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Isolation and characterization of a novel tyrosinase produced by Sahara soil actinobacteria and immobilization on nylon nanofiber membranes

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Highlights

- Isolation of a novel highly stable tyrosinase from extremophile bacteria and biochemical characterization.
- Genome sequencing and assignment of the strain to *Streptomyces cyaneofuscatus* sp.
- Bacterial tyrosinase with high temperature and neutral and alkaline pH activity
- Substrate specificity with higher diphenolase than monophenolase activity

- Immobilization of the tyrosinase on electrospun nylon nanofiber membranes

Abstract

In the present study different actinomycete strains were collected and isolated from algerian Sahara soil with the aim to select novel enzymes with promising features for biotechnological applications. The Ms1 strain was selected, amongst the others, for its capability to produce melanin in different solid media. Ms1 chromosomal DNA was sequenced and the strain assigned to *Streptomyces cyaneofuscatus* sp. A tyrosinase (MW ~ 30 kD) encoding sequence was identified and the corresponding enzyme was isolated and biochemically characterized. The tyrosinase showed the highest activity and stability at neutral and alkaline pH and it was able to oxidize l-DOPA at T = 55° C and pH 7. The enzyme showed variable stability in presence of various water-miscible organic solvents, while it was inactivated by reducing agents. The tyrosinase activity was unaffected by NaCl and enhanced by different cations . Furthermore, the enzyme showed a higher specificity for diphenols than monophenols showing a higher diphenolase than monophenolase activity. Finally, tyrosinase was stabilized by immobilization on nylon nanofiber membranes with a payload of 82% when 1% glutaraldeyde was used. Taken all together, these results show that the enzyme displays interesting properties for biotechnological purposes.

Keywords

Bacterial tyrosinase, *Streptomyces cyaneofuscatus*, biochemical characterization, nanofiber immobilization

1. Introduction

Tyrosinases (monophenol, dihydroxyphenylalanine - l - (DOPA): dioxygen oxidoreductase, E.C.1.14.18.1) belong to the Type 3 copper containing proteins, where the two antiferromagnetically coupled copper ions are coordinated by six histidine residues (Solomon et al. 1992; Huber and Lerch 1988). These enzymes are ubiquitously diffuse in nature and are usually associated with melanin production to protect microorganisms mainly against UV radiation damages (Plonka and Grabacka 2006; Brenner and Hearing 1992; Claus and Decker 2006). Tyrosinases use molecular oxygen to catalyze two different reactions: the orto-hydroxylation of monophenols (monophenolase, cresolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase, cathecolase activity) with the concomitant reduction of oxygen to water. The macromolecular melanins are non-enzymatically produced by the polymerization of the reactive quinones (Claus and Decker 2006). Tyrosinases comprise the activity of Cathechol oxidases (EC. 1.10.3.1) and with the structurally different Laccases (EC.1.10.3.2) are grouped under the family name of "polyphenol oxidases". Laccases are enzymes lacking monophenol hydroxylase activity (Pogni et al. 2015). Streptomycetaceae are Gram-positive soil bacteria with mycelial growth. Some members of this family are involved

in lignin degradation, melanin formation as well as in the production of antibiotics and other secondary metabolites [5]. Different type of tyrosinases are produced by *Streptomyces* spp., with 40% of them producing melanin-like exopigments on tyrosine containing agar media (Claus and Decker 2006; Yang and Chen 2009; Le Roes-Hill et al. 2015). Bacterial tyrosinases may be divided in five types depending on different molecular organization or on the presence of a caddy protein for enzyme activity (Fairhead and Thöny-Meyer 2012). Amongst them, tyrosinase from *Bacillus megaterium* is a secreted small monomeric enzyme with an autonomous catalytic domain, while tyrosinases from *Streptomyces castaneoglobisporus* and *Streptomyces antibioticus* and the membrane bound tyrosinase from *Marinomonas mediterranea* requires an accessory protein for enzyme activity (Sendovski et al. 2011; Kohashi et al. 2004; Betancourt et al. 1992; López-Serrano et al. 2004). Since mono- and diphenols are the typical substrates for tyrosinases, these enzymes can be applied in different biotechnological fields ranging from wastewater treatment and biosensors to food and material functionalization (Fairhead and Thöny-Meyer 2012; Faccio et al. 2012; Richter et al. 2015; Zaidi et al. 2014; Rahman et al. 2016). To improve performance and economic viability enzymes are generally immobilized onto different supports as immobilization can improve the stability and reusability allowing better handling and usage (Cantone et al. 2013; Cowan and Fernandez-Lafuente 2011; Durán et al. 2012; Hwang and Gu 2013; Mateo et al. 2007; Rodrigues et al. 2013; Garcia-Galan et al. 2011; Guzic et al. 2014; Sheldon and van Pelt 2013). To date the most used tyrosinase is the commercial one produced by the mushroom *Agaricus bisporus* (Wichers et al. 2003; Subrizi et al. 2014). However, this tyrosinase is not the best choice for biotechnological applications due to its low heat and solvent stability. Differently bacterial tyrosinases have shown a greater stability and many studies review their applicability in different industrial fields (Claus and Decker 2006; Fairhead and Thöny-Meyer 2012; Faccio et al. 2012). In this study five actinomycete strains, Ms1, M51, O5, H1 and 10 were collected and isolated from Algerian Sahara soil. Ms1 strain, that was isolated from M'Sila city soil, was selected and characterized among others for its capability to produce melanin exopigments in different solid media and was assigned to *Streptomyces cyaneofuscatus* sp. Tyrosinase from this strain was isolated and biochemically characterized (different pHs, temperatures, substrates, solvent stability, inactivating agents). Eventually, the immobilization of the tyrosinase on electrospun nylon nanofiber membranes was optimized to enhance its stability and reusability.

2. Material and methods

All chemicals and mushroom tyrosinase from *Agaricus bisporus* were obtained from Sigma Aldrich (Milano, Italy) and used without further purification. All actinomycete strains were collected and isolated from Algerian Sahara soil. The bacterial 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) (Shirling and Gottlieb 1966), and as stock cultures in 20% (v/v) glycerol at − 80 °C.

2.1 Screening for tyrosinase activity

Two culture media were used for the detection of melanin production: (a) Tyrosine agar (peptone 0.5%, beef extract 0.3%, agar 2%, and l-tyrosine 0.5%, pH 7.0), where the growth was observed after incubation at 30 °C for 2–3 days

(Raval et al. 2012), and (b) Modified Phenoxazinone Production Medium (MPPM), containing (g/L) 10.0 glycerol, 10.0 glucose, 10.0 soya flour, 5.0 casamino acids, 5.0 yeast extract, 4.0 $CaCO_3$, 15.0 bacteriological agar, pH 7.0, and 1 ml of trace salts solution (g/100 ml: 1.0 $FeSO_4$, 0.9 ZnSO4, 0.2 $MnSO_4$) where the growth was observed after incubation at 30 °C for 7 days. The plates were monitored for the production of a dark brown to black pigment as an indication of tyrosinase positive organism.

Liquid media with the same content of the solid ones were used, supplemented with five drops of chloroform and 4 ml of 100 mM filter sterilized $CuSO_4 \cdot 5H_2O$ as inducers for the production of tyrosinase enzyme under agitation at 220 rpm in Erlenmeyer flasks at 30°C for 24-48 hours.

2.2 Scanning Electron Microscopy (SEM) analysis

The microscopical analysis of the Ms1 strain was performed by scanning electron microscopy (SEM) using a Phenom G2 pure 134 desktop apparatus working in the magnification range (20–17,000).

2.3 Tyrosinase production and partial purification

Pre-cultures of Ms1 strain were prepared by adding 100µl of stock culture of Ms1 strain to 10 ml of MPPM, pH 7.0, and incubating for 48 h at 30 °C with shaking at 220 rpm. 20 ml of the pre-culture inoculum were then used to inoculate 400 ml of MPPM (in a 2 L shake flask, final concentration 5%), supplemented with 4 ml of 100 mM filter sterilized $CuSO_4 \cdot 5H_2O$ (final concentration 1 mM). After a 24 h incubation, the culture was centrifuged at 10,000 × *g* for 5 min at 4 °C and the supernatant was used for the purification of the produced extracellular tyrosinase (Le Rose-Hill et al. 2015). The culture supernatant was subjected to ammonium sulfate precipitation by using the procedure reported in: http://www.encorbio.com/protocols/AM-SO4.htm. The culture supernatant with a 65% saturation level, was stirred at 25 °C for 1 h at room temperature and centrifuged at 10,000 × *g* for 10 min at 4 °C. The pellet was suspended in 50 mM potassium phosphate buffer (pH 6.5) and dialyzed at 4 °C for 24 h against ice-cold 50 mM potassium phosphate buffer, pH 6.5. 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 solution was dissolved in 50 mM potassium phosphate buffer, pH 6.5, and applied to a DEAE Sephadex TM A-50 (GE Healthcare) using batch technique to eliminate melanin. The enzyme was finally eluted with 50 mM potassium phosphate buffer, pH 6.5. Aliquots were withdrawn at different purification steps (culture filtrate, after 45% and 65% ammonium sulphate precipitation, dialyzed sample, and partially purified tyrosinase) and kept for further analysis.

The protein content of diverse aliquots was measured by Bradford's method (Bradford 1976).

2.4 Determination of tyrosinase activity and SDS-PAGE analysis

The enzyme activity was assayed using l-DOPA as substrate. A 10 mM solution of l-DOPA in 50 mM potassium phosphate buffer (pH 6.5) was prepared and its oxidation by tyrosinase was monitored at 475 nm (ε = 3600 M^{-1} cm^{-1}) (Le Roes-Hill et al. 2015) using a Shimadzu UV-1601 spectrophotometer reader. One unit of enzyme activity

was defined as the amount of enzyme required to oxidize 1 µmol of substrate per minute at 20 ± 2 °C (Raval et al. 2012; Le Roes-Hill et al. 2015). Carrying out tyrosinase assay, the enzyme activity was determined by:

U/ml = ΔA/min x 1/ε x (total volume of solution/enzyme volume) x dilution factor

where:
ΔA/min is the change in abs/min, ε = extinction coefficient of the substrate in $mM^{-1}cm^{-1}$ and the dilution factor = 100 (10 µl of 1 ml used in assay).

