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ORIGINAL ARTICLE

Characterization of a thermotolerant laccase produced by *Streptomyces* sp. SB086

Tatiana Alves Rigamonte Fernandes · Wendel Batista da Silveira · Flávia Maria Lopes Passos · Tiago Domingues Zucchi

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Abstract Laccases have become desirable enzymes for application in many industrial processes. Nowadays, most of these enzymes are obtained from fungi. Among prospective studies for bacterial laccase genes, some have included actinomycetes, but only a few studies have characterized the enzyme produced. Thus, we have isolated a laccase-producing actinomycete from forest soil under restoration process and further aimed to characterize its produced enzyme. The isolate SB086 was assigned to the *Streptomyces* genus by a combination of phenotypical, chemical and phylogenetic properties. Our data indicate that the bacterium produces a thermotolerant laccase. The maximum activity was obtained in the pH range 4.0–5.0 and at 50 °C in reaction mixture containing 5 mM $CuSO_4$; thermal stability was noted at 60 °C and 70 °C—a well-desired characteristic for industry. The active enzyme presented a high molecular mass (over 100 kDa) and was less sensitive to inhibition by metal ions than generally described for bacterial laccases. Our findings support in silico data of bacterial laccase secretion, and reinforce the view that actinomycetes may be a rich source of laccase for industrial application.

Keywords Actinobacteria · Laccase · Lignin degradation · Multicopper oxidase

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T. A. R. Fernandes · T. D. Zucchi (✉)
Laboratório de Microbiologia Ambiental, Embrapa Meio Ambiente, Rodovia SP 340, Km 127,5, CP 69, Jaguariúna, SP 13820-000, Brazil
e-mail: tiago_zucchi@hotmail.com

T. A. R. Fernandes · W. B. da Silveira · F. M. L. Passos
Departamento de Microbiologia, BIOAGRO, Universidade Federal de Viçosa, Av. PH Rolfs, s/n, Campus Universitário, Viçosa, MG 36571-000, Brazil

Introduction

The search for alternative energy supplies has received increasing efforts recently, and bioethanol production has been highlighted as a promising option, mainly because this fuel is obtained from microbial fermentation of an abundant renewable source: lignocellulose biomass. For fermentation to occur, the complex structure of the polymer has to be degraded by biological pre-treatment with a complex of enzymes, in which laccases play a crucial role (Hatakka 1994). Laccases (EC 1.10.3.2) are multicopper oxidases capable of taking electrons from a broad range of organic and inorganic compounds, even non-aromatic ones, concomitantly with molecular oxygen reduction, producing water (Claus 2004). The broad substrate range of laccase makes the enzyme attractive for industrial application in numerous fields besides energy supply, e.g., in the pulp and paper industry for pulp delignification; in the food industry for elimination of undesirable phenolic components in fruit juices, beer and wine; in the cosmetics and pharmaceutical industry for polymers, hair dyes and drug production; and also in bioremediation, for bleaching of textile effluents, phenols removal from residuary water of factories, pesticide degradation and decontamination of aromatic polycyclic aromatic hydrocarbons (PAHs) from soil (Schroeder et al. 2008; Piscitelli et al. 2010).

The physiological roles of microbial laccases are known to involve a variety of processes, such as endospore pigmentation, morphogenesis, stress response and copper homeostasis (Martins et al. 2002; Sharma et al. 2007; Piscitelli et al. 2010). The molecular structure of the enzyme generally presents four domains in the active site, containing three types of copper centers: type 1, which confers blue color to the purified enzyme and catalyzes the removal of electrons from substrate; and types 2 and 3, which form a group that activates molecular oxygen (Claus 2004). Furthermore, the nucleotide sequence conservation of the domains makes possible to design specific

primers for taxonomic groups (Luis et al. 2004; Kellner et al. 2008; Ausec et al. 2011).

