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Isolation, Cloning and Characterization of a Tyrosinase with Improved Activity in Organic Solvents from *Bacillus megaterium*

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Key Words

Bacillus megaterium · L-Tyrosine · Tyrosinase · Monophenolase activity · Diphenolase activity · Organic solvents

Abstract

A tyrosinase-expressing bacterium was isolated from soil, and extracellular enzymatic activity was induced by the presence of tyrosine and $CuSO_4$. Amplification of the 16S rDNA genes revealed a high similarity with *Bacillus megaterium*. The enzyme was over-expressed in *Escherichia coli* BL21 and purified using an affinity column. The tyrosinase was composed of 297 amino acids and was determined to be a monomer with a relative molecular mass of 31 kDa according to gel filtration. The K_m values for 3,4-dihydroxy-L-phenylalanine (L-DOPA) and L-tyrosine were 0.35 and 0.075 mM, respectively, and the k_{cat}/K_m values were $28.9 \cdot 10^3$ and $32.9 \cdot 10^3$ ($s^{-1} \cdot M^{-1}$). The maximum activity for both monophenolase and diphenolase was observed at 50°C and pH 7.0. Enzymatic activity was enhanced in the presence of 10–50% water-miscible organic solvents, which included ethanol, methanol, 2-propanol and dimethyl sulfoxide (DMSO). The activity in 30% DMSO was 170% of the activity in water and the enantioselectivity towards L-DOPA decreased by 40%. The residual activity following an incubation period of 17 h in 0–70% methanol was constant. This newly isolated and characterized tyrosinase may have potential applications in organic synthesis due to its high activity and stability at typically denaturing conditions.

Introduction

Tyrosinases (EC 1.14.18.1) are copper-containing enzymes which are ubiquitously distributed in all domains of life [Halaouli et al., 2006]. Tyrosinases are found in prokaryotic as well as in eukaryotic micro-organisms, and in mammals, invertebrates and plants. Molecular oxygen is used by tyrosinases to catalyze two different enzymatic reactions: (1) the orthohydroxylation of monophenols to *o*-diphenols (monophenolase activity) and (2) the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). The active quinones polymerize spontaneously to the macromolecular melanin [Decker and Tuczek, 2000; Seo et al., 2003]. Tyrosinases belong to a larger group of proteins named type-3 copper proteins, which include the catechol oxidases that exhibit only catecholase activity, and the oxygen-carrying hemocyanins from mollusks and arthropods [Decker and Tuczek, 2000; Halaouli et al., 2006]. Tyrosinase is perhaps the most thoroughly studied enzyme of this family due to its role in skin pigmentation and melanoma [Yu and Chang, 2004], as well as undesired browning in fruits and vegetables [Martinez and Whitaker, 1995; Seo et al., 2003].

Although much of the work on tyrosinases and their applications has been done with mushroom tyrosinase, there are some examples of well characterized bacterial tyrosinases. They were first described in several species of *Streptomyces* [Katz et al., 1983; Lerch and Ettinger, 1972], but the enzyme has also been reported in other species such as *Rhizobium, Symbiobacterium thermophi-*

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lum, Pseudomonas maltophilia, Sinorhizobium meliloti, *Marinomonas mediterranea, Thermomicrobium roseum, Bacillus thuringiensis*, and *Pseudomonas putida* F6 [Claus and Decker, 2006; Dalfard et al., 2006; Liu et al., 2004; McMahon et al., 2007; Ruan et al., 2005]. Recently, a unique tyrosinase with a high tyrosine-hydroxylation/dopa-oxidase ratio was discovered in *Ralstonia solanacearum* [Hernàndez-Romero et al., 2006]. The first crystal structure of a tyrosinase was determined recently by Matoba et al. [2006] for an enzyme from *Streptomyces castaneoglobisporus,* contributing new insights on the structure-function relationship.

Tyrosinases demonstrated usefulness in numerous biotechnological applications. Their main application is in the detoxification of phenol-containing waste water and contaminant soils [Burton, 2003a; Duran and Esposito, 2000; Duran et al., 2002]. The phenolic compounds are transformed by the enzyme to quinones which auto-oxidize to form insoluble polymeric compounds that precipitate from water [Girelli et al., 2006]. Another application is the synthesis of chemicals of commercial importance such as 3,4-dihydroxy-L-phenylalanine (L-DOPA), the preferred drug for treatment of Parkinson's disease [Ates et al., 2007; Burton, 2003b]. Hydroxytyrosol, a potent antioxidant abundant in olives, was also synthesized with tyrosinase from tyrosol [Espin et al., 2001]. Chen et al. [2002] reported the novel use of tyrosinase for the in vitro conjugation of the protein gelatin to the polysaccharide chitosan. Finally, biosensors based on tyrosinases were designed for measuring phenols, polyphenols and pesticides [Abhijith et al., 2007; Tanimoto de Albuquerque and Ferreira, 2007].

It is now well established that enzymes function in organic solvents providing advantages, such as higher substrate solubility, reversal of hydrolytic reactions and modified enzyme specificity, which result in new enzyme activities. As a result, enzymatic catalysis in organic solvents has a variety of applications, which include chiral resolution of pharmaceuticals, synthesis of fine chemicals and enantio- and regioselective polymerization [Schmid et al., 2001; Yang and Russell, 1996]. Mushroom tyrosinase has been well studied in pure organic solvents [Kermasha and Tse, 2000; Kermasha et al., 2001] and aqueous media containing water miscible-solvents [Ito and Oda, 2000]. Tyrosinase from *Streptomyces* sp. REN-21, characterized as organic solvent resistant, had 44% of the activity of the control in the presence of 50% ethanol, while mushroom tyrosinase exhibited only 6% of the activity under the same conditions [Ito and Oda, 2000].

This work describes the isolation of a novel tyrosinase-producing bacterium from soil, the cloning of the tyrosinase gene into *Escherichia coli* and the subsequent purification and characterization of the enzyme. The increased activity of the purified tyrosinase in the presence of water-miscible organic solvents makes this enzyme unique compared with other tyrosinases reported to date.