To determine the specific activity (U/mg), the enzyme activity (U/ml) was divided by the total protein concentration (mg/ml) as determined by Bradford's assay.

To check the homogeneity of the protein, and to determine its molecular weight, the different aliquots were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli method (Laemmli 1970). A 5% stacking gel and a 12.5% separating gel were used. The gel was poured using the Invitrogen Mini-Gel system. 15 µl of each tyrosinase sample was mixed with 5 µl of loading buffer, the samples were boiled for 5 min and then loaded on the gel; 5 µl of the Spectra multicolor broad range protein ladder (Fermentas) was loaded on the same gel and used as molecular weight standard. The gel was run at 100 V in electrophoresis buffer and was stained with pre-made Thermo Scientific Page Blue protein staining solution inside the microwave for 2 min on a rocking shaker. Excess stain was removed by washing the gel with distilled water for 10 min.

2.5 Effect of pH and temperature on enzyme activity

To determine the optimal pH value for l-DOPA oxidation by tyrosinase, 50 mM sodium acetate buffers in the pH range 3 – 5.5 with increments of 0.5, 50 mM potassium phosphate buffers in the pH interval 6 – 7.5 with 0.5 increments and 50 mM Tris–HCl buffers in the pH range 8 – 10 with 0.5 increments were used. The pH stability was obtained mixing equal volumes of enzyme and 100 mM buffer solutions and incubating the samples at 4 °C for 24 h. The residual enzymatic activity after 24 h was determined through the standard l-DOPA assay.
l-DOPA oxidation by tyrosinase was determined spectrophotometrically at different temperatures. The absorbance at 475 nm was monitored in the interval of 5 - 70 °C with 5 °C increments. The tyrosinase was incubated with l-DOPA in a temperature-controlled system setting the values at 30, 40, 50, 60 and 70 °C. The experiments were performed in triplicate; the samples were taken off the spectrometer after 15, 30, and 90 minutes and immediately refrigerated in ice. The reference sample was an aliquot of the enzyme stored at 4 °C. The residual activity was determined by measuring the ability of different samples to oxidize l-DOPA at 20 ± 2 °C. All measurements were performed using a Shimadzu UV-1601 spectrophotometer reader.

2.6 Substrate specificity

The activity of the enzyme was determined on various substrates including: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), caffeic acid, guaiacol, pyrogallol, resorcinol, p-cresol and L-tyrosinase and compared with l-DOPA. All substrates (Sigma) were analysed at a final concentration of 1mM, starting from stock solutions 10mM in phosphate buffer 50 mM pH=6.5. The enzyme : substrate molar ratio was 1:100. The UV-Vis measurements were performed at room temperature using a PerkinElmer Lambda 900 spectrophotometer. The spectra were recorded after the addition of the substrate (t = 0) and at different reaction times.

2.7 Enzyme activity in presence of inactivating and reducing agents, cations and organic solvents

The tyrosinase activity for the oxidation of l-DOPA was analysed in presence of different compounds such as: EDTA, l-ascorbic acid, l-cysteine, NaCl, Sodium Dodecyl Sulphate (SDS) and sodium metabisulphite. The concentrations of the agents were in the range: 0.01 - 10 mM. The activity in the presence of cations such as: Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} with a 1mM concentration was also tested. The various water-miscible organic solvents used to test the l-DOPA oxidation were acetone, acetonitrile, dimethylsulfoxide, ethanol and methanol. They were added to the substrate/enzyme solution immediately before performing the assay. The final concentrations of the solvent mixtures were from 10 to 50 %, v/v. The oxidation of l-DOPA in the absence of organic solvent was the reference control. The enzyme stability was achieved incubating the tyrosinase at 4 °C for 20 h in the presence of various water-miscible organic solvents (same ratios reported above). K_m and V_{max} were obtained monitoring the oxidation of l-DOPA in phosphate buffer at pH 7. The substrate concentrations for the assay were 10 – 500 mM and a fixed concentration of tyrosinase (1 U/ml) was used. The experiments were carried out at 20 ± 2 °C using a Shimadzu UV-1601 temperature-controlled spectrophotometer.

2.8 Genomic DNA isolation, genome sequencing and annotation

Chromosomal DNA was purified from Ms1 strain according to the following protocol: briefly, Ms1 strain was cultivated in Jasenka medium (10% w/v Saccarose, 3% w/v Tryptone soya broth, 3% w/v Yeast extract, 1% $MgCl_2$) at 30 °C for 40 h, and mycelium was harvested from 1 ml of culture by centrifuging for 10 minutes at 10000 *x g*. Mycelium was dissolved in 500 µl of SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris HCl pH 7.5, 50 mg/ml lysozyme) and incubated at 37 °C for 1 h. SDS at the final concentration of 1%, 30 µg of Proteinase K and 25 µg of RNAse were added and the lysate was incubated at 55 °C for 2 h with gentle shaking. NaCl concentration was adjusted to 1.4 M and DNA was extracted and deprotinated by adding 1 volume of phenol-chloroform 1:1 pH 8.0 and by centrifuging for 10 min at 10000 *x g*. Precipitated DNA was obtained by adding 2 volumes of ice cold ethanol and was then air dryed and dissolved in distilled water. DNA concentration was determined using a Qubit® Fluorometer (Invitrogen), according to the manufacturer instructions. Whole genome sequencing was performed by Novogene Bioinformatics Technology Co. L.t.d. (Hong Kong) using the HiSeq platform (Illumina, Inc., San Diego, CA) with a 2 x 250-bp paired-end approach; reads were assembled using SPAdes version 3.10.0 (Bankevich et al. 2012), with a genome raw coverage of ca. 175x. Ms1 genome was annotated using the on line server RAST version 2.0 (Rapid Annotation using Subsystem Technology, http://rast.nmpdr.org/) (Aziz et al. 2008). Sequence comparison was carried out using the BLAST online tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Identification to

the species level of the Ms1 strain was performed with the JSpecies software version 1.2.1 (Richter and Rosselló-Móra 2009) using the default settings for Average Nucleotide Identity calculation with the blast algorithm (ANIb) and with the genome-to-genome distance comparison (GGDC) method using the GGDC 2.1 online tool (http://ggdc.dsmz.de) (Meier-Kolthoff et al. 2013); results from the GGDC 2.1 online tool were interpreted according to the Formula 2, as recommended for draft genomes.

Putative tyrosinase encoding sequences were identified in the Ms1 strain genome with the BLAST online tool, using the MelC-, MelD- and GriEF-encoding sequences from *S. griseus* subsp. *Griseus* NBRC 13350 strain genome (GenBank accession no. AP009493.1). The enzymes theoretical MW was calculated using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/) available on the Expasy platform. Amino acid identity was analyzed using the Clustal Omega online tool (Sievers et al. 2011) available at the EMBL-EBI platform (http://www.ebi.ac.uk/Tools/msa/clustalo/). The presence of a signal peptide for the export via the TAT (Twin-Arginine Translocation) pathway was investigated in the MelC1 encoding sequence using the TatP 1.0 server (Bendtsen et al. 2005). The MelC1/MelC2 encoding operon nucleotide sequence was submitted to GenBank with the accession no. **MF288894**)

2.9 Tyrosinase immobilization on nylon nanofiber membranes

The tyrosinase was immobilized on a surface of 24 mm^2 (3x8 mm) for nanofiber carriers following the procedure reported in Fatarella et al. 2014. Hydrolyzed nylon (in HCl 3M for 2h at 25 °C) was rinsed out with 1 mM potassium phosphate buffer at pH 7 and reacted with 1 – 5 % (v/v) solution of glutaraldehyde in potassium phosphate buffer (pH 7) and kept for 20 min incubation time at room temperature. Then, glutaraldehyde-modified nylon was washed off with the potassium phosphate buffer and incubated for the coupling reaction with different amounts of enzyme (1 and 3 mg) for 12 hours at room temperature. Eventually, the unbound enzyme was rinsed out with buffer. The washing phosphate buffer was kept for the activity measurement. To determine the optimum immobilization conditions, were used: (a) glutaraldehyde concentration (1 – 5 %), (b) protein concentration (1 – 3 mg/mL) with a contact time of 12 h. The enzyme immobilization efficiency (EF) was calculated using the following relationship:

$$EF(\%) = A_i / A_f \times 100$$

where A_f is the specific activity of the free enzyme solution and A_i is the specific activity of the immobilized enzyme which is equal to the difference between the specific activity of the free enzyme (A_f) and the specific activity of the unbound enzyme in the washing buffer. Enzyme specific activity is calculated determining the ΔAbs using a Shimadzu UV-1601 spectrophotometer reader.

Once the immobilization conditions were optimized, the stability and reusability measurements were performed at room temperature. For the storage stability studies the nylon immobilized enzyme was stored in semi-dry condition at 4 °C without any buffer and the enzyme activity was checked for 6 days. Each time the supported nanofiber carrier was dipped in a fresh solution of 10 mM l-DOPA in potassium phosphate buffer (pH 7). After each assay the support was washed with buffer at pH 7. For the reusability test the activity assay was repeated for six consecutive runs using the same sample in a phosphate buffer at pH 7.

