

Accepted Manuscript

Title: Expression and characterization of a thermostable organic solvent-tolerant laccase from *Bacillus licheniformis* ATCC 9945a

Author: Nikola Lončar Nataša Božić Zoran Vujčić



PII: S1381-1177(16)30096-0
DOI: <http://dx.doi.org/doi:10.1016/j.molcatb.2016.06.005>
Reference: MOLCAB 3380

To appear in: *Journal of Molecular Catalysis B: Enzymatic*

Received date: 27-4-2016
Revised date: 8-6-2016
Accepted date: 10-6-2016

Please cite this article as: Nikola Lončar, Nataša Božić, Zoran Vujčić, Expression and characterization of a thermostable organic solvent-tolerant laccase from *Bacillus licheniformis* ATCC 9945a, *Journal of Molecular Catalysis B: Enzymatic* <http://dx.doi.org/10.1016/j.molcatb.2016.06.005>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Expression and characterization of a thermostable organic solvent-tolerant laccase from *Bacillus licheniformis* ATCC 9945a

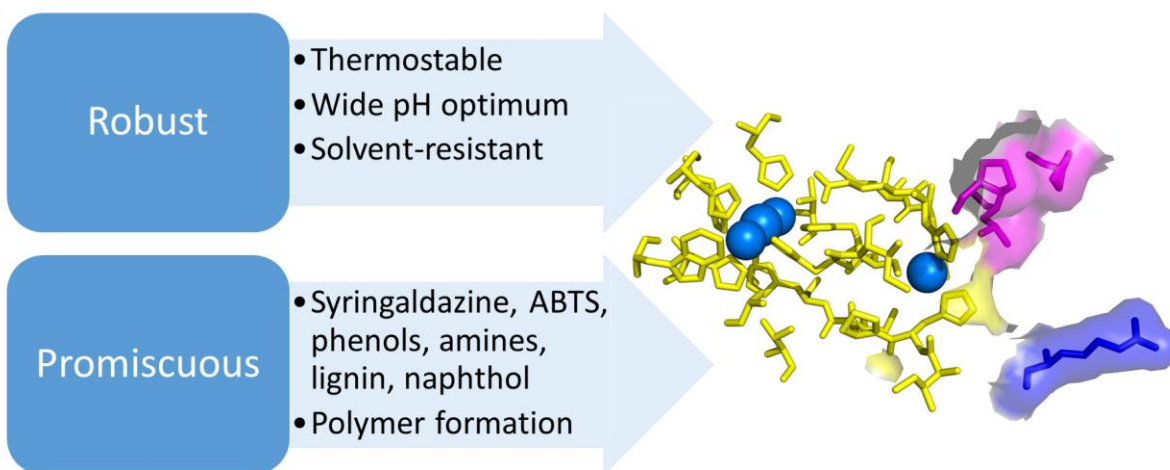
Nikola Lončar^{1*}, Nataša Božić², Zoran Vujčić¹

¹Faculty of Chemistry, University of Belgrade, Studentski trg 12-16, Belgrade, Serbia

²Institute of Chemistry, Technology and Metallurgy-Center of Chemistry, University of Belgrade, Studentski trg 12-16, Belgrade, Serbia

*Corresponding author: Tel.: 381-11-3282393, E-mail: nloncar@chem.bg.ac.rs

Graphical Abstract



Highlights

- *Bacillus licheniformis* 9945a laccase is overexpressed in *E. coli* with yield 50 mg/L.
- Temperature optimum of laccase is 90°C and pH optimum is 7.0.
- Enzyme is thermostable with a melting temperature of 79°C at pH 7.0.
- Presence of organic solvents reduces melting temperature but activity remains impaired.
- Lignin model compounds are dimerized after one electron oxidation of phenolic group.

Abstract

Bacterial laccases have proven advantages over fungal and plant counterparts in terms of wider pH optimum, higher stability and broader biocatalytic scope. In this work, *Bacillus licheniformis* ATCC 9945a laccase is produced heterologously in *Escherichia coli*. Produced laccase exhibits remarkably high temperature optimum at 90°C and possess significant thermostability and resistance to inactivation by organic solvents. Laccase has an apparent melting temperature of 79°C at pH 7.0 and above 70°C in range of pH 5.0-8.0, while having half-life of 50 min at 70°C. Presence of 10% organic solvents such as acetonitrile, dimethylformamide, dimethylsulfoxide or methanol reduces melting temperature to 45-52°C but activity remains practically unimpaired. With 50% of acetonitrile and methanol laccase retained

~40% of initial activity. EDTA and 300 mM sodium-chloride have positive effect on activity. Enzyme is active on syringaldazine, ABTS, phenols, amines, naphthol, lignin and lignin model compounds and mediates C-C bond formation *via* oxidative coupling after one electron oxidation of phenolic group. Successful polymerization of 2-naphthol was achieved with 77% conversion of 250 mg/L 2-naphthol in only 15 min which may further expand substrate scope of this enzyme towards polymer production and/or xenobiotics removal for environmental applications.