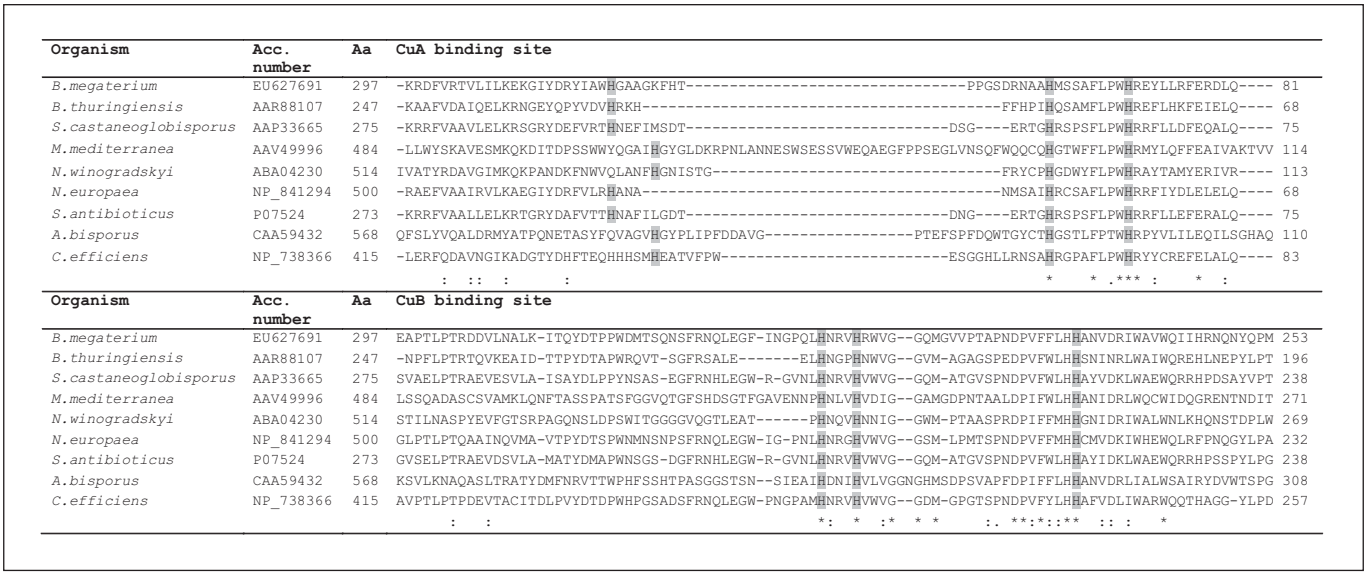
Results and Discussion

Nowadays, there is an increasing interest in using tyrosinases in industrial applications [Selinheimo et al., 2007] due to their ability to convert monophenols into diphenols which possess beneficial attributes as pharmaceutical drugs and food additives [Halaouli et al., 2006]. Our aim was to discover and characterize a novel bacterial tyrosinase with biotechnologically interesting features using functional-based screening in soil samples.

Isolation and Identification of a Tyrosinase-Expressing Bacterial Strain

A bacterium capable of forming melanin from L-tyrosine was isolated from soil samples collected from different areas in Israel. Diluted samples were streaked on selection plates (MMB) containing 0.1% L-tyrosine and glucose, and black colonies suspected of melanin production were isolated and grown again on L-tyrosine-containing plates and on similar plates without tyrosine (negative control). One strain, named VS1, consistently formed black colonies on L-tyrosine-containing plates, but was white on plates without tyrosine. Similar results were obtained in liquid culture; however, the addition of 0.2 mM $CuSO_4$ was required to obtain black pigment formation. The bacteria had the morphology of a rod shape. Monophenolase and diphenolase activities of the extracellular liquid broth were determined by monitoring the oxidation of L-tyrosine or L-DOPA to dopachrome at 475 nm (results not shown). It was observed that the addition of a catalytic amount of L-DOPA (0.025 mM) to L-tyrosine suppressed the lag period characteristic of tyrosinase transforming tyrosine [Dalfard et al., 2006; Halaouli et al., 2005]. Activity measurements were also performed using reverse-phase high-performance liquid chromatography, and the conversion of L-tyrosine to L-DOPA was confirmed by comparing the product peak with authentic standards and the UV absorbance spectrum (data not shown).

rRNAs are essential elements in protein synthesis which are conserved in all living organisms and, there-



Organism	Acc. number	Aa	CuA binding site	
B.megaterium	EU627691	297	-KRDFVRTVLILKEKGIYDRYIAWHGAAGKFHT----------PPGSDRNAAHMSSAFLPWHREYLLRFERDLQ----	81
B.thuringiensis	AAR88107	247	-KAAFVDAIQELKRNGEYQPYVDVHRKH----------FFHPIHQSAMFLPWHREFLHKFEIELQ----	68
S.castaneoglobisporus	AAP33665	275	-KRRFVAAVLELKRSGRYDEFVRTHNEFIMSDT----------DSG----ERTGHRSPSFLPWHRRFLLDFEQALQ----	75
M.mediterranea	AAV49996	484	-LLWYSKAVESMKQKDITDPSSWWYQGAIHGYGLDKRPNLANNESWSESSVWEQAEGFPPSEGLVNSQFWQQCQHGTWFFLPWHRMYLQFFEAIVAKTVV	114
N.winogradskyi	ABA04230	514	IVATYRDAVGIMKQKPANDKFNWVQLANFHGNISTG----------FRYCPHGDWYFLPWHRAYTAMYERIVR----	113
N.europaea	NP_841294	500	-RAEFVAAIRVLKAEGIYDRFVLRHANA----------NMSAIHRCSAFLPWHRRFIYDLELELQ----	68
S.antibioticus	P07524	273	-KRRFVAALLELKRTGRYDAFVTTHNAFILGDT----------DNG----ERTGHRSPSFLPWHRRFLLEFERALQ----	75
A.bisporus	CAA59432	568	QFSLYVQALDRMYATPQNETASYFQVAGVHGYPLIPFDDAVG----------PTEFSPFDQWTGYCTHGSTLFPTWHRPYVLILEQILSGHAQ	110
C.efficiens	NP_738366	415	-LERFQDAVNGIKADGTYDHFTEQHHHSMHEATVFPW----------ESGGHLLRNSAHRGPAFLPWHRYYCREFELALQ----	83

Organism	Acc. number	Aa	CuB binding site	
B.megaterium	EU627691	297	EAPTLPTRDDVLNALK-ITQYDTPPWDMTSQNSFRNQLEGF-INGPQLHNRVHRWVG--GQMGVVPTAPNDPVFFLHHANVDRIWAVWQIIHRNQNYQPM	253
B.thuringiensis	AAR88107	247	-NPFLPTRTQVKEAID-TTPYDTAPWRQVT-SGFRSALE-------ELHNGPHNWVG--GVM-AGAGSPEDPVFWLHHSNINRLWAIWQREHLNEPYLPT	196
S.castaneoglobisporus	AAP33665	275	SVAELPTRAEVESVLA-ISAYDLPPYNSAS-EGFRNHLEGW-R-GVNLHNRVHVWVG--GQM-ATGVSPNDPVFWLHHAYVDKLWAEWQRRHPDSAYVPT	238
M.mediterranea	AAV49996	484	LSSQADASCSVAMKLQNFTASSPATSFGGVQTGFSHDSGTFGAVENNPHNLVHVDIG--GAMGDPNTAALDPIFWLHHANIDRLWQCWIDQGRENTNDIT	271
N.winogradskyi	ABA04230	514	STILNASPYEVFGTSRPAGQNSLDPSWITGGGGVQGTLEAT------PHNQVHNNIG--GWM-PTAASPRDPIFFMHHGNIDRIWALWNLKHQNSTDPLW	269
N.europaea	NP_841294	500	GLPTLPTQAAINQVMA-VTPYDTSPWNMNSNPSFRNQLEGW-IG-PNLHNRGHVWVG--GSM-LPMTSPNDPVFFMHHCMVDKIWHEWQLRFPNQGYLPA	232
S.antibioticus	P07524	273	GVSELPTRAEVDSVLA-MATYDMAPWNSGS-DGFRNHLEGW-R-GVNLHNRVHVWVG--GQM-ATGVSPNDPVFWLHHAYIDKLWAEWQRRHPSSPYLPG	238
A.bisporus	CAA59432	568	KSVLKNAQASLTRATYDMFNRVTTWPHFSSHTPASGGSTSN--SIEAIHDNIHVLVGGNGHMSDPSVAPFDPIFFLHHANVDRLIALWSAIRYDVWTSPG	308
C.efficiens	NP_738366	415	AVPTLPTPDEVTACITDLPVYDTDPWHPGSADSFRNQLEGW-PNGPAMHNRVHVWVG--GDM-GPGTSPNDPVFYLHHAFVDLIWARWQQTHAGG-YLPD	257