3. Results

Eight actinomycete strains from Algerian Saharan soil were tested and screened for their ability to produce melanin on MPPM and tyrosine agar solid medium. Preliminary qualitative screening assays for the enzyme production were carried out in Petri dishes. The formation of the extracellular melanin pigments was observed on the reverse side of the Petri dishes during 7 days of cultivation. Of note, only 5 of the tested strains produced extracellular melanoid pigments (**Fig. 1**). In addition, the strains were also investigated for the extracellular tyrosinase production by cultivation in broths supplemented with chloroform for tyrosine broth and filter sterilized $CuSO_4 \cdot 5H_2O$ for MPPM broth as inducers. Again, also in this case, melanin was produced by the same 5 strains. The Ms1 strain showed high yield of tyrosinase production especially on MPPM, and was selected for further studies. The morphological analysis of this strain is reported in **Fig. 2**. The micrograph showed the presence of spore chains with open loops, hooks and spirals, suggesting that the strain belongs to *Streptomyces* genus.

3.1 Strain identification and tyrosinase encoding sequence analysis

The whole genome sequence of Ms1 strain was obtained using an Illumina HiSeq platform by means of a 2 x 250-bp paired-end approach. 5,732,580 reads were obtained and assembled into 38 scaffolds (400 - 1,530,988 bp in size), with a total length of 8,180,953 bp, an N50 of 692,752 bp and an L50 of 4. The GC content was ca. 71%.

Comparison of the assembled Ms1 genome scaffolds with publicly available *Streptomyces* genus whole genome sequences showed the highest nucleotide identity (99%) with *Streptomyces cyaneofuscatus* strain NRRL B-2570 (GenBank accession no. JOEM01000000.1). Ms1 identification as belonging to the *S. cyaneofuscatus* species was confirmed by calculating the ANIb and by the GGDC method. Ms1 whole genome was compared with the genome sequences of its closest homologue *S. cyaneofuscatus* strain NRRL B-2570, of representative strains of the *Streptomyces griseus* group, including *S. luridiscabiei* strain NRRL B-24455 (GenBank accession no. LIQV01000000.1) and *S. griseus* subsp. *griseus* NBRC 13350 (GenBank accession no. AP009493.1), of a more distantly related *Streptomyces* spp. (*S. albus* subsp. *albus* strain NRRL B-16041 (GenBank accession no. JNWW01000000.1). The highest ANIb was observed with *S. cyaneofuscatus* strain NRRL B-2570 (99.4%), while identity with the remaining strains was overall lower (range 86% - 91%), being always lower than the 94% - 96% cut-off value currently accepted for species identification (Richter and Rosselló-Móra 2009). The genome-to-genome distance was calculated by means of the GGDC method, that simulates *in silico* the DNA-DNA hybridization (DDH) technique; the in silico DDH similarity between Ms1 and *S. cyaneofuscatus* strain NRRL B-2570 genome scaffolds was estimated as 95.7% while similarity of Ms1 with the remaining tested genomes was always under the 70% cut-off value that indicates the species boundary (range 33% - 66.3%).

Sequence comparison revealed the presence of three operons encoding enzymes homologous to bacterial tyrosinases and their cofactors. Two of them encoded enzymes homologous to the MelD1 and MelD2 and to the GriE and GriF intracellular enzymes of *S. griseus* subsp. *griseus* NBRC 13350 (Yang and Chen 2009), with an amino acid identity of 61% and 85% and of 85% and 97% with the former and the latter enzymes, respectively. The remaining operon encoded for MelC1 and MelC2 homologues, displaying high identity to the extracellular MelC1-MelC2 tyrosinases produced by some *Streptomyces* spp. (Yang and Chen 2009). The Ms1 MelC2 tyrosinase showed the highest amino

acid identity with the corresponding enzyme of *S. cyaneofuscatus* strain NRRL B-2570 (99.6%), differing for a single aminoacid (a leucine to valine substitution), while it was more distantly related to the prototype tyrosinase enzyme of *S. antibioticus* IMRU3720 (79%) (Bernan et al. 1985) and to the corresponding enzyme of *S. griseus* subsp. *griseus* NBRC 13350 (61%) (Yang and Chen 2009) (**Fig. 3**).

Multiple sequence alignment of the MelC2 tyrosinase sequences confirmed the presence, in MelC2 from the Ms1 strain, of the conserved histidine residues involved in the coordination of the copper ions Cu_A and Cu_B (Tsai and Lee 1998) (**Fig. 3**). As described for other bacterial tyrosinases, the Ms1 *melC* operon included an additional gene encoding for the MelC1 chaperon-like protein, that is likely needed for the MelC2 enzyme secretion and binding of copper ions. Analysis of the Ms1 MelC1 protein revealed the presence of a signal peptide for protein secretion via the TAT pathway and of the Tat motif (data not shown). The theoretical MW of MelC2 protein was predicted to be 30761.49 Da.

3.2 Tyrosinase purification and biochemical characterization

The enzyme was partially purified by ammonium sulphate precipitation, which was a critical purification step, followed by an ion exchange step. Protein concentration, total and specific activity of samples from each purification step were calculated and reported in **Table1**. The total yield of the enzyme was 21 %, (purification fold of 6.8). The tyrosinase production was reproducible each time with a maximum level of 13 mg/l with optimum production at 30 °C for an incubation time of 24 hours after inoculation and agitation of 220 rpm. As shown in **Fig. 4**, the SDS-PAGE analysis of the partially purified enzyme revealed the presence of a major protein band with an estimated MW of 30 kDa, in accordance with the theoretical molecular mass value previously deduced from the predicted amino acid sequence of the MelC2 protein.

The kinetic parameters, K_m= 1.4582 mM, V_{max}= 351 μg $L^{-1}min^{-1}$, for the oxidation of l-DOPA in phosphate buffer at pH 7 were determined. The tyrosinase was able to oxidize l-DOPA across a range of temperature from 50 to 70 °C, with an optimal temperature of 55 °C (**Fig. 5(a)**); enzyme stability over time was optimal at 40 °C (incubation times up to 90 minutes) (more than 100%) while an activity decrease (around 70 %) was observed, after 90 min of incubation at 50 °C (residual activity 23.5 % ± 0.1), and was completely abolished at 60 °C after 15 min of incubation (**Fig. 5(b)**). The oxidation of l-DOPA was performed with high enzyme activity at neutral and basic pH with the optimal pH in the range 6.5 - 7.5 (**Fig. 6(a**)). A small activity decrease at pH > 7 and a quick activity drop at pH < 6 were detected. The enzyme retained 100 % of its activity after 24 h of incubation in buffer solutions in the pH 5 - 10 range (**Fig. 6(a**)). At pH 4 the residual enzyme activity decreased up to 7.7 ± 0.2 % with a complete loss of activity at pH 3 (**Fig. 6(b)**).

The substrate specificity of the tyrosinase on various mono-, di- and triphenols was tested. The enzyme was able to oxidize caffeic acid, *p*-cresol and pyrogallol while ABTS, resorcinol and guaiacol were not oxidized and the activity on tyrosine was practically not detectable (**Table 3**). A lag phase was observed for all the oxidized compounds. A clear preference for the oxidation of diphenols was shown (**SI Fig. 1S**).

The tyrosinase activity in the presence of various water miscible organic solvents was affected to a different extent depending on type and concentration of organic solvents, as shown in **Fig. 7a**. The activity decreased in parallel with

the increase of the amount of organic solvent, ranging from 20 to 50 %. When 10 % (v/v) of acetone was used, a small decrease in the enzyme activity was observed while a significant drop (around 40 % of the remaining activity) was evident with all the other acetone concentrations. With different methanol percentages only a small inactivation effect on the tyrosinase activity was detected, showing that nearly half of the activity was maintained for 50% (v/v) methanol (**Fig. 7a**). In general, the enzyme was able to oxidize l-DOPA in presence of all the tested organic solvents (**Fig. 7b**). Over 50 % residual activity was observed in presence of 30 % (v/v) methanol, 20 % (v/v) acetone, 10 % (v/v) ethanol, and > 70 % residual activity in presence of 10 % (v/v) acetone.

The inactivation effect of reducing agents and cations on the enzyme activity is reported in **Table 2**. Total inactivation of the enzymatic activity occurred in presence of 0.05 mM l-cysteine, 0.1 mM sodium metabisulphite and 0.1 mM l-ascorbic acid. A marked inactivation was observed also with 0.05 mM SDS, with a residual activity of 22 ± 5%. On the other hand, a limited inactivation effect was observed with 10 mM EDTA, with a residual activity of 66 ± 1.4%, while the enzyme activity was maintained with 1 mM NaCl. Tyrosinase activity was enhanced in general by all the cations tested, apart for Ca^{2+} and Fe^{2+}, that had a limited effect (residual activity 97 ± 4% and 96 ± 0.1%, respectively) (**Table 2**).

The stability of bacterial *S. cyaneofuscatus* tyrosinase versus the fungal *A. bisporus* tyrosinase was tested at room temperature (**Fig. 8**). In general the bacterial tyrosinase was more stable than the fungal one and retained more than 50 % of activity after an incubation time up to 6 days. In particular, a marked difference was observed for an incubation time up to 4 days, with the residual activity of tyrosinase being more than double compared to that of the fungal tyrosinase (80 % vs 30 % respectively).

Eventually the enzyme was immobilized on electrospun nylon nanofiber membranes. The optimal efficiency of immobilization was reached when the enzyme concentration was 1 mg/ml with 1 % of glutaraldehyde and an immobilization efficiency of 82 % (**Table 4**). Then the storage stability and reusability of the enzyme were tested (**Fig. 9a**). The immobilized enzyme showed an increased stability when stored at 4° C compared to the free form after an incubation time of 6 days (**Fig. 9a**). The reusability of the supported enzyme was tested at room temperature but a strong decrease of activity after three cycles was detected (**Fig. 9b**).

