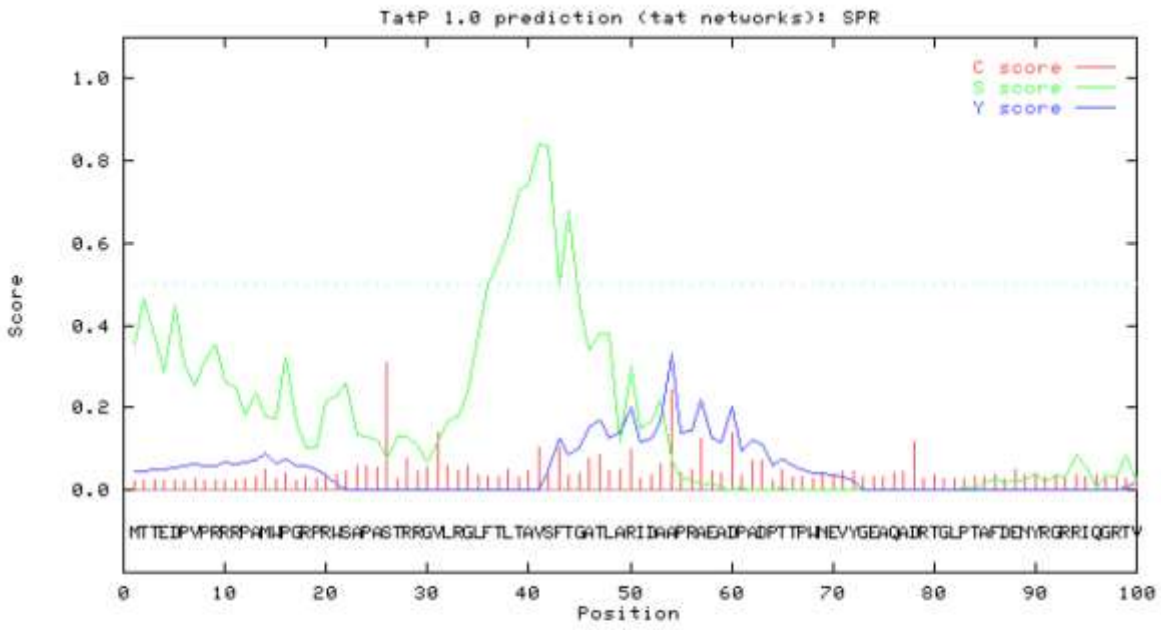


Fig. S1: Multiple sequence alignments for the identification of the Cu$_A$ (a) and Cu$_B$ (b) co-ordination sites. Histidine residues involved in the copper co-ordination are indicated by arrows. MelC2 sequences used in the alignment are as follow: *S. polyantibioticus* (this study; KR030067), *S. pharetrae* (this study; KR030068), *Streptomyces lividans* TK24 (EFD69188.1), *Streptomyces coelicolor* A3(2) (EFD69188.1), *Streptomyces sviceus* ATCC 29083 (EDY61099.2), *Streptomyces acidiscabies* 84-104 (ZP_10456104.1), *Streptomyces avermitilis* MA-4680 (NP_826539.1), *Streptomyces venezuelae* ATCC 10712 (YP_006880197.1), *Streptomyces clavuligerus* ATCC 27064 (EDY52544.1) and *Streptomyces griseus* subsp. *griseus* NBRC 13350 (YP_001822039.1).