Fig. 1. Amino acid sequence alignment at the CuA and CuB regions of tyrosinase from *B. megaterium*, with the proteins displaying the highest sequence similarity. Tyrosinase from *Agaricus bisporus* has been included as an important model tyrosinase [Hernàndez-Romero et al., 2006]. Conserved histidine residues directly involved in copper binding are marked in the shaded background. Asterisks (*) denote conserved residues in all sequences. Alignment was generated using CLUSTAL-W software (http://www.ebi.ac.uk/clustalw).

fore, used for species identification. The 16S rDNA sequence obtained from the melanin-producing bacteria revealed high similarity (>99%) with *Bacillus megaterium* species using a BLAST program and the NCBI databases *(*http://www.ncbi.nlm.nih.gov*)*. The identity of the bacteria was also confirmed by biochemical assays. To date, there are several reports on tyrosinase from *B. thuringiensis* strains [Dalfard et al., 2006; Liu et al., 2004; Ruan et al., 2005], and there is a putative tyrosinase gene located on a 208-kb plasmid of *B. cereus* 10987 [Rasko et al., 2004]. There is no description of a tyrosinase from *B. megaterium*, making tyrosinase-VS1 a novel enzyme. Extracellular tyrosinases have been reported previously in bacteria and fungi. For example, some strains of *Streptomyces* have extracellular tyrosinases that are secreted with the assistance of a helper protein [Claus and Decker, 2006]. This protein is also needed for the incorporation of copper and activation of the apotyrosinase. Recently, it was discovered that the filamentous fungus *Trichoderma reesei* has a secreted tyrosinase that is processed by cleavage of a 20-kDa peptide from its C-terminus [Selinheimo et al., 2006]. As the *B. megaterium* tyrosinase was successfully cloned in an active form in *E. coli*, it is assumed that there is no helper protein for this enzyme.

Cloning and Overexpression of the tyr *Gene from* B. megaterium

The putative sequence of *B. megaterium* tyrosinase was disclosed to us by Dr. Jibin Sun from the Helmholtz Centre for Infection Research in Braunschweig, Germany, who is engaged in sequencing the entire *B. megaterium* genome [Sun et al., 2006]. Primers which included the *Nco*I and *Bgl*II restriction sites and a His_6-tag in the C-terminus were designed and used to amplify the gene from the genomic DNA. The PCR product was cloned into the pET9d vector and transformed into *E. coli* BL21 (DE3) cells. The cloned gene was overexpressed efficiently using the T7 RNA polymerase expression system. The new *E. coli* transformants harboring pET9d/tyr exhibited strong intracellular tyrosinase activity indicating the successful cloning of the gene. In addition, sequence analysis confirmed correct insertion of the gene into the plasmid. Alignment of the CuA and CuB regions of the *B. megaterium* tyrosinase with similar tyrosinases is presented in figure 1. *B. megaterium* tyrosinase contains the 6 conserved histidine residues involved in the binding of the copper pair [Hernàndez-Romero et al., 2006; Lopez-Serrano et al., 2002; Matoba et al., 2006]. Furthermore, the typical distance of H-x(8)-H in CuA and H-x(3)-H in CuB [Claus and Decker, 2006; Olivares et al., 2002] was

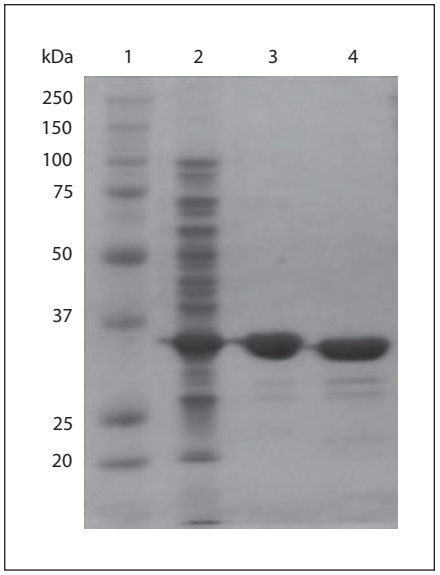


Fig. 2. Electrophoresis of *B. megaterium* tyrosinase under the denaturing conditions of SDS-PAGE (12% acryl amide gel). Gel lane 1: Molecular mass marker; gel lane 2: crude cell extract from *E. coli* BL21(DE3)pET9d/tyr; gel lane 3: His_6-tagged tyrosinase eluted from N_i (II)-bound affinity column; gel lane 4: His_6-tagged tyrosinase eluted from Superdex 200 26/10 gel filtration column. It is estimated that following the N_i (II)-bound affinity column purification step tyrosinase accounts for approximately 95% of protein in the solution.

kept. The closet homologues of the *B. megaterium* tyrosinase are the bacterial *Nitrosomonas europaea* (43% identity) and *B. thuringiensis* (42% identity), while the identity to *Agaricus bisporus* tyrosinase (mushroom tyrosinase) is only 15%.

Purification of Active His_6-tag Tyrosinase

The enzyme was purified in one step using a Ni(II)-bound affinity column. 43 mg of purified enzyme were obtained from 0.5 liter of cell culture, with a 9-fold increase of the specific activity in the preparation. The purified tyrosinase appeared as a single band on SDS-PAGE (fig. 2). The molecular mass of the purified *B. megaterium* tyrosinase was approximately 35 kDa as determined by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis; confirming the calculated value), and 31 kDa as determined by size exclusion chromatography (results not shown). The latter value suggests that the tyrosinase is active in the cell in the form of a monomer. Tyrosinases originating from *Streptomyces nigrifaciens*, *Streptomyces glaucescens*, *P. putida* F6 and *B. thuringiensis* are all monomers, while *Vibrio tyrosinaticus,* and *T. roseum* tyrosinases are dimers [Kong et al., 2000; Liu et al., 2004; McMahon et al., 2007].

The addition of a gel filtration step in the present work did not increase the purity of the protein as evidenced by the SDS gel in figure 2. A combination of the N_i (II)-bound affinity column and gel filtration was used to purify tyrosinase from *T. reesei* and remove dark brown impurities [Westerholm-Parvinen et al., 2007]. The protein content obtained for tyrosinase from *B. megaterium* was high (43 mg from 0.5 liter of culture) compared to *S. castaneoglobisporus* and *T. reesei* tyrosinases (12 mg from 1.25 liters of culture and 24 mg from 1 liter of culture, respectively) suggesting good overexpression and high growth yield. The purification yield was slightly lower than the *S. castaneoglobisporus* procedure (88 vs. 102%) but higher than the *T. reesei* tyrosinase purification procedure (88 vs. 54%, respectively).

pH and Temperature Dependency

The catalytic properties of the purified *B. megaterium* tyrosinase were investigated by following dopachrome appearance at 475 nm, using 1 mM L-DOPA and 1 mM L-tyrosine as substrates. For monophenolase activity, the pH optimum was determined over a range of pH 4.0–10.0; and the pH range for diphenolase activity was 4.0–7.0, as L-DOPA spontaneously converts to dopachrome at pH values above 7.5 (data not shown). The optimum pH of the enzyme was found to be 7.0 for the monophenolase and diphenolase activities, corresponding to that of other bacterial tyrosinases such as *P. putida* F6 (pH 7.0), *Streptomyces* sp. (pH 6.8) [McMahon et al., 2007] and fungal tyrosinase from *Pycnoporus* strains [Halaouli et al., 2005], while the optimum pH values for the diphenolase activities of *B. thuringiensis* and *T. roseum* were 9.0 and 9.5, respectively [Kong et al., 2000; Liu et al., 2004]. In addition, in both enzymatic reactions, the enzyme exhibited less than 50% of its maximum activity below pH 6.0 (data not shown). The effect of temperature on purified *B. megaterium* tyrosinase was determined at temperatures ranging from 10 to 70°C. The thermal stability of the enzyme was determined at 25, 37, 50, 60 and 70°C by incubating the enzyme at the different temperatures for 1 h and determining the residual activity in 50 mM potassium phosphate buffer (pH 7.0) at room temperature (25°C). According to the results presented in figure 3, the optimal temperature for this enzyme is approximately 50°C. The enzyme was fully stable below 50°C, but rapidly lost its activity at 70°C. Thus, *B. megaterium* tyrosinase is quite stable at high temperatures in comparison with tyrosinases from other sources such as *P. putida* F6