4. Discussion

Tyrosinases have many potential applications in biotechnology, ranging from materials functionalization (Wichers et al. 2003; Faccio et al. 2012), biosynthesis of new dyes (Warner and Stoler 2011), wastewaters bioremediation by removal of phenolic compounds (Marino et al. 2011; Saratale et al. 2011) and biosensors (Rahman et al. 2016). However, many studies have been performed using commercially available mushroom tyrosinase. On the search of novel enzymes for industrial applications, extremophilic bacteria are suited for the isolation of more robust enzymes with higher stability and activity. In this context a screening for tyrosinase production has been performed on different actinomycete strains, collected and isolated from Algerian Sahara soil. The isolates produced brown coloration, due to melanin production, in both tyrosine agar plate and tyrosine broth revealing the ability to produce tyrosinase (Le Roes-Hill et al. 2015; Dalfar et al 2006).

Ms1 strain showed a high production of tyrosinase and was selected for further characterization and for enzyme production. The strain was identified as belonging to *S. cyaneofuscatus* species, that is included in the *S. griseus* clade; tyrosinase production has never been documented for this species, that has been described from both soil and marine ecosystems (Dalfar et al. 2006; Souagui et al. 2015; Braña et al. 2015; Zothanpuia et al. 2017). Tyrosinases in *Streptomyces* are encoded by the bicistronic operon *melC*. *melC2* encodes for the tyrosinase (MelC2) and remains inactive until it is activated and secreted with the help of MelC1, that usually acts as a co-factor for copper incorporation and secretion of tyrosinase (Yang and Chen 2009). Nevertheless, for the secreted bacterial tyrosinase by *B. megaterium*, the accessory protein MelC1 is not required (Sendovski et al. 2011). *melC* operon in Ms1 strain includes a gene encoding for the MelC1 chaperon-like protein, as reported for the majority of *Streptomyces* spp., that is apparently secreted due to the presence of a signal peptide for protein secretion via the TAT pathway and of the Tat motif. This evidence supports the hypothesis of a role of MelC1 in MelC2 secretion also in Ms1 strain, as already observed for other MelC1-MelC2 proteins (Yang and Chen 2009).

The tyrosinase production was reproducible with a production level around 4.6 mg/ml which is higher than that previously observed for *S. albus* (2.3 mg/ml) (Dolashki et al. 2009). Higher production levels of tyrosinase have been reported only when the enzyme is overexpressed in native or in an expression host (Bubacco et al. 2000; Guo et al. 2015; Ito et al. 2000). As shown in **Fig. 4**, the SDS-PAGE analysis of the partially purified enzyme revealed the presence of a major protein band with a MW of approximately 30 kDa. It is in agreement with the theoretical molecular weight of MelC2 protein (30761.49 Da), falling within the expected size range for *Streptomyces* tyrosinases (29 – 35 kDa) (Claus and Decker 2006; Faccio et al. 2012; Dolashki et al. 2009). The *Streptomyces* tyrosinases show also a certain heterogeneity reflected by different K_m values and temperature stabilities. The determined kinetic parameters (K_m = 1.4582 mM, V_{max}= 351 μg $L^{-1}min^{-1}$) were in agreement with data reported in literature for the oxidation of l-DOPA by other bacterial tyrosinases (Le Roes-Hill et al. 2015).

The tyrosinase exhibited interesting properties, being able to oxidize l-DOPA across a range of temperature from 45 to 60 °C, with an optimum of stability at 55 °C (**Fig. 5a**). In fact, enzymes produced by thermophiles (organisms growing in warm habitat, like hot desert soils), are able to operate at or near the growth temperature of these organisms (T_{opt} ~ 60°C) (Cowan and Fernandez-Lafuente 2011). The enzyme stability was tested at different incubation times, with the maintenance of 100 % of enzyme activity at a temperature of 40 °C for the entire range of incubation times and a stepwise activity decrease at 50 °C along with the increase of incubation time. An activity loss was detected for all the incubation times at 60 °C and 70 °C (**Fig. 5b**). These characteristics allow the enzyme to be used for applications requiring higher temperatures up to 40 °C. Furthermore, the l-DOPA was oxidized by the tyrosinase at pH range from 6.5 to 7.5, with the optimal pH value around 7 (**Fig. 6a**). Up to a 55% reduction in activity at pH > 9 and a quick drop at pH < 5 with rapid enzyme inactivation were observed (Le Roes-Hill et al. 2015; Dolashki et al. 2009). The enzyme was stable, retaining 100% of its activity, after 24 h of incubation in presence of buffer solutions ranging from pH 5-10 (**Fig. 6b**). Tyrosinases are able to catalyze different reactions at neutral or basic pH differently from other polyphenol oxidases like fungal laccases which work mainly at acidic pHs (Pogni et al. 2015; Fatarella et al. 2014).

Tyrosinases from diverse sources show different substrate specificity with usually much lower specific activity for hydroxylation of monophenols than for oxidation of diphenols (i.e plant and bacterial tyrosinases) (Selinheimo et al. 2007, Selinheimo et al. 2009, Hernandez-Romero at al. 2006). UV-Vis absorption spectra of the oxidation product of mono-, di- and triphenols by *S. cyaneofuscatus* tyrosinase are reported in **SI Fig. 1S**. Among monophenols, *p*-cresol was oxidized while guaiacol was not oxidized, probably for the steric hindrance caused by the methoxy group next to the phenolic hydroxyl group. ABTS and the diphenolic compound resorcinol, typical substrates for the enzyme Laccase, were not oxidized, while the diphenolic compound caffeic acid and the triphenol pyrogallol were oxidized. The much higher affinity for diphenols compared to monophenols was also shown by the practically not detectable activity for tyrosine (SI Fig.1S). These data pointed out the low monophenolase/diphenolase ratio and give us useful informations for the development of future tyrosinase-based applications.

Many reaction processes need to be performed in presence of organic solvents. The tyrosinase was able to oxidize l-DOPA in the presence of various water miscible organic solvents retaining around 50 % of activity with 50 % of acetone and methanol (**Fig. 7a**). Furthermore, the enzyme, when incubated for 20 h at 4 °C, retained significant activity (up to 60 %) in presence of solvent concentrations as high as 50 % (i.e. 50 % of residual activity with 30 % acetone and 50 % acetonitrile and ca. 60 % of residual activity with 30 % methanol) (**Fig. 7b**). The enzyme activity was also tested in presence of different cations, reducing agents and surfactant (**Table 2**). Limited inactivation effect was observed with EDTA, while a stronger inactivation was recorded for SDS and some reducing agents (l-ascorbic acid, l-cysteine and sodium metabisulphite). The inactivation effect of reducing agents on tyrosinase activity is known and l-ascorbic acid is used for controlled reaction for the synthesis of l-DOPA or to reduce the brownish effect in food preparation (Pialis et al. 1996; Jang and Moon 2011). In fact, increased amounts of l-ascorbic acid inhibit not only the oxidation of l-DOPA to dopachrome but determine also the irreversible inactivation of the enzyme.

When compared to *A. bisporus* fungal tyrosinase, the widely used and commercially available enzyme, the bacterial tyrosinase showed always a higher stability at room temperature, further supporting to the suitability of this enzyme for a commercial application (**Fig. 8**).

The immobilization procedure enhances the enzyme stability and facilitates its reusability enabling the industrial use. Nonetheless, at industrial level, relatively few processes with immobilized enzymes are commercialized (Di Cosimo et al. 2013). In literature many review and papers deal with enzymatic immobilization showing different strategies and chemical/mutagenetic methods for the preparation of the biocatalysts (Fernando-Lafuente 2009, Rodrigues et al. 2013, Mateo et al. 2007, Guzic et al. 2014, Hwang and Gu 2013). Mushroom tyrosinase was used for the l-DOPA synthesis supported on zeolite (Seetharam and Saville 2002), as cross-linked enzyme aggregates (CLEASs) (Xu et al. 2012), on nylon membranes in a batch reactor (Pialis et al. 1996) or as immobilized form on polyamide tubular membrane and on carbon nanotubes (Algieri et al. 2012, Subrizi et al. 2014). Preliminary experiments on the immobilization of the tyrosinase on nylon carriers functionalized with glutaraldehyde have been performed in this study. There are diverse advantages in using nanofibers as scaffold for enzymatic immobilization: they present high surface-to-volume ratio, the high porosity and interconnectivity of the electrospun nanofibers provides them a low hindrance for mass transfer, nanofibers can be modified for an efficient enzymatic

immobilization (Hwang 2013, Wang 2009). Furthermore enzyme-nanofiber membranes can be recovered easily and can be used in a continous reactor system. Previously the enzyme Laccase was immobilized by our group on the same support showing high payload, good storage stability and reusability (Fatarella et al. 2014). The nanofibers were hydrolyzed and the enzyme was covalently bound to the support preactivated with glutaraldeyde. Glutaraldeyde is one of the most common and used bifunctional crosslinker for enzymatic immobilization (Barbosa et al. 2014). It is able to react with itself and as a protein crosslinker can be used to produce the chemical aggregation of the enzyme. In the present study different concentrations of glutaraldeyde were tested to optimize the enzymatic immobilization. The higher payload (82 %) of the enzyme was obtained with the lower amounts of protein (1 mg/ml) and glutaraldehyde (1 %). The storage stability was enhanced for the immobilized form of the enzyme compared with the free one even if a decrease in the enzymatic activity was detected during the monitoring period. The reusability experiments showed a reduction in activity after three cycles. The low value of the enzymatic activity can be justified by the non enzymatic formation of melanin as final product. This can determine the fouling of the membrane surface for melanin precipitation with the consequently activity reduction.