Keywords: Laccase, *Bacillus licheniformis*, oxidation, thermostable enzyme, lignin.

1. Introduction

Laccases (benzenediol/oxygen oxidoreductases; EC 1.10.3.2) belong to the class of multi-copper oxidases able to oxidize a wide range of substrates *via* four electron reduction of molecular oxygen to water. Substrate scope of laccases includes phenols and polyphenols, nonphenolic substrates such as aromatic and biogenic amines, arylamines, anilines and some cyanide complexes of metals [1,2]. First and the most famous bacterial laccase is thermostable CotA originating from spore coat of *Bacillus subtilis* [3]. CotA performed more efficient degradation of organochlorine insecticides, lindane and endosulfan than fungal laccase and has shown potential for bioremediation of xenobiotics [4]. Recent publications demonstrate high interest in laccases, whether chemical modification for pH and thermostabilization is investigated [5], synthesis of fine chemicals and the modification of biopolymers [6,7] or completely new applications such as first enzymatic Achmatowicz reaction [8].

Our knowledge on bacterial laccases is still scarce and new enzymes have to be characterized in order to expand available biocatalysts toolbox and to broaden knowledge about them [9]. In this work, *Bacillus licheniformis* ATCC 9945a laccase (*Bli*Lacc) is overexpressed and purified. Biochemical characterization indicated that *Bli*Lacc is stable in wide range of conditions and may fit in well among biocatalysts used in organic chemistry. Ability of C-C coupling of phenolic compounds is demonstrated together with possibility of lignin modification and polymer formation from simple building blocks thus suggesting this enzyme as a promising tool for synthetic chemistry.

2. Experimental

2.1. Chemicals

All reagents and solvents were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA). Phusion master mix, GeneJet plasmid miniprep kit and GeneJet gel extraction kit were purchased from Thermo Scientific.

2.2. Strains, culture conditions and plasmids

Bacillus licheniformis (ATCC® 9945aTM) was used in this study as a source organism of the *Lacc* gene. *E. coli* TOP 10 was used for general cloning purposes and as the expression host. All strains were grown in Luria–Bertani (LB) broth at 37°C, while for expression of recombinant laccase terrific broth (TB) was used. Plasmid pBad*NdeI*His was used for the expression of the gene. pBad*NdeI*His is a pBAD/Myc-HisA-derived expression vector (Invitrogen) in which the *NdeI* site is removed and the *NcoI* site is replaced by *NdeI*.

A set of two oligonucleotide primers was designed based on the putative outer spore coat (*CotA*) gene sequence (CP005965.1|:747254-748795). The PCR fragment was amplified using primers GGG AAC CAT ATG AAA CTT GAA AAA TT and ACT GAA TTC TTG ATG ACG AAT ATC CG for cloning between *NdeI* and *EcoRI* to give pBAD-*Lacc*His construct which enables use of affinity purification on Ni-Sepharose. Restriction sites in primers are underlined.

2.3. Expression of laccase: Cell cultivation and fractionation.

Host cells carrying pBAD-*Lacc*His plasmid were grown overnight at 37°C in LB medium containing ampicillin (100 µg/mL). The culture was diluted (1%) into fresh TB medium containing ampicillin (100 µg/mL). Expression of *BliLacc* was induced with 0.02% arabinose and carried out at 17°C for 48 h. Copper-chloride (final concentration 2 mM) was added in extracting buffer (50 mM KPi pH 7.8, 300 mM NaCl, 2 mM CuCl₂, 0.5% Triton X-100). Cell free extract was incubated 1 h at 50°C and clarified by centrifugation at 16000 rpm for 1 h. Clear supernatant was loaded on a Ni²⁺-Sepharose HP equilibrated with 50 mM KPi buffer pH 7.8 with 0.3 M NaCl and incubated overnight at 4°C using nutating shaker. Non-specifically bound proteins were washed away stepwise with 2 column volumes of 25 mM imidazole in starting buffer followed by elution with 250 mM imidazole in the same buffer. Collected fractions were

analyzed by activity measurement and reducing SDS-PAGE. Pure fractions were concentrated using an Amicon stirring cell equipped with a 10 kDa cut-off membrane.