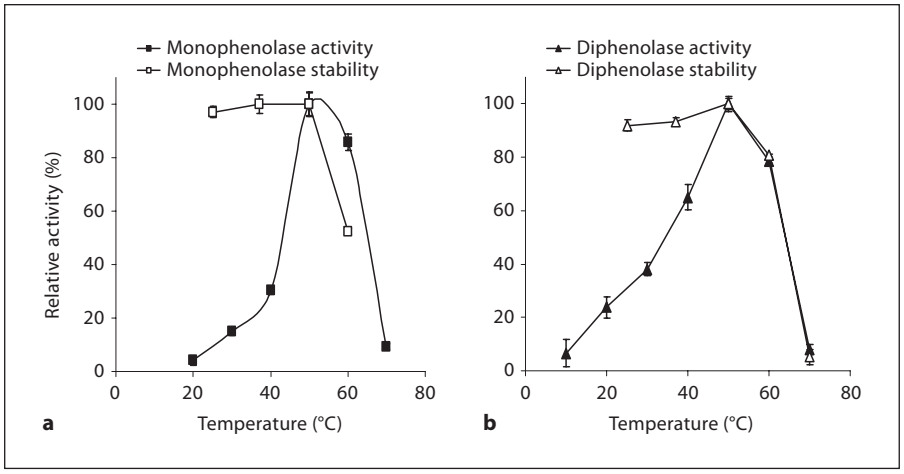


Fig. 3. Effect of temperature on tyrosinase monophenolase activity and stability (**a**) and diphenolase activity and stability (**b**). For diphenolase activity, the reaction medium contained 1.04 μg·ml^{-1} tyrosinase and 2 mM L-DOPA. For monophenolase activity, the reaction medium included 1.04 μg·ml^{-1} tyrosinase and 1 mM L-tyrosine. The temperature was varied from 10 to 68°C at pH 7.0 under the standard reaction conditions. For the thermal stability tests, the enzyme was incubated at different temperatures for 1 h and the residual activity was determined under the standard reaction conditions. The highest activity was defined as 100% (29.3 μmol·min^{-1}·mg^{-1} and 25.4 μmol·min^{-1}·mg^{-1} at 46°C in the activity test and 10.9 μmol·min^{-1}·mg^{-1} and 2.1 μmol·min^{-1}·mg^{-1} at 50°C in the stability test for diphenolase and monophenolase activity, respectively).

Table 1. Kinetic constants of purified tyrosinase from *B. megaterium* in the presence and absence of 30% DMSO

Medium	Substrate	K_m (mM)	V_{max} (μmol·min^{-1}·mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}·M^{-1})	$(k_{cat}/K_m)_L/(k_{cat}/K_m)_D$
Buffer	L-tyrosine	0.075	4.2	2.5	32.9·10^3	
	L-DOPA	0.35	17.2	10.1	28.9·10^3	5.7
	D-DOPA	1.18	10.2	6	5.1·10^3	
Buffer with 30% DMSO	L-DOPA	0.73	36.2	21.3	29.2·10^3	4.1
	D-DOPA	1.7	20.6	12.12	7.1·10^3	

Reaction conditions: an amount of 1.14 μg purified enzyme was assayed in 1 ml of 50 mM potassium phosphate buffer (pH 7.0) containing substrate concentrations ranging from 0.1 to 3.0 mM L- or D-DOPA, or 0.02–2.0 mM for L-tyrosine.

and *T. reesei* (30°C) [Dalfard et al., 2006; McMahon et al., 2007; Selinheimo et al., 2007]. The values obtained are comparable with tyrosinase from *Rhizobium etli*, displaying optimum activity at pH 7.0 and 50°C [Cabrera-Valladares et al., 2006].

Kinetic Constants of B. megaterium *Tyrosinase*

The kinetic parameters of *B. megaterium* tyrosinase were determined for L-tyrosine, L-DOPA and D-DOPA in a buffer and in 30% dimethyl sulfoxide (DMSO; table 1). The enzyme exhibited typical Michaelis-Menten kinetics on L-DOPA and D-DOPA, and substrate inhibition kinetics on L-tyrosine (data not shown). This finding matches recent reports for tyrosinase from the edible mushroom *A. bisporus*, which showed a decrease in the maximum reaction rate with increasing phenol concentrations [Selinheimo et al., 2009]. The K_m value for L-tyrosine is lower than for L-DOPA, while the reaction rate (k_{cat}) is higher for L-DOPA. The enzyme exhibits higher specificity for L-tyrosine as inferred from the k_{cat}/K_m value. This trend was observed for other bacterial tyrosinases such as *R. solanacearum*, *S. castaneoglobisporus*, *B. thuringiensis and P. putida* F6, as well as for fungal tyrosinases such as *T. reesei* [Hernàndez-Romero et al., 2006; McMahon et

Table 2. Substrate specificity of *B. megaterium* tyrosinase

Substrate (2.5 mM)	Relative activity
Monohydroxyphenols	
L-Tyrosine	35.6
D-Tyrosine	20.3
DL-Tyrosine	20.6
p-Coumaric acid	6.1
o-Coumaric acid	<1
p-Hydroxybenzoic acid	<1
Tyramine	2.5
Phenol	<1
3-Aminophenol	3.9
Vanillic acid	0.0
1-Naphthol	0.0
2,6-Dimethoxyphenol	0.0
Dihydroxyphenols	
L-DOPA	100.0
D-DOPA	34.0
DL-DOPA	78.0
Caffeic acid	60.9
(+)-Catechin	60.8
(±)-Catechin	58.7
Catechol	10.3
Chlorogenic acid	20.1
Epicatechin	23.4
Resorcinol	0.0
Trihydroxyphenols	
Pyrogallol	99.9
Phloroglucin	1.6

Values represent percentages. Reaction conditions: *B. megaterium* tyrosinase activity was measured using 1.88 μg enzyme and 2.5 mM of each substrate in 50 mM potassium phosphate buffer (pH 7.0). Relative activity is presented with the activity on L-DOPA set as 100%.