5. Conclusions

A novel tyrosinase from extremophile bacteria with high stability and activity has been isolated and characterized by a *Streptomyces* strain isolated from the algerian Sahara soil. The strain, able to produce melanoid exopigments in different solid media and named Ms1, was identified as belonging to *S. cyaneofuscatus* sp. by whole genome sequencing. The *melC* operon coding for the MelC2 Ms1 tyrosinase was identified. Biochemical characterization of the enzyme was performed showing that the enzyme was able to oxidize l-DOPA with an optimal activity at 55 °C and pH 7. The tyrosinase is also able to oxidize the substrate in presence of different concentrations of water-miscible organic solvents. It was inactivated in presence of cysteine, sodium metabisulphite, ascorbic acid, whereas a limited inactivation effect was observed with EDTA. The enzyme was immobilized on nylon nanofiber membranes and its reusability was tested. The activity decrease after three reuses, was probably due to the formation of melanin. This aspect has to be taken into account for the use of the supported enzyme. Furthermore, the enzyme greater specificity for the oxidation of diphenols (diphenolase activity) than for the hydroxylation of monophenols (monophenolase activity), points out that the production of synthetic melanins might be more appropriate for future tyrosine based applications rather than its use for crosslinking reactions.

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References

Algieri, C., Donato, L., Bonacci, P., Giorno, L., 2012. Tyrosinase immobilized on polyamide tubular membrane for the l-DOPA production: total recycle and continuous reactor study. Biochem. Eng. J. 66, 14-19.

Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., Zagnitko, O., 2008. The RAST server: rapid annotations using subsystems technology. BMC Genomics. 9, 1471-2164-9-75.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19, 455-477.

Barbosa, O., Ortiz, C., Berenguer-Murcia, Á., Torres, R., Rodrigues, R.C., Fernandez-Lafuente, R., 2014. Glutaraldehyde in bio-catalysts design: a useful crosslinker and a versatile tool in enzyme immobilization. RSC Adv. 4, 1583-1600.

Bendtsen, J.D., Nielsen, H., Widdick, D., Palmer, T., Brunak, S., 2005. Prediction of twin-arginine signal peptides. BMC Bioinformatics 6, 167.

Bernan, V., Filpula, D., Herber, W., Bibb, M., Katz, E., 1985. The nucleotide sequence of the tyrosinase gene from *Streptomyces antibioticus* and characterization of the gene product. Gene 37, 101-110.

Betancourt, A.M., Bernan, V., Herber, W., Katz, E., 1992. Analysis of tyrosinase synthesis in *Streptomyces antibioticus*. Microbiology 138, 787-794.

Bradford, M.M,. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Braña, A.F., Fiedler, H.P., Nava, H., González, V., Sarmiento-Vizcaíno, A., Molina, A., Acuña, J.L., García, L.A., Blanco, G., 2015. Two *Streptomyces* species producing antibiotic, antitumor, and anti-inflammatory compounds are widespread among intertidal macroalgae and deep-sea coral reef invertebrates from the central Cantabrian Sea. Microbial. Ecol. 69, 512-524.

Brenner, M., Hearing, V.J., 1992. The protective role of melanin against UV damage in human skin. Photochem. Photobiol. 84, 539-549.

Bubacco, L., Vijgenboom, E., Gobin, C., Tepper, A.W.J.W., Salgado, J., Canters, G.W., 2000. Kinetic and paramagnetic NMR investigations of the inhibition of *Streptomyces antibioticus* tyrosinase. J. Mol. Cat. B-Enzymatic 8, 27-35.

Cantone, S., Ferrario, V., Corici, L., Ebert, C., Fattor, D., Spizzo, P., Gardossi, L., 2013. Efficient immobilisation of industrial biocatalysts: criteria and constraints for the selection of organic polymeric carriers and immobilization method. 42, 6262-6276.

Claus, H., Decker, H., 2006. Bacterial tyrosinases. Syst. Appl. Microbiol. 29, 3-14.

Cowan, D.A. and Fernandez-Lafuente, R., 2011. Enhancing the functional properties of thermophilic enzymes by chemical modification and immobilization. Enzyme Microb. Technol. 49, 326-346.

Dalfar, A.B., Khajeh, K., Soudi, M.R., Naderi-Manesh, H., Ranjbar, B., Sajedi, R.H., 2006. Isolation and biochemical characterization of laccase and tyrosinase activities in a novel melanogenic soil bacterium. Enzyme Microb. Tech. 39, 1409-1416.

Di Cosimo, R., McAuliffe, J., Poulose, A.J., Bohlmann, G., 2013. Industrial use of immobilized enzymes. Chem. Soc. Rev. 42, 6437-6474.

Dolashki, A., Gushterova, A., Voelter, W., Tchorbanov, B., 2009. Purification and characterization of tyrosinases from *Streptomyces albus*. Z. Naturforsch. 64c, 724-732.

Durán, N., Rosa, M.A., D'Annibale, A., Gianfreda, L., 2002. Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review. Enzyme Microb. Technol. 31, 907-931.

Faccio, G., Kruus, K., Saloheimo, M., Thöny-Meyer, L., 2012. Bacterial tyrosinases and their applications. Process Biochem. 47, 1749-1760.

Fairhead, M., Thöny-Meyer, L., 2012. Bacterial tyrosinases: old enzymes with new relevance to biotechnology. New Biotechnol. 29, 183-191.

Fatarella, E., Spinelli, D., Ruzzante, M., Pogni, R., 2014. Nylon 6 film and nanofiber carriers: preparation and laccase immobilization performance. J. Mol. Cat. B-Enzymatic 102, 41-47.

Fernandez-Lafuente R., 2009. Stabilization of multimeric enzymes: strategies to prevent subunit dissociation. Enzyme Microb. Technol. 45, 405-418.

Garcia-Galan, C., Berenguer-Murcia, Á., Fernandez-Lafuente, R., Rodrigues, R.C., 2011. Potential of different enzyme immobilization strategies to improve enzyme performance. Adv. Synth. Catal. 353, 2885-2904.

Goudjal, Y., Toumatia, O., Yekkour, A., Sabaou, N., Mathieu, F., Zitouni, A., 2014. Biocontrol of *Rhizoctoni asolani* damping-off and promotion of tomato plant growth by endophytic *actinomycetes* isolated from native plants of Algerian Sahara. Microbiol. Res. 169, 59-65.

Guo, J., Rao, Z., Yang, T., Man, Z., Xu, M., Zhang, X., Yang, S.T., 2015. Cloning and identification of a novel tyrosinase and its overexpression in *Streptomyces kathirae* SC-1 for enhancing melanin production. FEMS Microbiol. Lett. 362, 1-7.

Guzic, U., Hupert-Kocurek, K., Wojcieszynska, D., 2014. Immobilization as a strategy for improving enzyme properties-application to oxidoreductases. Molecules 19, 8995-9018.

Hernandez-Romero, D., Sanchez-Amat, A., Solano, F., 2006. A tyrosinase with an abnormally high tyrosine hydroxylase/dopa oxidase ratio. FEBS J.273, 257-270.

Huber, M., Lerch, K., 1988. Identification of two histidines as copper ligands in *Streptomyces glaucescens* tyrosinase. Biochemistry 27, 5610–5615.

Hwang, E.T. and Gu, M.B., 2013. Enzyme stabilization by nano/microsized hybrid materials. Eng. Life Sci. 13(1), 49-61.

Ito, M., Oda, K., 2000. An organic solvent resistant tyrosinase from *Streptomyces* sp. REN-21: purification and characterization. Biosci. Biotechnol. Biochem. 64, 261-267.

Jang, J.H., Moon, K.D., 2011. Inhibition of polyphenol oxidase and peroxidase activities on fresh-cut apple by simultaneous treatment of ultrasound and ascorbic acid. Food Chem. 124, 444-449.

Kohashi, P.Y., Kumagai, T., Matoba, Y., Yamamoto, A., Maruyama, M., Sugiyama, M., 2004. An efficient method for the overexpression and purification of active tyrosinase from *Streptomyces castaneoglobisporus*. Protein Expr. Purif. 34, 202-207.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lee, N., Lee, S.H., Baek, K., Kim, B.G., 2015. Heterologous expression of tyrosinase (MelC2) from *Streptomyces avermitilis* MA4680 in *E. coli* and its application for ortho-hydroxylation of resveratrol to produce piceatannol. Appl. Microbiol. Biotechnol. 99, 7915–7924.

Le Roes-Hill, M., Palmer, Z., Rohland, J., Kirby, B.M., Burton, S.G., 2015. Partial purification and characterization of two actinomycete tyrosinases and their application in cross-linking reactions. J. Mol. Cat. B-Enzymatic 122, 353–364.

López-Serrano, D., Solano, F., Sanchez-Amat, A., 2004. Identification of an operon involved in tyrosinase activity and melanin synthesis in *Marinomonas mediterranea*. Gene 342, 179-187.

Marino, S.M., Fogal, S., Bisaglia, M., Moro, S., Scartabelli, G., De Gioia, L., Spada, A., Monzani, E., Casella, L., Mammi, S.L., 2011. Investigation of *Streptomyces antibioticus* tyrosinase reactivity toward chlorophenols. Arch. Biochem. Biophys. 505, 67-74.

Mateo, C., Palomo, J.M., Fernandez-Lorente, G., Guisan, J.M., Fernandez-Lafuente, R., 2007. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microb. Tech. 40, 1451-1463.

Meier-Kolthoff, J.P., Auch, A.F., Klenk, H.P., Göker, M., 2013. Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinformatics 14, 1471-2105-14-60.

Pialis, P., Jimenez Hamann, M.C., Saville, B.A., 1996. l-DOPA production from tyrosinase immobilized on nylon 6,6. Biotechnol. Bioeng. 51, 141-147.

Plonka, P.M., Grabacka, M., 2006. Melanin synthesis in microorganisms-biotechnological and medical aspects. Acta Biochim. Pol. 53, 429-443.

Pogni, R., Baratto, M.C., Sinicropi, A., Basosi, R., 2015. Spectroscopic and computational characterization of laccases and their substrate radical intermediates. Cell. Mol. Life Sci. 72, 885-896.

Rahman, S.F., Min, K., Park, S.H., Park, J.H., Yoo, J.C., Park, D.H., 2016. Selective determination of dopamine with an amperometric biosensor using electrochemically pretreated and activated carbon/tyrosinase/nafion-modified glassy carbon electrode. Biotechnol. Bioproc. Eng. 21, 627-633.