Since the discovery of laccase in 1883, it was thought for a century that only plants and fungi synthesized this enzyme. Although it is nowadays known that bacteria are also producers, most laccases studied recently are still obtained from fungi (Piscitelli et al. 2010). It is possible that this bias is due to the rooted idea that bacterial laccases occur mainly intracellularly or bound to spores (Sharma et al. 2007); this retention of the enzyme would, a priori, make bacterial laccases less suitable for industrial applications. However, this idea has long been contested, e.g., since Alexandre and Zhulin (2000) reported the presence of signal peptides in bacterial laccase sequences. More recently, it has been revealed that 76 % of 1,200 putative genes for laccase-like enzymes possess signal peptides (Ausec et al. 2011), indicating an extracellular destination and corroborating earlier studies.

Among prospective studies for bacterial laccase genes, some have included actinomycetes, e.g., *Streptomyces griseus* (Endo et al. 2002), *S. lavendulae* (Suzuki et al. 2003) and *S. cyaneus* (Arias et al. 2003), but only a few proceed to enzyme characterization. Besides *Streptomyces*, hypothetical laccase-like genes can be found in over 20 actinomycetes genera in the NCBI (National Center for Biotechnology Information) database. Since *Actinomycetales* has been recognized as a producer of unique biomolecules, with the predicted proteins varying enormously in chain length, this group has also been considered as a reliable source for prospecting for enzymes with new biochemical properties. Therefore, our main aim was to characterize laccase produced by an actinomycete isolated from soil in order to investigate its potential for industrial applications.

Materials and methods

Isolation, selection and maintenance of laccase-positive actinobacteria

Actinobacteria isolates were recovered from humic acid agar plates (HVA; Hayakawa and Nonumura 1987) that had been inoculated with a suspension of reforestation soil collected in the city of Santa Bárbara d'Oeste, State of São Paulo, Brazil, and incubated at 28 °C for 21 days. The isolates were maintained on oatmeal agar plates (ISP medium 3; Shirling and Gottlieb 1966), and glucose-yeast extract agar plates (GYEA; Gordon and Mihm 1962) at 28 °C. Mycelial fragments were maintained in glycerol solution (20 % v/v) at −80 °C. Laccase production was screened by inoculating the isolates on oatmeal agar amended with 0.01 % guaiacol, which acquires a reddish-brown coloration after oxidative polymerization by laccase (Coll et al. 1993). The positive isolates were pre-inoculated in liquid GY medium for 3 days at 28 °C. The pre-inoculum (1 mL) was then used to inoculate basal mineral medium supplemented with 0.025 % vitamin-free casaminoacids (Sigma, St. Louis, MO) (Crawford 1978) and 0.5 % wheat bran. After 4 days incubation at 28 °C, the culture was centrifuged (15,000 *g*; 10 min at 4 °C) and the supernatant was tested for laccase activity.

Enzyme assay

Laccase activity was further assayed by determining the oxidation of 5 mM ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; Sigma-Aldrich] in 0.1 M McIlvaine buffer (pH 5.0). The increase in absorbance at 436 nm was monitored with a Shimadzu UV-1601 PC spectrophotometer, and the temperature of the reaction mixture was controlled in water bath. Laccase activity in the crude or partially purified extracts was assessed in a 990 μL reaction mixture containing 5 mM ABTS as a substrate, 330 μL of 0.1 M McIlvaine buffer, and 330 μL enzyme extract. The progress of the reaction was monitored at 436 nm for 20 min. The activity was expressed as units of activity (1 U laccase activity was defined as 1 U change in the absorbance reading at 436 nm per 1 min). An initial assay at 30 °C was conducted with positive results from guaiacol test; the supernatant from the previously selected isolate, namely SB086, was chosen for enzyme characterization.

Characterization of laccase

The best conditions for laccase activity were estimated by assaying the culture supernatant within the temperature range 20–70 °C and pH range 3–8 with 10 mM McIlvaine buffer. Thermal stability was estimated by pre-treating the reaction mixture at 60 °C or 70 °C for intervals from 20 to 180 min, then transferring to a 50 °C bath for the reads of ABTS oxidation. The effects of metal ions ($CuSO_4$, $MnSO_4$, $MgSO_4$, $ZnSO_4$, $CaCl_2$, $FeSO_4$ and K_2SO_4) at concentrations ranging from 0 to 20 mM were also determined, with 0.1 M McIlvaine buffer (at 50 °C, optimum pH 4.0).