Table 3. Effect of inhibitors on *B. megaterium* tyrosinase

Inhibitor	Concentration mM	Inhibition %
EDTA	1.0	27
	0.1	20
Glutathione	1.0	100
	0.1	42
β-Mercaptoethanol	1.0	100
	0.1	50
Sodium diethyldithiocarbamate	1.0	100
	0.1	60

Reaction conditions: Purified enzyme was incubated with each inhibitor (0.1 or 1 mM) at room temperature (25°C) for 2 min and the activity assay was initiated by the addition of 2.5 mM L-DOPA.

al., 2007; Selinheimo et al., 2006]. The K_m value of *B. megaterium* tyrosinase for L-DOPA (0.35 mM) is similar to the reported values for tyrosinase from *T. roseum* (0.18 mM) [Cabrera-Valladares et al., 2006; Kong et al., 2000] or *P. putida* F6 (0.33 mM) [McMahon et al., 2007], but lower than the value reported for the tyrosinase from *B. thuringiensis* (0.768 mM) [Liu et al., 2004], *S. castaneoglobisporus* (8.1 mM) [Kohashi et al., 2004] or *T. reesei* (3 mM) [Selinheimo et al., 2006]. The K_m value of *B. megaterium* tyrosinase for L-tyrosine (0.075 mM) is similar to tyrosinase from *P. putida* F6 (0.23 mM) [McMahon et al., 2007] and mice (0.09–0.11 mM) [Garcia-Molina et al., 2006; Olivares et al., 2002], but lower than the value reported for tyrosinase from *Vibrio tyrosinaticus* (3.1 mM) [Cabrera-Valladares et al., 2006]. Thus, these results show that tyrosinase from *B. megaterium* displays a K_m value for L-DOPA and L-tyrosine similar to that of enzymes from other organisms. However, the k_{cat} value of *B. megaterium* tyrosinase for L-DOPA (10.1 s^{-1}) is lower than the values reported for other tyrosinases [Garcia-Molina et al., 2007]. Tyrosinase from *A. bisporus* has a k_{cat} value 1 order of magnitude higher (107 s^{-1}), and tyrosinase from *Streptomyces glaucescens* is 2 orders of magnitude more active (1,440 s^{-1}). This may be indicative of steric hindrance in the catalytic site of *B. megaterium* tyrosinase [Olivares et al., 2002].

Substrate Specificity and Inhibitors

Several monophenols, dihydroxyphenols and trihydroxyphenols were used to investigate the substrate specificity of the enzyme (table 2). *B. megaterium* tyrosinase had very low activity towards monohydroxyphenols but substantial activity towards dihydroxyphenols. In addition, L-isomers of DOPA and tyrosine were much better substrates for *B. megaterium* tyrosinase than the corresponding D-isomers (table 2). Very low oxidation of *o*-coumaric acid is in accordance with the literature regarding orthophenolic compounds, which are poor substrates for tyrosinases presumably because of steric hindrance [Selinheimo et al., 2006, 2007]. Low oxidation activity towards catechol is remarkably different from most tyrosinases from other sources such as *S. glaucesens* and *T. roseum* [Kong et al., 2000; Lerch and Ettinger, 1972], but is comparable with reports for *B. thuringiensis* tyrosinase [Liu et al., 2004]. The results may denote differences in the substrate binding pockets of these groups of tyrosinases [Selinheimo et al., 2007].

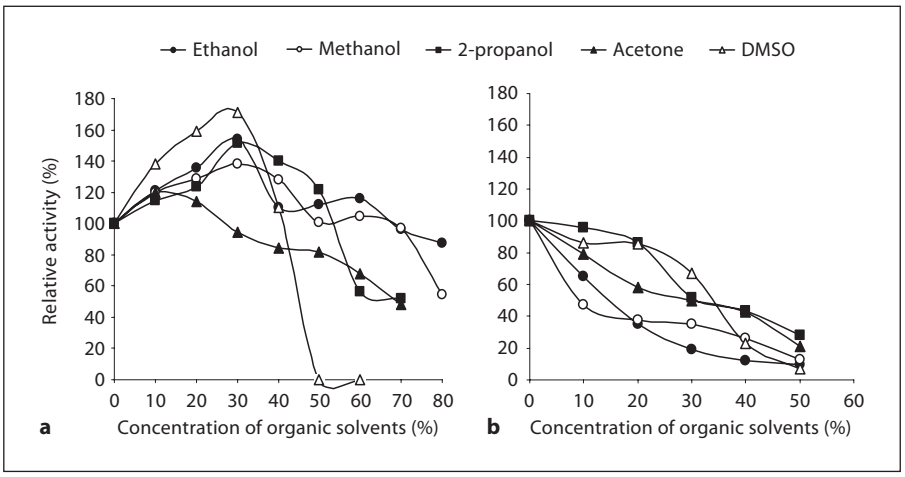


Fig. 4. Effect of organic solvents on the diphenolase activity of *B. megaterium* tyrosinase (**a**) and commercial *A. bisporus* tyrosinase (**b**). The reaction medium contained 1 mM L-DOPA and 1.1 μg·ml-1 *B. megaterium* tyrosinase or 0.5 $\mu g \cdot ml^{-1}$ *A. bisporus* tyrosinase. One hundred percent activity indicates the activity of each enzyme in 50 mM potassium phosphate buffer pH 7.0.

To further characterize the enzyme, various potential inhibitors of *B. megaterium* tyrosinase were examined. The inhibition was determined by measuring the enzymatic activity on 2.5 mM L-DOPA in the presence of the inhibitor at two concentrations (table 3). *B. megaterium* tyrosinase was inhibited strongly by glutathione, β-mercaptoethanol and sodium diethyldithiocarbamate, but EDTA did not inhibit the enzyme very efficiently. Thiol compounds, such as glutathione, inhibit the enzyme and may also affect the subsequent nonenzymatic reactions by reducing the quinones to diphenols (therefore retarding browning). These findings are comparable with other tyrosinases from bacterial, fungal and plant origins [Kong et al., 2000; Selinheimo et al., 2007]. Low inhibition of *B. megaterium* tyrosinase by EDTA is similar to other tyrosinases from other organisms such as *T. reesei* [Selinheimo et al., 2006; Selinheimo et al., 2007].

Effect of SDS on B. megaterium *Tyrosinase Activity*

It has been reported that SDS, an anionic detergent, is an activating agent of the enzyme. Recent experiments have confirmed that the activation is accompanied by an interaction between the detergent and the enzyme, resulting in a conformational change in the enzyme that improves the accessibility of the substrates to the active site without directly affecting its integrity [Gandia-Herrero et al., 2005]. The effect of SDS on *B. megaterium* tyrosinase was studied by incubating the enzyme solution with different concentrations of SDS (0.02–1%) at 50°C for 2 min and initiating the activity assay by adding 1 mM L-DOPA or L-tyrosine. The residual activity was determined relative to L-DOPA or L-tyrosine activity without the SDS addition at 50°C (results not shown). The diphenolase activity was increased by 20% in the presence of 0.02% SDS, while the presence of SDS concentrations above 0.5% inhibited the enzyme. The monophenolase activity was nearly constant in the presence of <0.1% SDS and deteriorated greatly at levels >0.5% similar to the diphenolase activity. Tyrosinases from the South African clawed frog *Xenopus laevis* [Wittenberg and Triplett, 1985] and *A. bisporus* [Espin and Wichers, 1999] have been shown to be activated by detergents. Among bacterial tyrosinases, SDS activation was observed for *Bacillus* sp. tyrosinase [Dalfard et al., 2006] and tyrosinase from *M. mediterranea* [Lopez-Serrano et al., 2002], but was not useful for *B. thuringiensis* tyrosinase [Liu et al., 2004]. Further evaluation of this activation phenomenon on *B. megaterium* tryrosinase is currently being investigated in our lab.