Raval, K.M., Vaswani, P.S., Majumder, D.R., 2012. Biotransformation of single aminoacid L-tyrosine into a bioactive molecule l-DOPA. Int. J. Sci. Res. Pub. 2, 2250-3153.

Richter, M., Rosselló-Móra, R., 2009. Shifting the genomic gold standard for the prokaryotic species definition. Proc. Natl. Acad. Sci. USA 106, 19126–19131.

Richter, M., Schulenburg, C., Jankowska, D., Heck, T., Faccio, G., 2015. Novel materials through Nature's catalysts. Materials Today 18, 459-467.

Rodrigues, R.C., Ortiz, C., Berenguer-Murcia, Á., Torres, R., Fernandez-Lafuente, R., 2013. Modifying enzyme activity and selectivity by immobilization. Chem. Soc. Rev. 42, 6290-6307.

Saratale, R.G., Saratale, G.D., Chang, J.S., Govindwar, S.P., 2011. Bacterial decolorization and degradation of azo dyes: a review. J. Taiwan Inst. Chem. Eng. 42, 138-157.

Seetharam, G., Saville, B.A., 2002. l-DOPA production from tyrosinase immobilized on zeolite. Enzyme Microb. Tech. 31, 747-753.

Selinheimo, E., NiEidhin, D., Steffensen, C., Nielsen, J., Lomascolo, A., Halaouli, S., Record, E., O'Beirne, D., Buchert, J., Kruus, K., 2007. Comparison of the characteristics of fungal and plant tyrosinases. J. of Biotech. 130, 471-480.

Selinheimo, E., Gasparetti, C., Mattinen M., Steffensen, C., Buchert, J., Kruus, K., 2009. Comparison of substrate specificity of tyrosinases from *Trichoderma reesei* and *Agaricus bisporus*. Enzyme Microb. Tech. 44, 1-10.

Sendovski, M., Kanteev, M., Ben-Yosef, V.S., Adir, N., Fishman, A., 2011. First structure of an active bacterial tyrosinase reveal copper plasticy. J. Mol. Biol. 405, 227-237.

Sheldon, R.A. and van Pelt, S., 2013. Enzyme immobilization in biocatalysis: why, what and how. Chem. Soc. Rev., 42, 6223-6235.

Shirling, E.B., Gottlieb, D., 1966. Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16, 313-340.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J.D., Higgins, D.G., 2011. Fast scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 11, 539.

Solomon, E.I., Baldwin, M.J., Lowery, M.D., 1992. Electronic structures of active sites in copper proteins: contributions to reactivity. Chem. Rev. 92, 521-542.

Souagui, Y., Tritsch, D., Grosdemange-Billiard, C., Kecha, M., 2015. Optimization of antifungal production by an alkaliphilic and halotolerant actinomycete, *Streptomyces* sp. SY-BS5, using response surface methodology. J. Mycol. Med. 25, 108-115.

Subrizi, F., Crucianelli, M., Grossi, V., Passacantando, M., Pesci, L., Saladino R., 2014. Carbon nanotubes as activating tyrosinase supports for the synthesis of catechols. ACS Catal. 4, 810-822.

Tsai, T.Y., Lee, Y.H., 1998. Roles of copper ligands in the activation and secretion of *Streptomyces* tyrosinase. J. Biol. Chem. 273, 19243-19250.

Warner, J.C., Stoler, E.J., 2011. Color composition containing an aromatic compound and tyrosinase, United States Patent. Appl. US 2011/0113571 A1.10.11.15.

Wichers, H.J., Recourt, K., Hendriks, M.,·Ebbelaar, C.E.M., Biancone, G., Hoeberichts, F.A., Mooibroek, H., Soler-Rivas, C., 2003. Cloning, expression and characterization of two tyrosinase cDNAs from *Agaricus bisporus*. Appl. Microbiol. Biotechnol. 61, 336-341.

Xu, D., Chen, J., Yang, Z., 2012. Use of cross-linked aggregates as catalyst for synthesis of l-DOPA. Biochem. Eng. J. 63, 88-94.

Yang, H.Y., Chen, C.W., 2009. Extracellular and intracellular polyphenol oxidases cause opposite effects on sensitivity of *Streptomyces* to phenolics: a case of double-edges word. Plos One 4, e7462.

Zaidi, K.U., Ali, A.S., Ali, S.A., Naaz, I., 2014. Microbial Tyrosinases: promising enzymes for pharmaceutical, food bioprocessing and environmental industry. Biochem. Res. Int. 2014, 854687.

Zothanpuia, Passari, A.K., Chandra, P., Leo, V.V., Mishra, V.K., Kumar, B., Singh, B.P., 2017. Production of potent antimicrobial compounds from *Streptomyces cyaneofuscatus* associated with fresh water sediment. Front. Microbiol. 8, 68.

Captions to Figures

Fig. 1 Melanin production by tyrosinase in agar (a) and MPPM (b) (Ms1, O5, H1, M51 and 10 from the left)

Fig. 2 Scanning Electron Micrography of Ms1 strain showing the typical straight chains spore arrangement of *Streptomyces* genus.

Fig. 3 Amino acid sequence alignment of Ms1 MelC2 tyrosinase (GenBank accession no. **MF288894**) with enzymes from different *Streptomyces spp*. (*S. cyaneofuscatus* NRRL B-2570, GenBank accession no. WP_030566324; *S. antibioticus* IMRU3720, GenBank accession no. WP_030787646.1; *S. griseus* NBRC 13350, GenBank accession no. WP_012379204.1). Different amino acids with respect to Ms1 MelC2 protein sequence are shaded in grey. Conserved histidine residues involved in binding of the Cu_A and Cu_B copper ions are indicated by a black dot

Fig. 4 SDS-PAGE analysis of Ms1 tyrosinase. Lane 1: culture filtrate; lane 2: proteins precipitated by ammonium sulfate with 45 % saturation; lane 3: proteins precipitated by ammonium sulfate with 65 % saturation; lane 4: proteins after dialysis; lane 5: molecular mass markers; lane 6 partially purified Ms1 Tyr eluted from DEAE Sephadex (circled band).

Fig. 5 (a) Optimal temperature and (b) thermostability of *S. cyaneofuscatus* tyrosinase. (a) The enzyme activity was detected at temperatures ranging from 5 to 70 °C as indicated in the graph. (b) The enzyme was incubated at different temperatures (30, 40, 50, 60 and 70 °C) and various incubation times (0, 15, 30 and 90 min). The activity was measured using the l-DOPA standard assay and 100% activity was set in a) at 20° C and in b) at 0 min.

Fig. 6 (a) Optimal pH and (b) pH stability of *S. cyaneofuscatus* tyrosinase. (a) The enzyme activity was detected at different pH values as indicated; (b) The residual activity was detected after incubation of the enzyme at different pH for 24 h at 4 °C, referring to the enzyme activity at pH 6.5.

Fig. 7 (a) Activity of *S. cyaneofuscatus* tyrosinase in the presence of various water-miscible organic solvents (0, 10, 20, 30, 40 and 50 % (v/v)) for l-DOPA oxidation. (b) Residual activity of *S. cyaneofuscatus* tyrosinase incubated for

20 h at 4 °C of water-miscible organic solvents (same percentages of (a)). The oxidation of l-DOPA in the absence of organic solvent was the reference control.

Fig. 8 Stability of *S. cyaneofuscatus* and *A. bisporus* tyrosinase for diverse incubation times at room temperature (T = 25 °C). All the data were normalized to the tyrosinase activity obtained with the l-DOPA assay at an incubation time t = 0.

Fig. 9 a) Storage stability of covalently immobilized (●) and free (■) *S. cyaneofuscatus* tyrosinase. T = 4° C and pH 7. b) Reusability of nylon nanofiber carrier-tyrosinase. The l-DOPA oxidation reaction was performed at room temperature. Experiments were performed in triplicate. The error bars indicate the standard deviation. All the data were normalized towards the activity of the first measurements (100%) activity.