Purification with acetone gradient and molecular weight estimation

Different volumes of cold acetone (−20 °C), providing the saturation ranges of 0–20 %, 20–40 %, 40–60 %, and 60–80 % acetone were added to the culture supernatant for sequential protein precipitation. At each acetone addition, samples were homogenized by inversion, incubated at 4 °C for 5 min, and the protein was harvested by centrifugation (13,000 *g*, 15 min, 4 °C). The supernatant was transferred to a new tube for the next acetone addition, and the pellet was completely dried, at room temperature. The pellet was, then, resuspended in distilled water to the initial sample volume.

For laccase molecular weight estimation, Amicon® Ultra Centrifugal Filter Devices (30, 50 and 100 kDa) for the Concentration and Purification of Biological Samples (Millipore, Bedford, PA) were used following manufacturer's protocol.

Taxonomy analysis of isolate SB086

Phylogeny

Genomic DNA was extracted from the selected SB086 isolate using PureLink™ Genomic DNA kit (Invitrogen) following the manufacturer's protocol. PCR amplification and 16S rRNA gene sequencing were achieved following the methods of Zucchi et al. (2011). The almost complete 16S rRNA gene sequence [1,371 nucleotides (nt)] was aligned manually using MEGA version 5 software (Tamura et al. 2011) against corresponding sequences of closely related type strains of *Streptomyces* species retrieved from the GenBank database using the EzTaxon-e server (Kim et al. 2012). Phylogenetic trees and bootstrap analysis were inferred following the procedures described by Zucchi et al. (2013). The root position of the neighbor-joining tree was inferred by using *Streptomyces albus* subsp. *albus* (GenBank accession no. J6216022) and *Streptacidiphilus albus* (GenBank accession no. AF074415) as outgroups.

Chemotaxonomy

Isolate SB086 was examined to establish whether it had a chemotaxonomic profile typical of the actinobacteria genus addressed by the phylogenetic analysis. Cell mass for fatty acid analysis was harvested from Trypticase Soy Broth (TSB; Difco, BD, Franklin Lakes, NJ) which had been incubated at 28 °C for 3 days. Fatty acids of the isolate were methylated, determined by gas chromatography (Hewlett Packard 6890) and analyzed using the standard Sherlock Microbial Identification (MIDI) system and the TSB version 6 database (Sasser 1990). The DNA base composition of the isolate was determined following the methods of Gonzalez and Saiz-Jimenez (2002).

Cultural and morphological properties

Cultural properties were determined using standard ISP medium (Shirling and Gottlieb 1966) after incubation of plates at 28 °C for 14 days. Isolate SB086 was examined for phenotypic properties known to be valuable in actinomycete systematics, and additional tests (pH and NaCl) were carried out on the isolate using GYEA as the basal medium, as described by Williams et al. (1983).

Results

Selection of laccase producer actinobacteria

A total of 25 strains isolated from forest soils under restoration process presented morphological features typical to members of actinomycetes. Only four isolates demonstrated the ability to oxidize guaiacol on oatmeal agar. Some of them, such as isolate SB086, naturally pigmented the growth medium (data not shown) making the detection of laccase production more difficult. All positive and doubtful positive isolates were further evaluated for laccase activity using ABTS as substrate.

Laccase characterization in crude extract

Isolate SB086 supernatant provided the most intense ABTS oxidation, indicating the highest laccase activity among the isolates (data not shown); therefore, this isolate was chosen for further laccase characterization. The laccase produced by SB086 was active between pH 4.0 and 7.0 (Fig. 1a) and was completely inactivated at pH 3.0 and 8.0. The optimum activity for this enzyme was observed both at pH 4.0 and 5.0, and enzyme activity decreased to 60 % at pH 6.0. The optimum temperature for SB086 laccase was observed at 50 °C (Fig. 1b). At lower (45 °C) and higher (55 °C) temperatures, the enzyme activity was reduced to around 75 %. At the highest temperature assayed, 70 °C, 14 % of activity was still detected. Considering the optimum of 50 °C, the thermal stability was evaluated at higher temperatures: 60 °C and 70 °C. The enzyme retained about 50 % of its activity after 20 min of pre-treatment at 60 °C or 70 °C, and about 15 % after 180 min at 60 °C or 60 min at 70 °C (Table 1).