Influence of Organic Solvents on B. megaterium *Tyrosinase Activity, Stability and Enantioselectivity*

The effect of organic solvents on the activity of tyrosinase from *B. megaterium* and mushroom tyrosinase was investigated using 1 mM L-DOPA as a substrate (fig. 4a). *B. megaterium* tyrosinase was activated in the presence of 10–30% of all miscible organic solvents examined. It retained full activity even in the presence of 70% ethanol and methanol. In contrast, mushroom tyrosinase lost its activity quite rapidly in the presence of the same solvents (fig. 4b). For instance, *B. megaterium* tyrosinase had 112 and 101% activity compared to that of the control in the presence of 50% ethanol and methanol, respectively (fig. 4a), while mushroom tyrosinase exhibited only 10 and 13% activity under the same conditions (fig. 4b). The kinetic constants were determined for the system con-

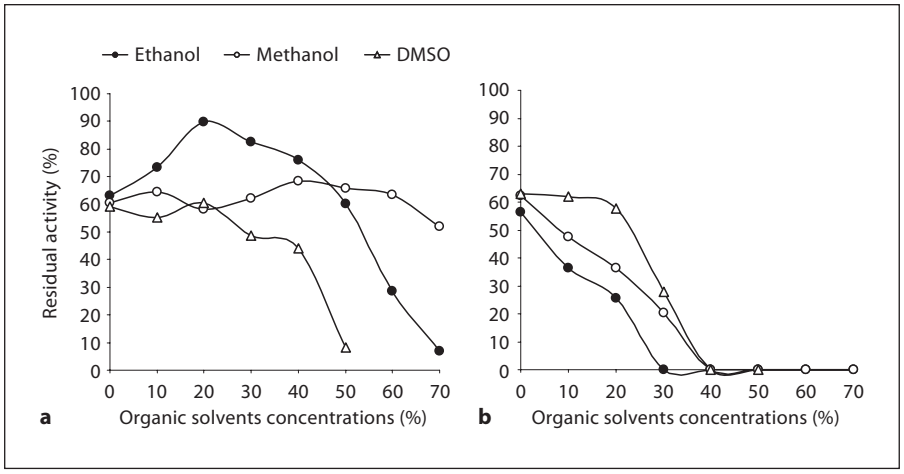


Fig. 5. Stability of *B. megaterium* (**a**) and commercial *A. bisporus* tyrosinase (**b**) in the presence of organic solvents. The reaction medium contained 1 mM L-DOPA and 1.75 μg·ml^{-1} *B. megaterium* tyrosinase or 0.9 μg·ml^{-1} *A. bisporus* tyrosinase, and was incubated at 30°C for 17 h. The residual activity was measured at 25°C. One hundred percent activity indicates the activity of each enzyme before incubation.

taining 30% DMSO in which the activation of tyrosinase was the most pronounced (170% activity; fig. 4). Both the K_m and the k_{cat} values increased 2-fold, thereby the selectivity factor, k_{cat}/K_m, remained unchanged (table 1). In the literature, there is an example of a tyrosinase from *Streptomyces* sp. REN-21 which exhibited high stability in the presence of organic solvents compared with mushroom tyrosinase [Ito and Oda, 2000]. However, only 40–50% of the original activity was found in the presence of 50% ethanol or 30% DMSO, while *B. megaterium* tyrosinase showed 112 and 170% diphenolase activity, respectively.

The effect of organic solvents on the stability of *B. megaterium* and *A. bisporus* tyrosinase was investigated by incubating the enzyme solution at 30°C for 17 h with various concentrations of organic solvents followed by the measurement of the residual activity (fig. 5). Under these conditions, *B. megaterium* tyrosinase was more stable than *A. bisporus* tyrosinase in all of the tested conditions, although the activity in the buffers of both enzymes was reduced by 40% compared to the activity without incubation (fig. 5). For example, *B. megaterium* tyrosinase had 44% activity to that of the nonincubated control in the presence of 40% DMSO (fig. 5a), while *A. bisporus* tyrosinase showed no activity under the same conditions (fig. 5b). Additionally, the enzyme retained its stability at methanol concentrations of 0–70%, which is very unusual for enzymes in general and was not seen with the solvent tolerant tyrosinase from *Streptomyces* sp. REN-21 [Ito and Oda, 2000]. High stability at 66% ethanol was reported for tyrosinase from grapes which lost only 4% diphenolase activity after 2 h of incubation [Valero et al., 1990]. The stability of *B. megaterium* tyrosinase at various temperatures in the presence of 30% DMSO was also examined by incubating the enzyme at 50, 60 and 70°C for 1 h and subsequently determining the residual activity. Under these conditions, *B. megaterium* tyrosinase was stable at 50°C (76% of the activity at buffer without DMSO) and rapidly lost its activity above this temperature (data not shown). Thus, a combination of both 30% organic solvent and a high temperature are still tolerable for the enzyme.

In general, organic solvents can affect the enzymatic activity and specificity via interactions with water, enzymes, substrates and products [Yang and Russell, 1996]. Kermasha et al. [2001] reported that among 100% methanol, acetone, heptanol and chloroform, the latter solvent was the only appropriate medium for commercial mushroom tyrosinase activity with catechin and vanillin as substrates since no activity was detected in the other solvents. In contrast, *B. megaterium* tyrosinase showed 97% and 48% diphenolase activity using L-DOPA as a substrate in the presence of 70% methanol and acetone, respectively. Mushroom tyrosinase exhibited higher activity in pure chloroform compared to an aqueous medium using phenolic substrates such as chlorogenic acid, but it is essential to emphasize the immiscible nature of chloroform [Kermasha and Tse, 2000; Tse et al., 1997]. It is well documented that hydrophobic solvents are usually better than hydrophilic ones, as the latter have a greater tendency to strip tightly bound water from the enzyme molecule [Carrea et al., 1995; Gupta and Roy, 2004; Kilbanov, 2001]. However, our findings indicate that hydrophilic solvents (up to 70% in water) are suitable media for *B. megaterium* tyrosinase activity, supposedly due to its hydrophobic nature [Matoba et al., 2006]. Ogel et al.

[2006] reported that DMSO and ethanol were found to be beneficial for increasing the activity of a crude cell extract containing a phenol hydroxylase from *Scytalidium thermophilum* with activity on catechol, but not on L-tyrosine. The identity of the enzyme was not elucidated; however, these findings show that there are other oxidizing enzymes that exhibit a similar characteristic behavior as tyrosinase from *B. megaterium*.

B. megaterium tyrosinase was found to be stereospecific towards the L-enantiomer of tyrosine and DOPA (table 2), similar to other tyrosinases from fungal and plant sources such as *T. reesei, Pycnoporus sanguineus*, *A. bisporus*, apples and potatos [Kawamura-Konishi et al., 2007; Selinheimo et al., 2006, 2007]. Tyrosinase from *Streptomyces* sp. REN 21 was also highly selective towards L-DOPA and L-tyrosine [Ito and Oda, 2000]. The K_m value of *B. megaterium* tyrosinase for the D-isomer was higher than for the L-isomer (1.18 vs. 0.35 mM, respectively), as was shown previously for tyrosinases from mushrooms, pears and strawberries. This observation is most likely due to the effect of a different spatial orientation of the D-isomer side chain [Espin et al., 1998a, b]. The V_{max} value of *B. megaterium* tyrosinase was higher for L-DOPA than for D-DOPA (17.2 $\mu M \cdot min^{-1} \cdot mg^{-1}$ vs. 10.2 $\mu M \cdot min^{-1} \cdot mg^{-1}$, respectively), presumably due to better orientation and affinity of the more strongly bound isomer in the enzyme-substrate complex resulting in more rapid electron-transfer steps during the catalytic cycle of the enzyme [Casella et al., 1991]. These findings are partly different from mushroom tyrosinase that exhibited lower K_m values for the L-isomer than for D-isomer, but the V_{max} values were comparable for both the L- and D-DOPA [Espin et al., 1998a]. It was suggested that the similar V_{max} values resulted from the fact that the spatial orientation of the ring substituents did not affect the electron donor capacity of the substrates which attack the copper atoms of the enzyme's active site [Espin et al., 1998a, b]. Tyrosinase from *Streptomyces* sp. REN-21 showed a similar trend to *B. megaterium* tyrosinase, i.e. an increased K_m value for D-DOPA accompanied by a decrease in k_{cat} value; however, the decline in selectivity (k_{cat}/K_m) was more dramatic (67-fold for *Streptomyces* tyrosinase vs. 6-fold for *B. megaterium* tyrosinase) [Ito and Oda, 2000].