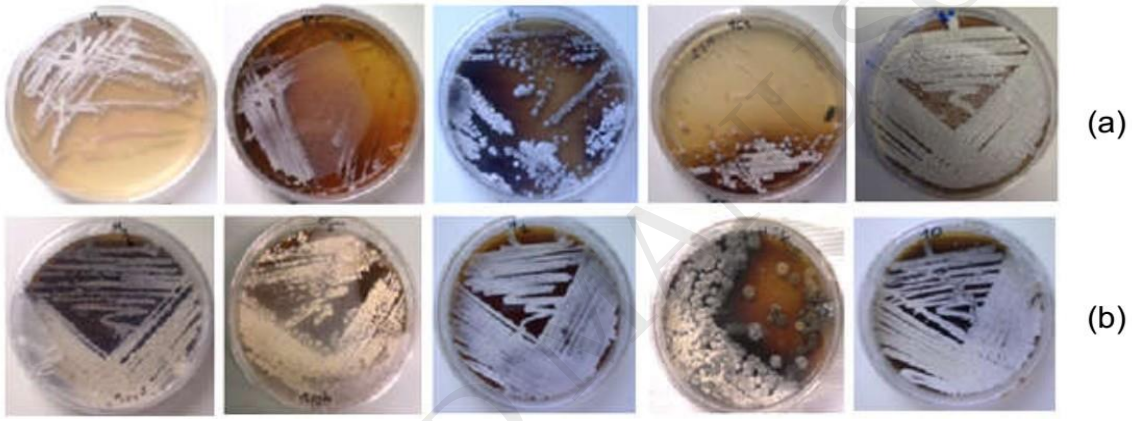

Fig. 1

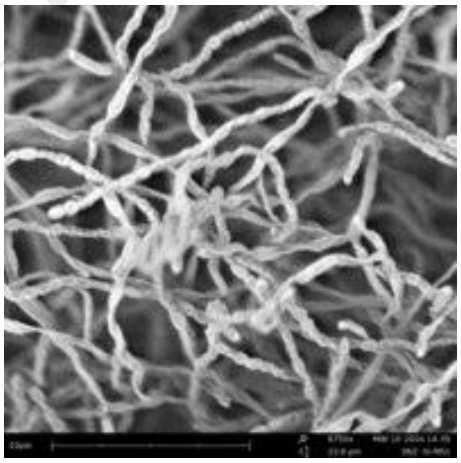

Fig .2

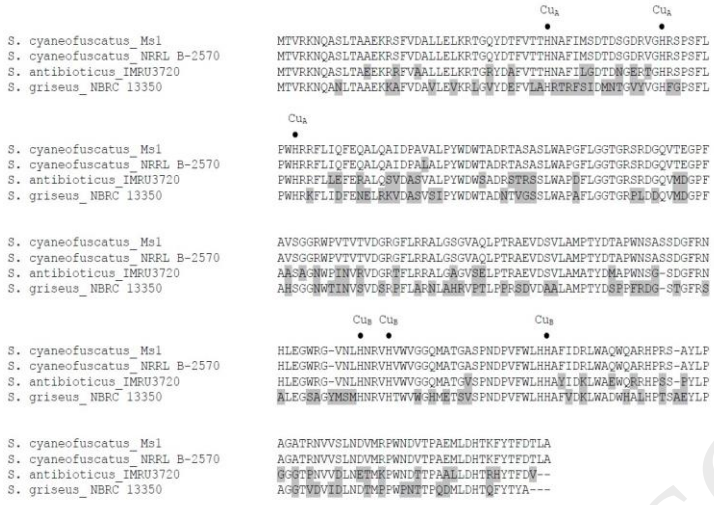

Fig. 3

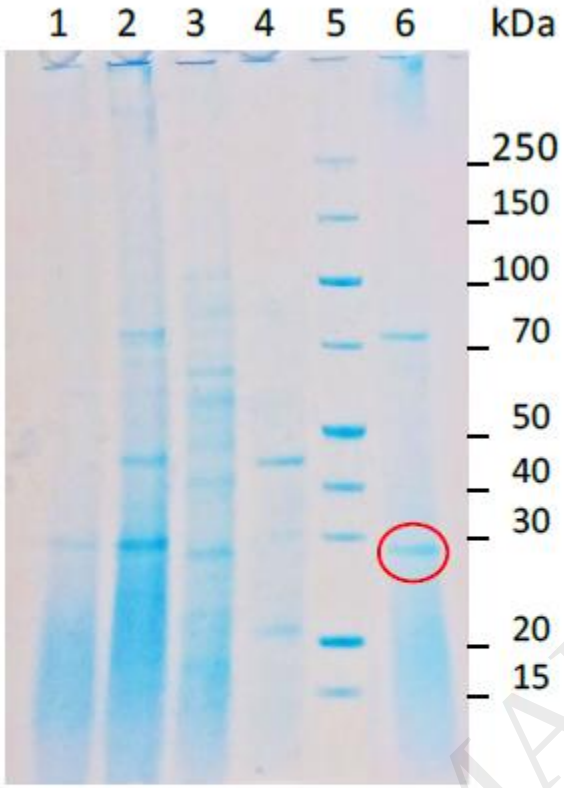

Fig. 4

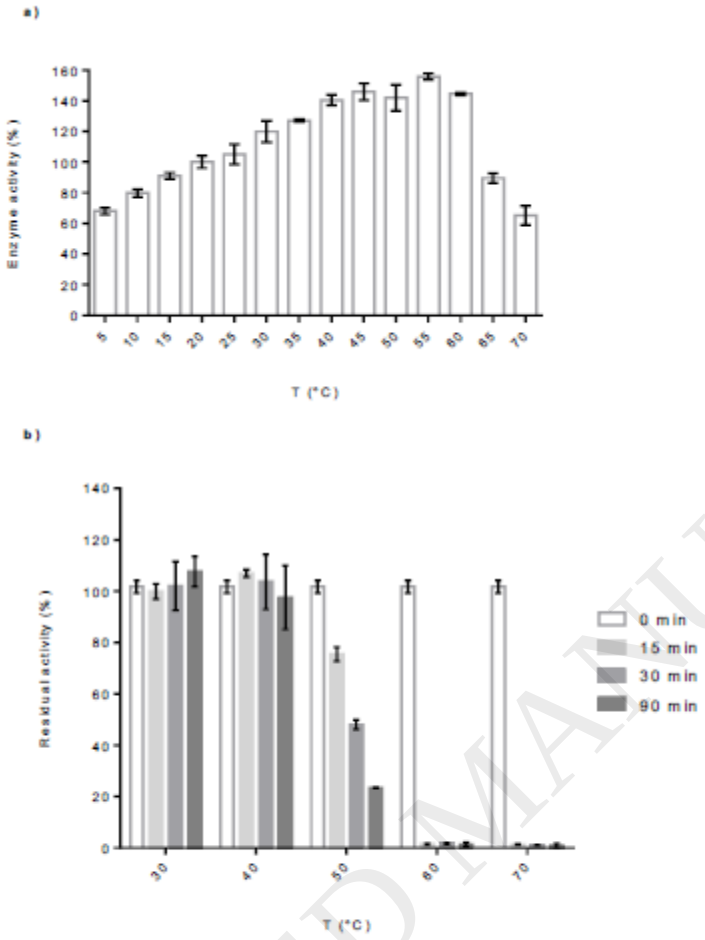

Fig. 5

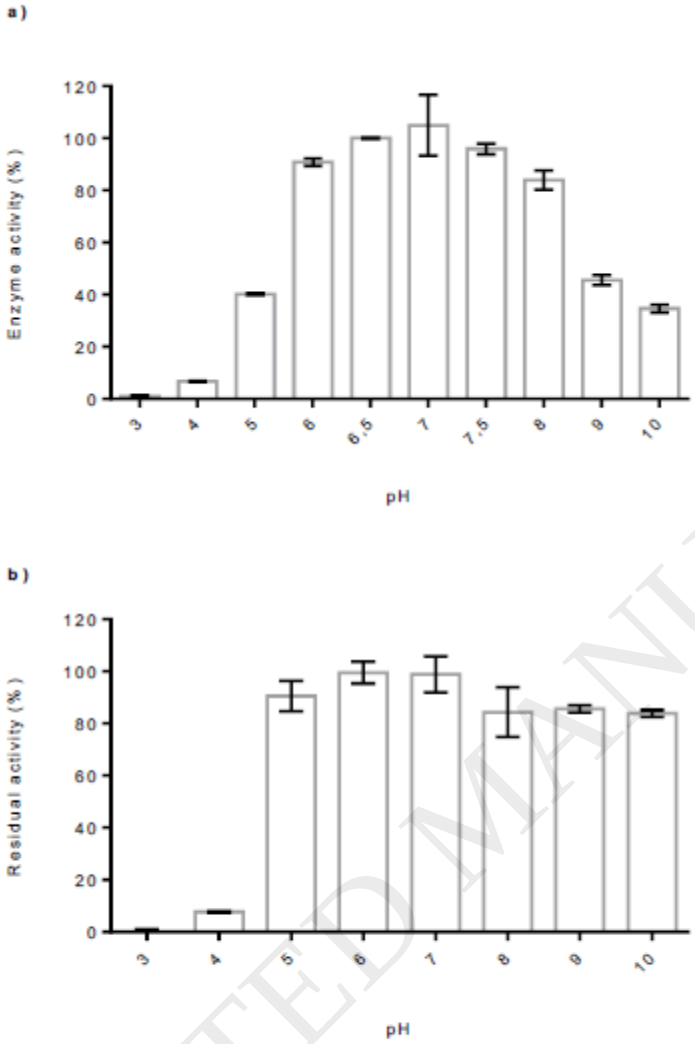

Fig. 6

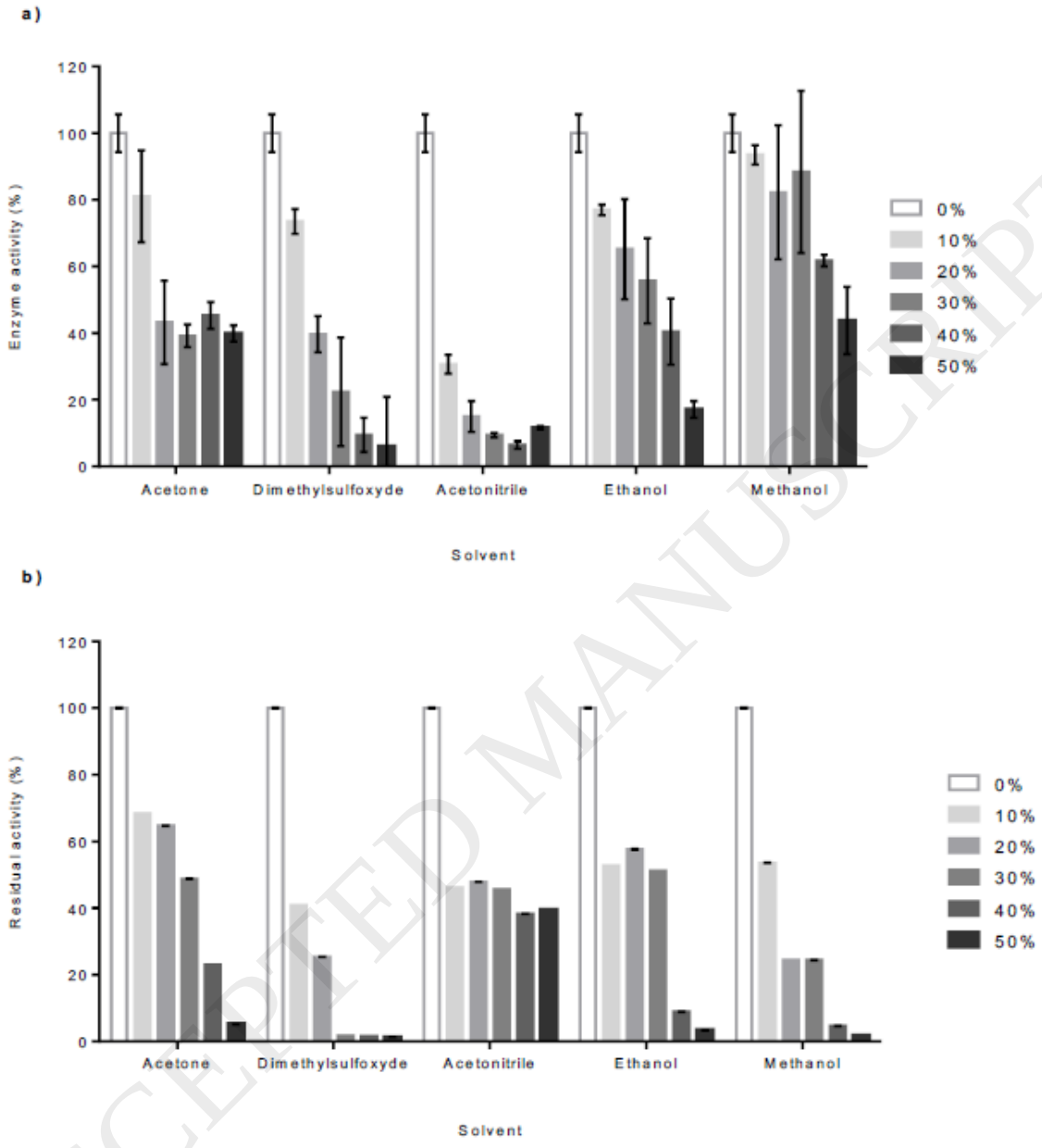

Fig. 7

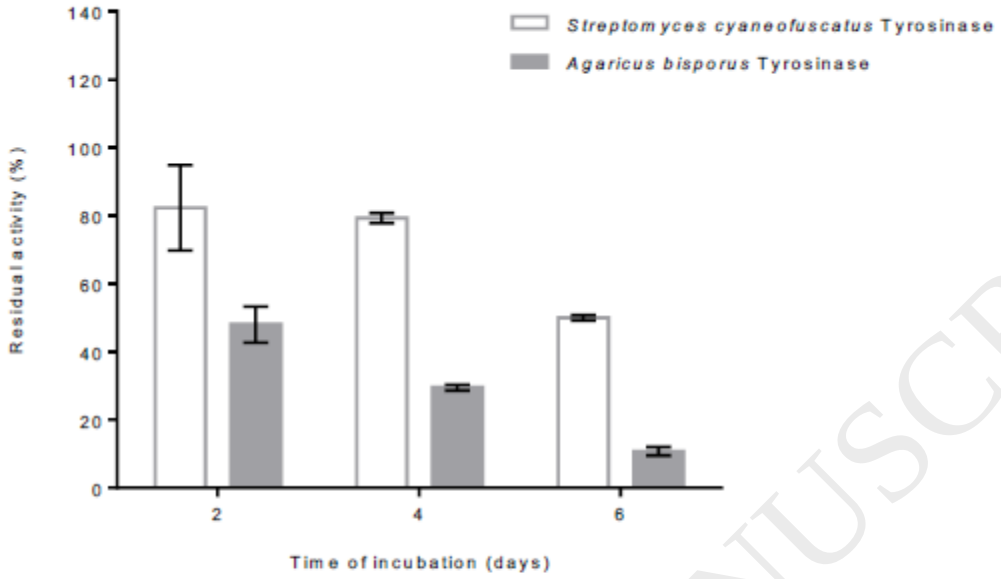

Fig. 8

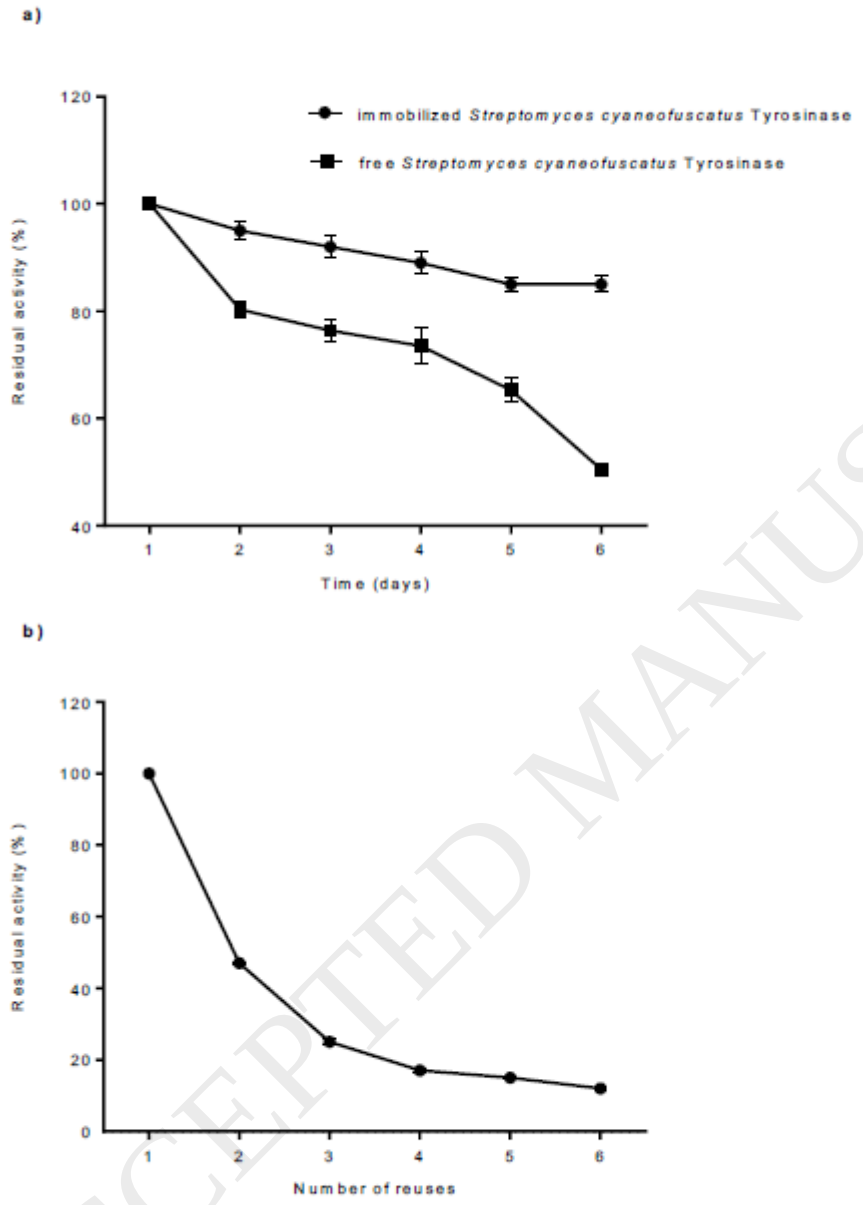

Fig. 9

Table 1. *S. cyaneofuscatus* tyrosinase: culture filtrate, ammonium sulphate precipitation and anion exchange chromatography

Sample	Total activity (U)	Protein amount (mg/ml)	Specific activity (U/mg)	Purification fold	Yield (%)
***Streptomyces* sp Ms1 Tyr culture filtrate**	1055	148.8	7	1	100
After 65% ammonium sulphate precipitation	310.9	41	7.6	1.1	29.5
After anion exchange Deae Sephadex A50	222	4.6	47.6	6.8	21.0

Table 2. Activity of *S. cyaneofuscatus* tyrosinase in presence of inactivating and reducing agents, and cations

Inactivating and , reducing agents or cations	Concentration (mM)	Residual activity (%)