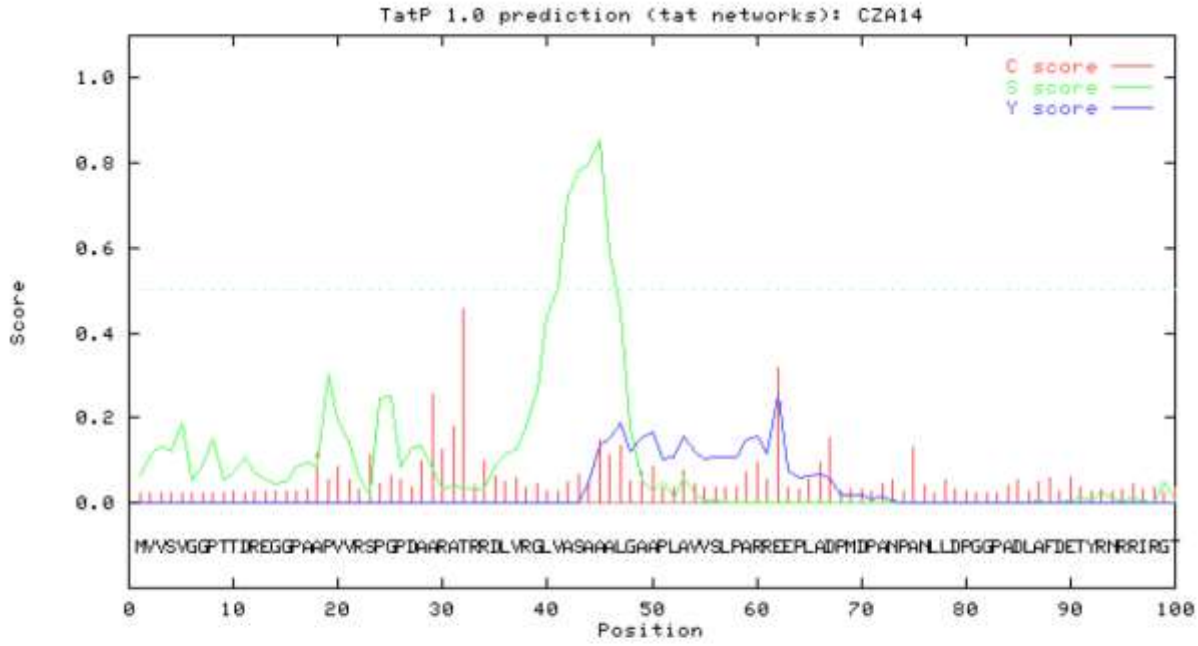
a



data

```
>SPR                    length = 100
# Measure  Position  Value  Cutoff  Tat signal peptide?
  max. C    26       0.309   0.51   NO
  max. Y    54       0.332   0.35   NO
  max. S    41       0.840   0.75   YES
  mean S     1-53    0.308   0.30   YES
  max. D     1-53    0.320   0.36   NO
# Most likely cleavage site between pos. 53 and 54: IDA-AP
```

b



data

```
>CZA14                length = 100
# Measure  Position  Value  Cutoff  Tat signal peptide?
  max. C    32       0.455   0.51   NO
  max. Y    62       0.256   0.35   NO
  max. S    45       0.856   0.75   YES
    mean S     1-61    0.161   0.30   NO
    max. D     1-61    0.209   0.36   NO
  # Most likely cleavage site between pos. 61 and 62: ARR-EE
```

Fig. S2: Analysis results for the presence of the TAT signal peptide in the MelC1 sequences of (a) SPRTyr (KR030065) and (b) CZA14Tyr (KR030066).

a

1 2 3 4 5 6 7 8

b

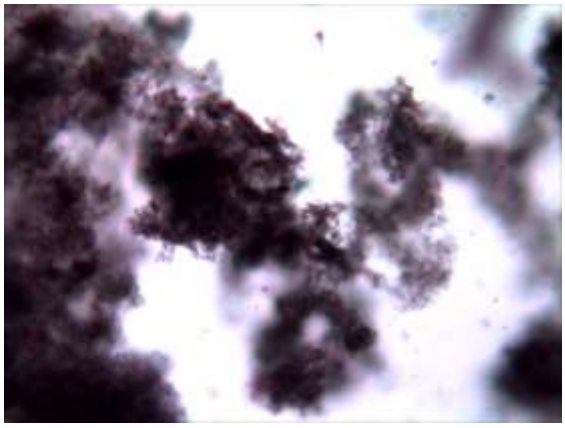
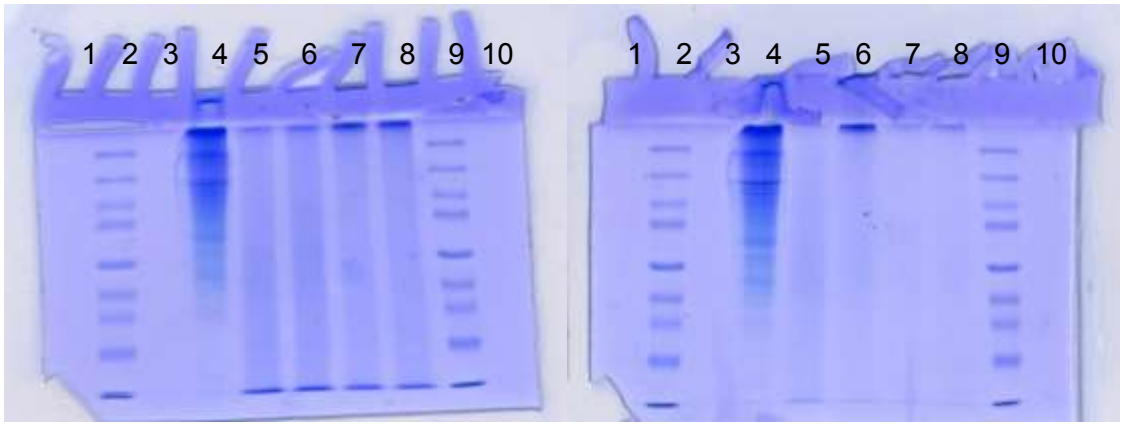