Laccase activity from SB086 was enhanced in the presence of the metals Cu, Ca, K, Mg, Mn and Zn, and inhibited by Fe. The highest effect on laccase activity was observed at 5 mM $CuSO_4$, which triggered a relative activity of 212.57 %. $MgSO_4$ and K_2SO_4 progressively enhanced enzyme activity, producing 186.39 % and 133.51 % of relative activity, respectively, at 20 mM. $ZnSO_4$ at 5 or 10 mM induced around 159 % of relative activity. Laccase activity was also enhanced with 1 mM $CaCl_2$ or $MnSO_4$, less enhanced with 5 mM $MnSO_4$ and inhibited by 5 mM $CaCl_2$. $FeSO_4$ intensely inhibited laccase activity even at the lowest tested concentration (Table 2).

Purification with acetone gradient and molecular weight estimation

After precipitation with cold acetone, laccase activity was observed only in the 60 % acetone saturation fraction. Laccase size was estimated to be above 100 KDa with Amicon® Ultra Centrifugal Filter Devices.

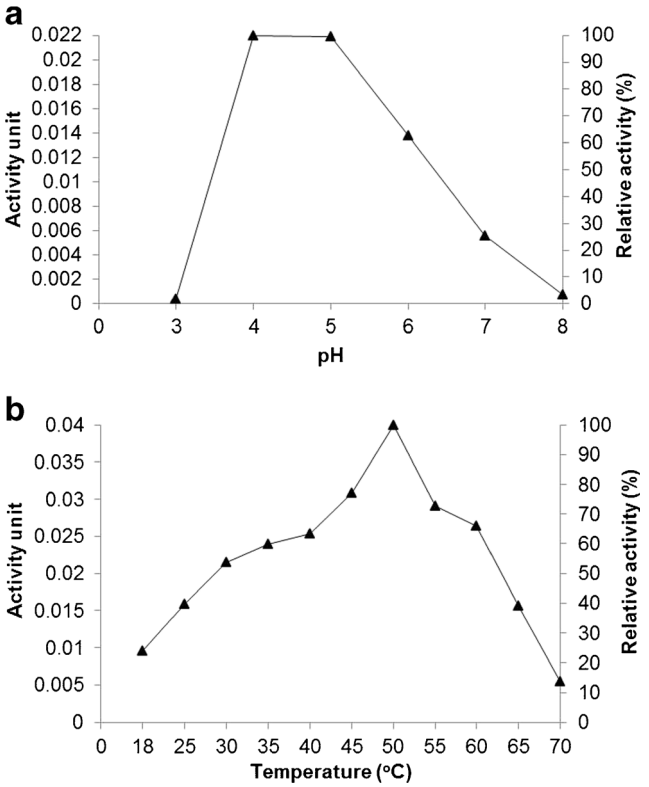

Fig. 1 Effect of **a** pH and **b** temperature on laccase activity from crude extract of *Streptomyces* sp. SB086, with McIlvaine buffer 0.1 M. Optimum pH was estimated at 50 °C, and optimum temperature was estimated at pH 4.0