Our findings also indicate that 30% DMSO influences the enantioselectivity of *B. megaterium* tyrosinase (5.7 in the absence of DMSO vs. 4.1 in the presence of DMSO as determined from the ratios of k_{cat}/K_m for the two enantiomers; table 1). To our knowledge, this is the first report to describe the influence of organic solvents on the enantioselectivity of tyrosinases, although there are numerous reports describing the influence of organic solvents on the selectivity of other enzymes [Carrea et al., 1995; Intra et al., 2005; Kawashiro et al., 1997]. For example, enantioselectivity of α-chymotrypsin in the transesterification of 3-hydroxy-2-phenylpropionate with propanol was changed from pro-S to pro-R simply by switching from 100% of one organic solvent to another [Wescott et al., 1996]. Another example is lipase from *Pseudomonas cepacia* which catalyzed hydrolysis of a prochiral diester and showed an opposite enantiomeric preference in isopropyl ether than in cyclohexane [Berglund, 2001]. Wescott et al. [1996] and Berglund [2001] ascribe the changes in enantioselectivity to the influence of the organic solvent on the substrate desolvation. Additionally, the effect of the organic solvent on enzyme selectivity was also shown to be highly dependent on the nature of both the enzyme and the solvent [Kawashiro et al., 1997]. The tyrosinase examined in the present work did not change its enantioselectivity in the presence of 30% ethanol. The effect of 30% methanol was subtle and was most pronounced in DMSO, indicating that the type of solvent is indeed highly influential as suggested previously [Kawashiro et al., 1997].

The newly isolated and characterized tyrosinase, showing up to 70% activation in the presence of water-miscible organic solvents, is currently being further evaluated for various biotechnological applications.

Experimental Procedures

Chemicals

L-DOPA was purchased from Acros (Geel, Belgium). D-DOPA, DL-DOPA, L-tyrosine, D-tyrosine, DL-tyrosine, *p*-coumaric acid, *p*-hydroxybenzoic acid, vanillic acid, 2,6-dimethoxyphenol, (+)-catechin, (±)-catechin, catechol, chlorogenic acid, epicatechin, resorcinol, phloroglucin, EDTA, glutathione, sodium diethyldithiocarbamate, kanamycin, and SDS were purchased from Sigma-Aldrich (Rehovot, Israel). *o*-Coumaric acid, phenol, 1-naphthol, caffeic acid, DMSO and pyrogallol were purchased from Merck (Whitehouse Station, N.J., USA). β-Mercaptoethanol was purchased from Spectrum (Gardena, Calif., USA). Methanol and ethanol were purchased from Bio Labs (Jerusalem, Israel). Acetone was purchased from Frutarom (Haifa, Israel). 2-Propanol was purchased from J.T. Baker (Deventer, The Netherlands). All materials used were of the highest purity available and were used without further purification.

Strains

B. megaterium was isolated from soil and stored in our lab, as described below. *E. coli* BL21 (DE3; Novagen, Darmstadt, Germany) was used as the host for plasmid pET9d. *E. coli* transformants were grown in a Luria-Bertani medium containing 0.025

mg/ml kanamycin. *A. bisporus* tyrosinase (mushroom tyrosinase) was purchased from Sigma-Aldrich (Rehovot, Israel, cat. No. T3824).

Isolation of a Tyrosinase-Expressing Bacterial Strain

Soil samples were collected from 10 different areas in Israel. 0.2 g of soil was dissolved in a 10-ml saline solution and a 1-ml solution was added to 25 ml of rich medium known to favor bacterial growth over fungal growth (tryptone 1%, glucose 0.2% and NaCl 0.5%). The flasks were incubated at 30°C overnight and serial diluted samples were streaked on MMB selection plates containing (% w/v): KH_2PO_4 (0.0351%), $MgSO_4 \cdot 7H_2O$ (0.015%), $(NH_4)_2SO_4$ (0.2%), glucose (0.2%), agar (1.5%) and 50 mM Tris buffer (pH 7.0). After sterilization, L-tyrosine (0.1%) and a trace elements solution (0.3%) were added to the medium. The trace elements solution contained (% w/v): $CuSO_4 \cdot 5H_2O$ (0.02%), $FeSO_4 \cdot 7H_2O$ (0.2%), $MnSO_4 \cdot H_2O$ (0.015%), $ZnSO_4 \cdot 7H_2O$ (0.014%), $CoCl_2 \cdot 6H_2O$ (0.026%), $NaMoO_4 \cdot 2H_2O$ (0.023%) and $CaCl_2 \cdot 2H_2O$ (0.061%). The plates were incubated at 30°C for 5–6 days. Melanin production was recorded by the appearance of black or black-brown color colonies. The selected strains were isolated and streaked again on L-tyrosine-containing MMB plates and on similar plates without tyrosine (negative control). The isolate which formed black colonies on tyrosine-plates and white colonies on plates without tyrosine was grown in a MMB liquid medium supplemented with 0.2 mM $CuSO_4$ at 30°C for 4 days. The broth was assayed for extracellular tyrosinase activity.

PCR Amplification and 16S rDNA Sequencing and Identification of the Bacterial Strain

Genomic DNA was extracted from the isolated bacteria using standard methods [Sambrook and Russell, 2001]. Universal 16S rDNA PCR forward primer (5′-GAGAGTTTGATCCTGGCTCAG-3′) and reverse primer (5′-CTACGGCTACCTTGTTGTTACGA-3′) were used in the amplification of 16S rDNA genes [Dalfard et al., 2006]. A DNA thermal cycler (*Apollo AC401; CLP,* San Diego, Calif., USA) was used and programmed as follows: (1) the 1st cycle consisting of 94°C for 5 min, 50°C for 2 min and 72°C for 3 min; (2) a run of 28 cycles with each cycle consisting of 45 s at 94°C, 90 s at 50°C and 3 min at 72°C; and (3) the last cycle consisting of 45 s at 94°C and 90 s at 50°C, followed by incubation at 72°C for 10 min. The amplification products were purified with a PCR clean-up kit (RBC, Taipei, Taiwan) and DNA sequencing was performed using the dideoxy chain termination technique (Multidisciplinary Laboratories, Technion, Haifa, Israel).

The obtained 16S rDNA sequence was deposited in GenBank under accession No. EU627691 for isolate VS1.

Biochemical characterization of the new strain was performed by Milouda Biological Industries Services (Akko, Israel).

Analytical Methods

Conversion of L-tyrosine to L-DOPA was determined by high performance liquid chromatography with an Agilent 1100-series instrument (Agilent Technologies, Santa Clara, Calif., USA) using an Eclipse XDB C18 column (5 μm, 4.6 × 150 mm; Agilent Technologies, Santa Clara, Calif., USA). An isocratic method comprising acetonitrile/water (0.1% formic acid) at a ratio of 2/98, respectively, was applied at 1 $ml \cdot min^{-1}$ for 13 min. A diode array detector was used at a fixed wavelength of 275 nm to monitor the reaction. Twenty microliters of filtered samples were injected into the column and under these conditions; L-DOPA eluted at 4.8 min and L-tyrosine at 7 min.