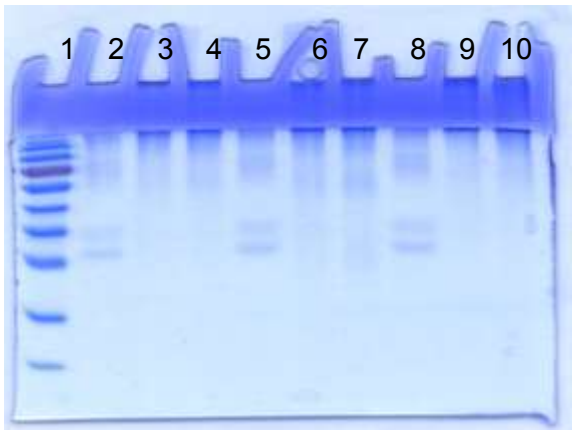
Fig. S3: Cross-linking of various proteins through the use of SPRTyr and CZA14Tyr. (a) Reaction vessels with cross-linking reactions in which gelatine was used as the target protein. Melanin formation and cross-linked material is clearly visible; (1: Gelatine; 2: Gelatine + SPRTyr; 3: Gelatine + CZA14Tyr; 4: Gelatine + L-DOPA/L-Tyrosine; 5: Gelatine + SPRTyr + L-DOPA; 6: Gelatine + SPRTyr + L-DOPA/L-tyrosine; 7: Gelatine + CZA14Tyr + L-DOPA; and 8: Gelatine + CZA14Tyr + L-DOPA/L-tyrosine). (b) Sample taken from the gelatine cross-linking experiment and viewed with an Olympus light microscope (x400 magnification) showing the extensive matrix of cross-linked material.

a

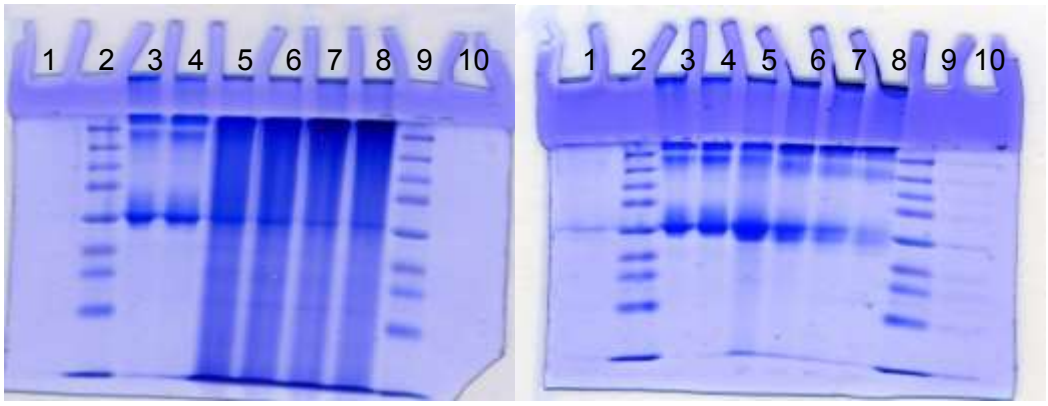


SPRTyr **CZA14Tyr**

b



c

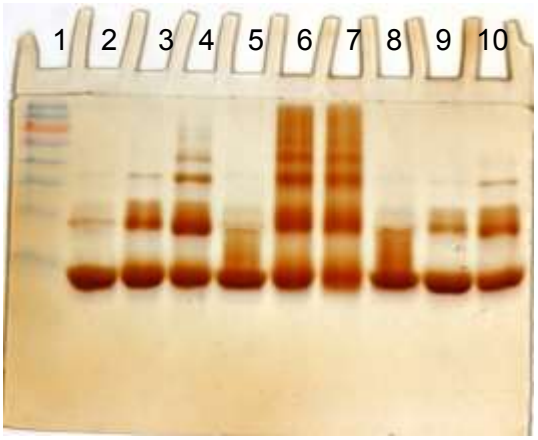


SPRTyr **CZA14Tyr**

Fig. S4: SDS-PAGE gels of the following cross-linking experiments: a Gelatine (for both gels 1, 3 and 10: Empty; 2 and 9: Protein ladder; 4: Gelatine only; 5: Gelatine + tyrosinase;