Characterization of isolate SB086

Isolate SB086 forms a distinct phyletic line that is associated with the *Streptomyces xanthophaeus* 16S rRNA subclade—a result supported by all of the tree-making algorithms and by a bootstrap value of 58 % (Fig. 2). The organism shares a 16S rRNA gene similarity of 99.3 % with the type strains of *Streptomyces xanthophaeus*, *Streptomyces spororaveus*, *Streptomyces avidinii*, *Streptomyces nojiriensis* and *Streptomyces subrutilus*, a value that corresponds to 9 nt difference out of 1,373 sites. The isolate was found to contain major amounts of anteiso-$C_{15:0}$ (23.34 %), iso-$C_{16:0}$ (15.65 %), $C_{16:0}$ (14.07 %) and iso-$C_{15:0}$ (12.33 %) (Online Resource, Table 1). The G+C contents of the DNA preparations of isolate SB086 was determined to be 69.8 %. All these characteristics are consistent with the classification of the strain in the genus *Streptomyces* (Williams et al. 1983; Manfio et al. 1995; Anderson and Wellington 2001; Kämpfer 2012).

Isolate SB086 was observed to form an extensively branched brownish substrate mycelium that bore an abundant light pink aerial mycelium on oatmeal agar. With a single exception (ISP 7), the isolate was observed to grow well on ISP and GYEA media (Online Resource, Table 2). Isolate SB086 can be distinguished readily from its nearest phylogenetic neighbors using a broad range of phenotypic properties, notably by its ability to grow in a broader range of different sugars (Table 3). Additional phenotypic properties of *Streptomyces* sp. SB086 are presented in Online Resource, Table 3.

Table 1 Thermal stability of laccase against pre-treatments at 60 and 70 °C

Time (min)	Relative activity (%)	
	Pre-treatment at 60 °C	Pre-treatment at 70 °C
0	100	100
20	46.4	46.7
30	44.4	20.6
60	26.4	14.3
90	28.7	nt
120	nt[a]	1.6
180	15.7	nt

[a] Not tested

Table 2 Effect of metal ions on laccase activity. Values represent the relative activity (activity of the control, without any metal ion added to the reaction mixture, was taken as 100 %) and are averages of two repetitions

Metal ion	1 mM	5 mM	10 mM	20 mM
$CuSO_4$	130.4	212.6	12.57	nt[a]
$CaCl_2$	124.1	86.4	nt	nt
K_2SO_4	97.4	103.1	125.1	133.5
$MgSO_4$	111.5	122	178	186.4
$MnSO_4$	138.2	112.6	nt	nt
$ZnSO_4$	108.4	158.1	159.7	nt
$FeSO_4$	8.4	8.9	nt	nt

[a] Not tested

Discussion

Bacterial isolates with known ability to degrade lignin belong to three classes: *Actinobacteria*, *Alphaproteobacteria*, and *Gammaproteobacteria*, including the isolates obtained from guts of termites and wood-boring beetles (Bugg et al. 2011). This information reinforces the potential of bacteria as a source of novel laccase enzymes, and also strengthens the need for extending knowledge in this research field. In this work, we focused on *Actinobacteria*, a group with still a small number of characterized laccases, and recognized for being a copious producer of unique biomolecules.

The wide range of substrates of laccase makes this enzyme attractive for many biotechnological applications in industry. In order to be adequate for industrial purposes, one of the desirable properties of enzymes is thermotolerance.