2.4. Biochemical characterization of recombinant laccase

For determination of Michaelis-Menten kinetic parameters the oxidation of SGZ ($\epsilon_{525} = 65,000 \text{ M}^{-1}\text{cm}^{-1}$) was tested using 0.15-20 μM substrate at 25°C in 100 mM TrisHCl pH 7.0 (Lu et al., 2012). pH optimum experiments were carried out as described above using set of 100 mM buffers (Na-acetate pH 3-5, MES pH 6, TrisHCl 7-8, glycine/NaOH pH 9-10). Temperature optimum was determined by preincubation of 1 mL 1 mM ABTS in 100 mM Na-acetate buffer pH 4.0 in dry bath at different temperatures. *BliLacc* was added and reaction stopped after 1 min by addition of trichloroacetic acid (TCA) [10].

ProtParam tool at ExPASy server (URL: <http://web.expasy.org/protparam/>) was used to calculate extinction coefficient for *BliLacc* of $71530 \text{ M}^{-1}\text{cm}^{-1}$ and an extinction coefficient at 610 nm of $4400 \text{ M}^{-1}\text{cm}^{-1}$ for Type 1 copper was used for the calculations of copper content [11].

The effects of inhibitors as well as organic solvents acetonitrile (AcN), dimethylformamide (DMF), dimethylsulfoxide (DMSO) or methanol (MeOH) on *BliLacc* activity were studied as described before with SGZ as substrate (Lu et al., 2012). *BliLacc* and inhibitors or organic solvents were incubated for 5 min at room temperature prior to addition of SGZ and determination of residual activity. Additionally, *BliLacc* was incubated with organic solvents for 5, 10, 20 and 30 min prior to activity measurement to confirm enzyme's stability upon prolonged incubation with cosolvent.

Screening of substrates was carried out at room temperature at pH 7 using 1 mM substrates and their characteristic wavelength maxima and extinction coefficients as indicated in Table 1. Stock solutions of substrates were made in DMSO and final concentration of DMSO in reaction mixture was kept under 5%. Oxidation of 2-naphthol was tested at different pH with 0.44 μM *BliLacc* and 250 mg/L 2-naphthol in a 0.5 mL reactions in 100 mM buffer (Na-acetate pH 3-5, MES pH 6, TrisHCl 7-8, glycine/NaOH pH 9-10). Reaction was incubated at 60°C for 15 min with shaking followed by stopping reaction at 100°C for 5 min. Resulting polymer was removed by centrifugation and supernatant analyzed by HPLC (*vide infra*) to quantify substrate depletion. Oxidation of ibuprofen, thioanisole and Cbz-ethanolamine was tested in 100 mM

TrisHCl pH 7.0 at 60 °C for 1 h and analysed by HPLC (*vide infra*). Controls were included in all experiments without enzyme and with enzyme inactivated by boiling at 100 °C for 10 min.

2.5. Thermal stability assays

Thermal stability was determined by measuring residual activity upon incubating 50 μ l aliquots of purified *Bli*Lacc (4.4 μ M) at 60°C and 70°C. Samples were withdrawn at specific time points, placed on ice and enzyme activity was determined spectrophotometrically using 1 mM ABTS in 100 mM Na-acetate buffer pH 4.0 as described above.

The ThermoFluor method was used to determine the apparent melting points of the laccase using an enzyme concentration of 1.0 mg/ml. For testing thermal stability of *Bli*Lacc in different pH 100 mM buffers were used (Na-acetate pH 4.0-5.0, MES pH 6.0, TrisHCl pH 7.0-8.0). Thermal stability in presence of 10% organic solvents was performed in 100 mM TrisHCl buffer pH 7.0. This method is based on the fluorescence increase upon binding of SYPRO Orange to hydrophobic protein surfaces that become exposed upon thermal protein unfolding or multimer dissociation [12]. The fluorescence of the Sypro Orange dye was monitored using a RT-PCR machine (CFX-Touch, Bio-Rad). The temperature was increased with 0.5°C per step, starting at 25°C and ending at 99°C, using a 10 s holding time at each step. The temperature at the maximum of the first derivative of the observed fluorescence was taken as the apparent melting temperature.

2.6. Ligninolytic activity

Activity on 5 mM guaiacylglycerol- β -guaiacyl ether in 100 mM TrisHCl buffer pH 7.0 at 65°C was followed by measuring substrate depletion using an HPLC. Thermo Dionex Ultimate 3000 HPLC system was fitted with a Grace Altima HP C18 column (2.1 x 100 mm, 3 μ m particles). Mobile phases consisted of 0.1% formic acid in water as component A and acetonitrile as component B. Gradient elution: 15% B for 3 min, 15 - 95% B in 13 min, 95% B for 5 min. The compounds were eluted at flow rate of 0.5 ml min⁻¹, and column was set at 30 °C. Injection volume was 10 μ L. Detector was set at 254 nm and 280 nm. Mass spectrometry product analysis was performed on Zorbax Eclipse XDB C18 column using Agilent 1100 series HPLC equipped with a diode array detector and an Agilent 6520 ESI-MS-TOF.