5: Gelatine + tyrosinase + L-DOPA; 6: Gelatine + tyrosinase + L-tyrosine; 7 and 8: Gelatine + tyrosinase + L-DOPA/L-tyrosine). b Casein (1: Protein ladder; 2: Casein only; 3: Casein + SPRTyr; 4: Casein + SPRTyr + L-DOPA/L-tyrosine; 5: Casein + L-DOPA/L-tyrosine; 6: Casein + CZA14Tyr; 7: Casein + CZA14Tyr + L-DOPA/L-tyrosine; 8: Casein only; 9: Casein + *S. antibioticus* tyrosinase; and 10: Casein + *S. antibioticus* tyrosinase + L-DOPA/L-tyrosine). c BSA (for both gels 1 and 10: Empty; 2 and 9: Protein ladder; 3: BSA only; 4: BSA + tyrosinase; 5: BSA + tyrosinase + L-DOPA; 6: BSA + tyrosinase + L-tyrosine; 7 and 8: BSA + tyrosinase + L-DOPA/L-tyrosine).

a



b

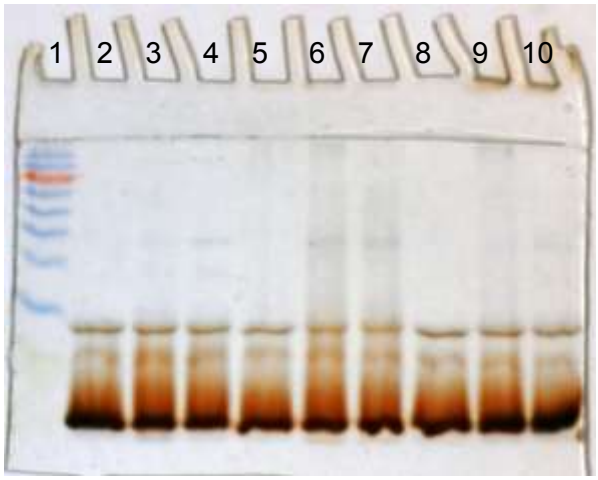
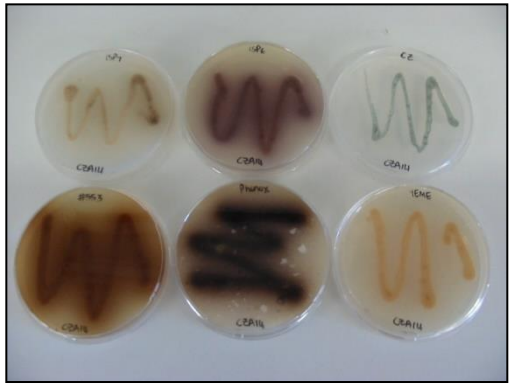
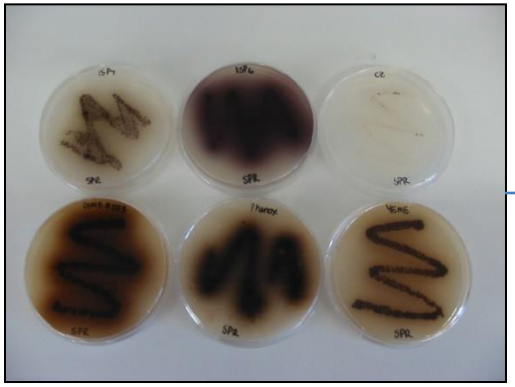


Fig. S5: SDS-PAGE gels of the following cross-linking experiments: a Horse heart cytochrome *c* (1: Protein ladder; 2: Horse heart cytochrome *c* only; 3: Horse heart cytochrome *c* + SPRTyr; 4: Horse heart cytochrome *c* + SPRTyr + L-DOPA; 5: Horse heart cytochrome *c* + L-DOPA/L-tyrosine; 6: Horse heart cytochrome *c* + SPRTyr + L-DOPA/L-tyrosine; 7: Horse heart cytochrome *c* + CZA14Tyr + L-DOPA/L-tyrosine; 8: Horse heart cytochrome *c* only; 9: Horse heart cytochrome *c* + CZA14Tyr; and 10: Horse heart cytochrome *c* + CZA14Tyr + L-DOPA). b Horse myoglobin (1: Protein ladder; 2: Horse myoglobin only; 3: Horse myoglobin + SPRTyr; 4: Horse myoglobin + SPRTyr + L-DOPA; 5: Horse myoglobin + L-DOPA/L-tyrosine; 6: Horse myoglobin + SPRTyr + L-DOPA/L-tyrosine; 7: Horse myoglobin + CZA14Tyr + L-DOPA/L-tyrosine; 8: Horse myoglobin only; 9: Horse myoglobin + CZA14Tyr; and 10: Horse myoglobin + CZA14Tyr + L-DOPA).

Streptomyces pharetrae CZA14^T



Streptomyces polyantibioticus SPR^T



Graphical Abstract

Genome sequencing Identification of genes involved in tyrosinase production

Tyrosinase production

Biochemical characterisation

Cross-linking reactions