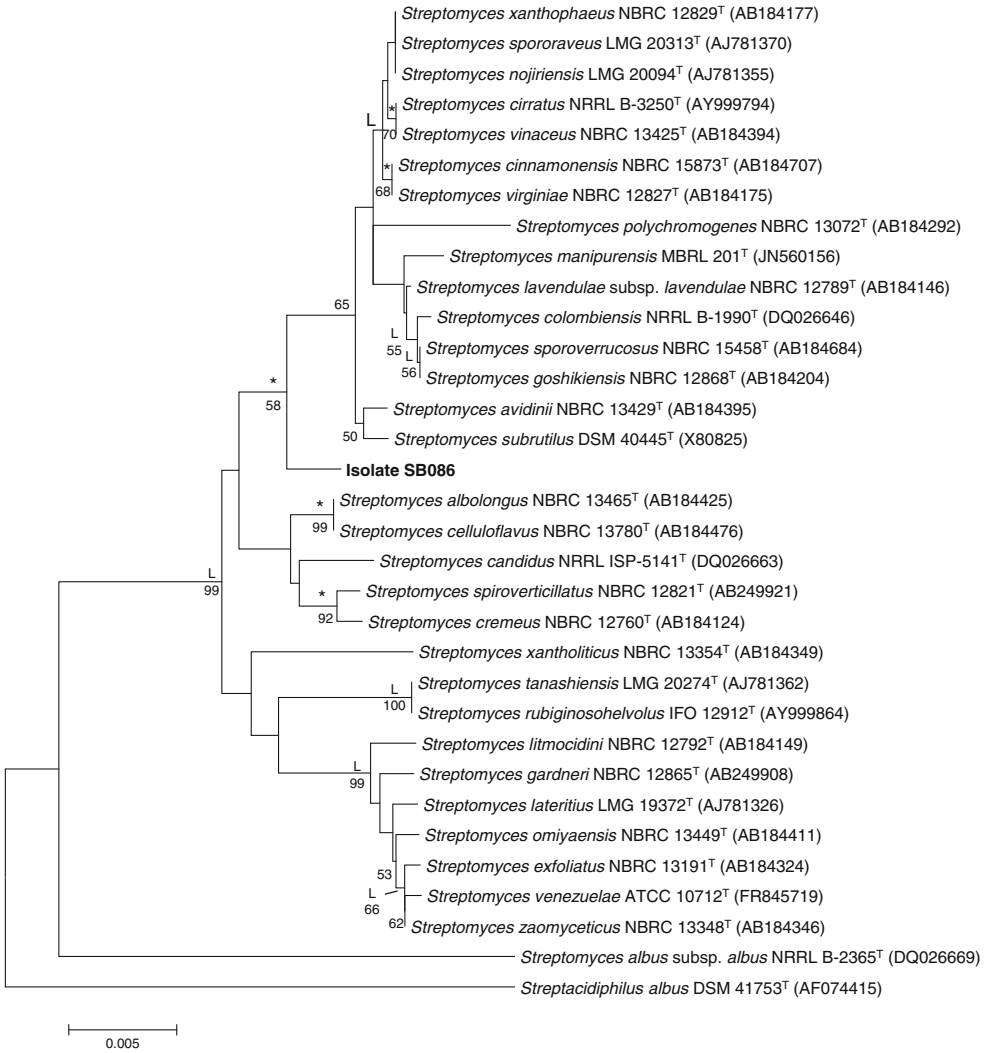

Fig. 2 Neighbor-joining tree based on nearly-complete 16S rRNA gene sequences (~1,400 bp) showing relationships between isolate SB086 and the type strains of phylogenetically close *Streptomyces* species. *Asterisks* indicate branches of the tree that were recovered with the maximum-likelihood and maximum-parsimony tree-making algorithms; *L* and *P* stand for branches that were also recovered using the maximum-likelihood or maximum-parsimony tree-making algorithms, respectively. *Numbers* at the nodes are percentage bootstrap values based on a neighbor-joining analysis of 1,000 resampled datasets; only values above 50 % are given. *Bar* 0.005 substitutions per nucleotide position

Streptomyces sp. SB086 laccase presented an optimum temperature of 50 °C, close to the average for microbial laccases of 55 °C (Strong and Claus 2011). The same 50 °C optimum was observed for the fungal *Pycnoporus coccineus* laccase (Suzuki et al. 2003), but its half-life at 70 °C was 100 min, while SB086 laccase half-life at 60 °C or 70 °C (pre-incubations) was about 20 min. In contrast, the most studied bacterial laccase, CotA, from *Bacillus subtilis* endospores, is an exception to this pattern, as its optimum activity is at 75 °C (Martins et al. 2002). The relatively high optimum temperature and thermal stability observed for the laccase produced by isolate SB086 and its optimum pH range is advantageous for industrial application, considering that cellulolytic enzymes generally require similar condition (temperature range from 40 to 50 °C, and pH 4 to 5; reviewed by Howard et al. 2003; Taherzadeh and Karimi 2007).