EDTA	0.1	99.1 ± 7.1
	0.2	98.6 ± 0.6
	0.5	96.5 ± 0.8
	1	90.2 ± 8.4
	5	89.0 ± 12.8
	10	66.1 ± 1.4
l-Ascorbic acid	0.01	88.0 ± 6.2
	0.05	3.1 ± 0.9
	0.1	1.8 ± 0.1
l-Cysteine	0.01	77.9 ± 13.3
	0.05	1.1 ± 0.0
	0.1	1.5 ± 0.4
Sodium metabisulphite	0.01	36.2 ± 10.3
	0.05	3.7 ± 0.20
	0.1	1.7 ± 0.0
NaCl	1	101.8 ± 4.2
SDS	0.01	82.7 ± 4.2
	0.05	22.6 ± 4.9
Ca^{2+}	1	97.1 ± 3.8
Co^{2+}	1	130.6 ± 1.0
Cu^{2+}	1	104.1 ± 7.5
Fe^{2+}	1	96.7 ± 0.1

Mg^{2+}	1	117.3 ± 7.6
Mn^{2+}	1	131.1 ± 0.2
Zn^{2+}	1	116.0 ± 2.9

Table 3: Absorption maxima (nm) in the UV-Vis absorption spectrum of the primary product formation by tyrosinase from mono-, di- and triphenolic substrates

Substrate	Wavelength (nm)	Active
l-DOPA	475	√
p-Cresol	400	√
Guaiacol	470	-
l-Tyrosine	475	-
Caffeic acid	480	√
Resorcinol	500	-
Pyrogallol	450	√
ABTS	420	-

Table 4. Enzyme Immobilization efficiency (EF%): 100 µL *S. cyaneofuscatus* tyrosinase for 12 h at room temperature and pH = 7

Protein amount (mg/ml)	Glutaraldeyde (%) 1% 3% 5%	EF (%)
1	x	82.8
	x	67.8
	x	74.5

3	x	49.9
	x	34.5
	x	46.0