3. Results

3.1. Production and purification of recombinant laccase

Full genome sequence of *Bacillus licheniformis* 9945a has been available as *B. licheniformis* strains are widely used in biotechnology for enzyme and antibiotics production [13]. This strain is known for thermostable amylase and for production of gamma-poly(glutamic acid) [14-16]. Genome mining identified one gene for outer spore coat laccase (CotA) (GenBank: AGN35164.1). Identified putative laccase has 66% identity in protein sequence with well known spore laccase originating from *Bacillus subtilis* (PDB: 1GSK_A) [3]. Different conditions for cultivation were attempted, namely expression in LB and TB media at 17 °C, 24 °C, 30 °C and 37 °C, and with varying inducer concentration. Additionally, growth in microaerobic conditions in presence of 0.2 mM and 2 mM copper was tested. Best result in terms of yield and activity of enzyme was obtained using TB medium and expressing enzyme at 17 °C for 48 h. 50 mg of purified *BliLacc* was obtained from 1 L of TB medium cultured in flasks (Fig. 1a). After purification and concentration by ultrafiltration solution of 220 µM *BliLacc* was obtained with intensive blue color. Enzyme remained soluble under these conditions. UV Vis spectra of 100 µM *BliLacc* shows characteristic peak at 606 nm that match blue color of Type 1 copper and shoulder at 330 nm for Type 3 copper (Fig. 1b). Using extinction coefficient known for several laccases we calculated concentration of 97.5 µM of copper Type 1 for 100 µM *BliLacc* determined by protein concentration measurement (Fig. 1b). This implies that *BliLacc* is expressed as fully loaded laccase under conditions described above.

3.2. Biochemical characterization of recombinant laccase

pH optimum of *BliLacc* with ABTS is around pH 3 while for SGZ it reaches its maximum at pH 7.0. Michaelis-Menten kinetics was determined at this pH and shown low K_m of only 4.06 ± 0.49 µM and k_{cat} of 1.99 ± 0.09 s⁻¹. Activity towards DMP was lower than towards both ABTS and SGZ and showed somewhat flat pH optimum line with preference for lower pH values (Fig. 2a). Temperature optimum reaches the peak at 90°C and decrease slightly at 100°C (Fig. 2b).

The apparent melting temperature ($T_{m\ app}$) for *BliLacc* was found to be 79 °C at pH 7.0 and remains higher than 70°C in the range of pH 5.0-8.0 (Fig. 3a). Effect of organic solvents on the $T_{m\ app}$ of *BliLacc* showed a trend of decreased melting temperature for 20-30 °C as compared to enzyme without organic solvents present. However, no significant discrimination can be made

among tested solvents. $T_{m\ app}$ is lowered from 79 °C to 45-52 °C, where *BliLacc* has lowest stability in presence of AcN and highest in presence of DMSO at pH 7.0 (Fig. 3b). Additionally, activity measurements were performed to check how activity is affected by organic solvents (Fig. 3c). Although apparent $T_{m\ app}$ is lowered in presence of 10% organic solvents, activity of *BliLacc* remained practically unaffected. DMSO and AcN have no influence on activity, DMF reduced it to 78% compared to control sample whereas in the presence of 10% MeOH enzyme exhibited 120% activity towards SGZ (Fig. 3c). In the presence of 50% organic solvents *BliLacc* retained significant percentage of initial activity: ~40% in case of AcN and MeOH whereas most of the activity is lost in the case of DMSO and DMF (Fig. 3c). Enzyme did not show different activity if incubated with cosolvents in range of 5-30 min.

High values for apparent melting temperature are good indication of the thermal stability of *BliLacc*. Upon incubation for 30 min at 60°C and 70°C, the enzyme retains 94% and 70% of its original activity, respectively. Half-life of *BliLacc* at 60°C is nearly 100 min whereas at 70°C it is 59 min (Fig. 4).

Thiol compounds such as L-cystein and dithiotreitol (DTT) completely inactivated laccase already at 1 mM concentration, whereas in the presence of 1 mM sodium-azide *BliLacc* showed 40% activity and 9% when 10 mM azide was used (Table 1). EDTA in a range of concentration 1-10 mM activated *BliLacc* to ~125%. Laccase retained 100% activity in the presence of both 100 mM and 300 mM NaCl.