The optimum pH of SB086 laccase (between pH 4.0 and 5.0) was similar to laccases of *Streptomyces cyaneus* (pH 4.5; Arias et al. 2003), *S. lavendulae* (pH 4.5; Suzuki et al. 2003) and most microbial laccases described, which have an acidic optimum pH. Considering that high activity at alkaline pH is desirable for many industrial applications (Strong and Claus 2011), this feature limits the range of possible applications of SB086 laccase to those where stability at acidic pH is required: for example, in wine industry for polyphenol elimination at pH around 2.5–4.0 (Madhavi and Lele 2009). Surprisingly, the small laccase from *Streptomyces coelicolor* presented highest activity at pH 9.4 with 2.6-dimethoxyphenol (2,6-DMP) as substrate (Machczynski et al. 2004).

SB086 laccase activity presented a new pattern of response to metal ions. It was stimulated by many metal ions and was inhibited only by iron. Conversely, laccase produced by *S. cyaneus* was inhibited by calcium and zinc (Arias et al. 2003), whereas *S. lavendulae* laccase was stimulated by 0.2 mM calcium or manganese ions, but was not affected by copper, zinc, magnesium and potassium ions (Suzuki et al. 2003). Copper and manganese inhibited *Sinorhizobium meliloti* laccase, while potassium ion stimulated it (Strong

Table 3 Cultural characteristics of *Streptomyces* sp. SB086 and the phylogenetically closest species

Characteristic	SB086	*S. xanthophaeus*	*S. spororaveus*	*S. avidinii*	*S. subrutilus*
Appearance in organic salt-starch agar (ISP-4)					
Spore-mass color	Light gray	Grayish yellowish pink to light grayish reddish brown	nd [a]	Grayish yellowish pink; light grayish reddish brown	Grayish yellowish pink
Substrate mycelium color	Undetermined	Very dark reddish gray to near black	nd	No distinctive pigments	
Soluble pigment	Purple	Traces of yellow	nd	No pigments	No distinctive pigments
Melanin production on tyrosine agar (ISP-7)	No growth	nd	nd	No pigment production	Only weakly or not at all
Growth on sole carbon sources					
D-Fructose	+	nd	-	+	+
Maltose	+	nd	+	nd	nd
Galactose	+	nd	-	nd	nd
D-Glucose	+	nd	+	+	+
L-Arabinose	+	-	-	-	-
D-Xylose	+	-	-	-	-
D-Mannitol	+	-	nd	-	+/-
Myo-Inositol	+	-	nd	-	-

[a] Information not determined in literature, to our knowledge

and Claus 2011). *Daedalea quercina* laccase was stimulated by copper, but inhibited by manganese ion (Baldrian 2004). Similarly to SB086, iron inhibited *S. cyaneus* and *Sinorhizobium meliloti* laccases, and both ferric and ferrous ions are recognized inhibitors of cellulolysis reactions (Tejirian and Xu 2010). After comparison, it can be noted that SB086 laccase was less sensitive to metal inhibition. Furthermore, the variable response to metal ions among laccases indicates that these are very heterogeneous enzymes in microbial sources.

The molecular weight of the active protein (≥ 100 kDa) indicates a non-monomeric organization—in line with several reported streptomycetes laccases that need to be at least dimeric to be active. For instance, *Streptomyces griseus* and *Streptomyces aviceus* produce trimerical laccases of 114 and 98 kDa, respectively (Endo et al. 2003; Gunne and Urlacher 2012) and *Streptomyces ipomoea* produces a dimerical laccase of 79 kDa (Molina-Guijarro et al. 2009).

Conclusion

In the presented *Actinobacteria* isolation from forest soil under restoration process, the isolate with highest laccase activity was found to belong to genus *Streptomyces*. Our data suggest that the isolate SB086 might be the nucleus of a new taxon. This isolate was found to synthesize and secrete a laccase-like enzyme with industrially interesting properties. Since most bacterial laccases are assigned only hypothetically, our findings are relevant for supporting in silico data of bacterial laccase secretion, and for reinforcing the view that *Bacteria* are a valuable source of laccases for industrial applications.

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