Cloning and Overexpression of the tyr *Gene from* Bacillus megaterium

The genomic DNA was extracted from *B. megaterium* by standard methods [Sambrook and Russell, 2001] and used as a template to amplify the *tyr* gene by PCR amplification. The putative sequence of *B. megaterium* tyrosinase was disclosed to us by Dr. Jibin Sun from the Helmholtz Centre for Infection Research and a pair of primers were designed to amplify the *tyr* gene containing a C-terminal His6-tag. The forward primer (*Nco*I front) containing the *Nco*I restriction site (underlined) was 5′-GAGGTTAAAC<u>CATGG</u>GTAACAAGTATAGAG TTAGAAAAAACG-3′. The reverse primer containing the 6-His and BglII restriction site (underlined) was 5′-CTGCTGTTTCT<u>AGATCT</u>GGTTAATGGTGGTGATGGTGATGTGAGGAACGTTTTGATTTTC-3. Primer *Nco*l front generates an *Nco*I restriction site within the *tyr* gene by changing the 2nd codon AGT to GGT, creating a serine to glycine change at the 2nd position in the translated product of the modified *tyr* gene. The PCR reaction was performed with BioTaq™ DNA polymerase (Bioline, London, UK) and carried out in a 200-μl reaction using a DNA thermal cycler (Apollo AC401; CLP, San Diego, Calif., USA). The PCR program had an initial denaturation step of 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 45 s at 52°C and 2 min at 72°C. This was followed by a final elongation step of 8 min at 72°C. The PCR product was purified using a PCR purification kit (Genomed GmbH, Löhne, Germany). The PCR product and the vector pET9d (Novagen, Darmstadt, Germany) were digested with *Nco*I and *Bgl*II, or with *Nco*I and *Bam*H I (Takara, Otsu, Japan), respectively, and then were ligated with T4 DNA ligase (Promega, Madison, Wisc., USA). The ligated plasmid pET9d/tyr was transformed into competent *E. coli* BL21 (DE3) using GeneZapper (Bio-Rad, Hercules, Calif., USA).

Purification of Active His_6-tag Tyrosinase

The His_6-tagged tyrosinase was purified in one step using an Ni(II)-bound affinity column (HisTrap HP; Amersham Biosciences, Giles, UK). *E. coli* BL21 (DE3) cells harboring pET9d/tyr were grown overnight at 37°C in 0.5 liter of TB medium (tryptone 1.2%, yeast extract 2.4%, glycerol 0.4% and potassium phosphate buffer 89 mM). The cells were harvested by centrifugation (8,000 *g* for 10 min at room temperature) and yielded 15.6 g of wet pellets, suspended in a binding buffer (20 mM sodium phosphate buffer, pH 7.5, 500 mM NaCl; and 20 mM imidazole) and then treated twice in a pressure cell press (French Press, Spectronic Instruments Inc., Rochester, N.Y., USA). The cell debris was removed by centrifugation (16,000 *g* for 20 min at 15°C), whereas the supernatant was applied to the Ni(II)-bound affinity column (previously charged with a Ni ion and equilibrated with a binding buffer).

Elution was performed with an appropriate buffer (20 mM sodium phosphate buffer, pH 7.5; 500 mM NaCl; and 500 mM imidazole). The fractions containing tyrosinase were collected and dialyzed against a phosphate buffer at 4°C (50 mM sodium phosphate buffer, pH 6.5, 0.02% sodium azide, 0.01 mM $CuSO_4$). Enzyme solutions were stored at –20°C without loss of activity.

Gel Filtration Analysis

Gel filtration analysis was performed using a Superdex 200 26/10 column (Pharmacia, Giles, UK). β-Amylase (200 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12 kDa; Sigma-Aldrich, Rehovot, Israel) were used as marker proteins for determining molecular mass.

Tyrosinase Activity Assay

Tyrosinase activity was assayed using L-DOPA and L-tyrosine as substrates [Kohashi et al., 2004]. An aliquot of the purified enzyme solution or liquid broth containing an extracellular enzyme was added to 50 mM potassium phosphate buffer pH 6.5 containing 2 or 1 mM L-DOPA or L-tyrosine and the formation of L-dopachrome ($\varepsilon = 3600$) was monitored by measuring the absorbance at 475 nm (Ultrospec 2100pro; GE Healthcare, Giles, UK). The total volume of the reaction mixture was 1 ml. Control experiments without an enzyme were performed with all the substrates. The rate of dopachrome formation was defined as the slope of the linear zone of absorbance versus the time plot. Specific activity was calculated as the ratio of the conversion rate and the total protein content was determined by the Bradford analysis method, with bovine serum albumin as a calibration standard (Bio-Rad Protein Assay Kit). All measurements were carried out in triplicate.

Influence of pH and Temperature on Tyrosinase Activity, Substrate Specificity, Inhibitor Studies and SDS Effect

Unless otherwise stated, activity was measured at room temperature (25°C) by using the colorimetric assay described above. The reaction medium (1.0 ml) contained 50 mM potassium phosphate buffer pH 7.0 and 1.88 $\mu g \cdot ml^{-1}$ of the enzyme with 1 mM L-tyrosine or 1 mM L-DOPA for monophenolase and diphenolase activities, respectively. Other conditions and reagents are detailed in the text and figure legends. All experiments were performed in triplicate.

The enzymatic activity at various pH values was determined by using the following buffers at 50 mM: acetic acid-sodium acetate (pH 4.0–5.0), potassium phosphate (pH 6.0–7.0) and glycine-NaOH (pH 8.0–10.0). For monophenolase activity, the reaction medium contained 2.08 $\mu g \cdot ml^{-1}$ tyrosinase and the pH optimum was determined over a range of pH 4.0–10.0, while for diphenolase activity, the reaction medium included 1.04 $\mu g \cdot ml^{-1}$ tyrosinase and the pH range was 4.0–7.0, due to spontaneous oxidation of the diphenol at alkaline pH.

Kinetic Analyses

The values of K_M and V_{max} for the tyrosinase were determined using the colorimetric assay described above (1 ml of final volume of 50 mM potassium phosphate buffer pH 7.0) employing 1.14 μg of a purified enzyme with increasing substrate concentrations ranging from 0.1–3.0 mM for L-DOPA and D-DOPA, or 0.02–2.00 mM for L-tyrosine. Measurements were performed in tetraplicate. The Michaelis-Menten curves for determination of the kinetic constants were obtained with Sigmaplot 11.0 (Systat Software Inc., San Jose, Calif., USA).

Influence of Organic Solvents on Tyrosinase Activity, Stability and Enantioselectivity

Determination of the organic solvents' influence on *B. megaterium* tyrosinase activity was carried out using the colorimetric assay described above. Ethanol, methanol, 2-propanol, acetone and DMSO were added directly to the 1-ml reaction cuvette. Final organic solvent concentrations ranged from 0 to 80% for ethanol, methanol and 2-propanol; 0–70% for acetone, and 0–60% for DMSO. The activity on L-DOPA or D-DOPA in buffer without the presence of organic solvents was defined as 100%. To observe the possibility of spontaneous oxidation of L-DOPA, control experiments without an enzyme were performed with all the organic solvents. All assays were performed in tetraplicate.

For stability tests, the enzyme solution was kept at 30°C for 17 h with various concentrations of organic solvents. Final organic solvent concentrations ranged from 0 to 70% for ethanol and methanol, and 0–50% for DMSO. Reactions were measured at room temperature (25°C) using 96-well plates (final volume 200 μl) using a multiplate reader (OPTImax tunable microplate reader; Molecular Devices, Sunnyvale, Calif., USA). The activity on L-DOPA in buffer or organic solvents before incubation was defined as 100%. All assays were performed in triplicate.

Acknowledgements

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