To explore substrate scope of *BliLacc*, a range of differently substituted phenolic substrates was tested. Table 1 gives an overview of substrate specificity of *BliLacc* and showed that enzyme is prevalently active on classical phenolic and amine substrates such as SGZ, o-dianisidine, ABTS and 2,6-DMP exhibiting similar turnover numbers (Table 2). However, *BliLacc* is also active on other substrates albeit with lower efficacy with the exception of *p*-phenylenediamine which appeared to be the most convenient substrate next to 2-naphthol. Removal of 2-naphthol was tested at concentration 250 mg/L in buffers with a pH range 3-8. Highest activity was shown to be at pH 8 where conversion of 77.35% was achieved *via* insoluble polymer formation after 15 min incubation at 60 °C. Ibuprofen, thioanisole and Cbz-ethanolamine were not susceptible to oxidation by this enzyme (data not shown).

3.3. Lignin model dimer oxidation

Measuring conversion of 5 mM guaiacylglycerol- β -guaiacyl ether with 1.1 μ M *Bli*Lacc showed 70.6% substrate conversion in 30 min and 92.5% in one hour at pH 7.0 and 65°C. After 90 min no substrate could be detected. However, observed products are not phenolic monomers meaning that the enzyme does not catalyze the cleavage of beta-ether bond. Using LC-MS two major peaks have been identified. Measured masses of products are M^+ 638.23688 Da and M^+ 956.34316 Da and calculated molecular formula of $C_{34}H_{38}O_{12}$ and $C_{51}H_{56}O_{18}$ which corresponds to the dimer and trimer of starting compound, respectively (Supporting information, Fig. S2 and Fig. S3). The dimer peak is predominant during the first hour of reaction while upon the longer incubation time the trimer peak increase and the orange-brown polymeric product precipitates.

4. Discussion

Bacterial laccases are in the focus of research in the past decade due to their increased stability as compared to fungal laccases [17-19]. However, production of bacterial laccases is problematic due to the formation of inclusion bodies, low expression levels and low copper loading. Addition of copper to the growth medium is possible but limited by copper toxicity to host organism. Different expression conditions for several laccases from *B. subtilis*, *Bacillus pumilus*, *Bacillus clausii* and other bacterial species demonstrate that a switch to oxygen-limited growth conditions after induction increased volumetric activity considerably [6,20]. In some cases, copper content of the recombinant laccase can be increased if the enzyme is coexpressed with copper chaperone CopZ [21]. *In vitro* refolding by dilution method was used after solubilization of inclusion bodies of laccase in 8M urea with 4 mM β -mercaptoethanol [22]. Introducing single mutations of Asp500 at C terminal loop that interacts with T1 copper was proven to increase expression level up to 3 fold [23]. Rational design was employed too to increase solubility and stability of bacterial laccases, where His-tag and residues 323-332 had to be deleted [17]. Extracellular expression has been used successfully for heterologous expression of *Bacillus* sp. enzymes in *E. coli* [24], so it might offer a solution for laccase expression too. In this work however, it was necessary to use lower temperature for expression but addition of copper decreased of active enzyme. Hence, different approach was used to increase yield of active *Bli*Lacc. Addition of 0.5% Triton X-100 to the extraction buffer gave high content of the extracted proteins. Copper addition during sonication and subsequent incubation at 50 °C for 1h

is proposed to allow copper loading into the apo-enzyme. High temperature is hypothesized to enable misfolded protein to bind copper and refold properly and in this case thermostability of bacterial laccase is very useful. From spectroscopic analysis it seems that purified laccase is fully copper-loaded with respect of Type 1 and Type 3 copper (Fig. 1b). Published extinction coefficient for Type 1 copper are in range of 4400-5700 $M^{-1}cm^{-1}$ [11,25]. For *BliLacc* extinction coefficient of laccase from *Streptomyces coelicolor* was used since it is a gram-positive bacterium as *B. licheniformis*, source of gene encoding *BliLacc*. Considering known obstacles in the production of these enzymes, obtained yield was still quite remarkable and this method is rather simple as first line of screening expression conditions compared to previously published workflows to obtain functional bacterial laccase.

The k_{cat} for SGZ is comparable with those described before for other *Bacillus* laccases such as $3.7 \pm 0.1 s^{-1}$ for CotA from *B. subtilis* [3]. Inactivation of bacterial and fungal laccases by thiols has been described earlier [18,26-28]. Laccases resistant to inhibition by high chloride concentration have advantage over fungal laccases for treatment of textile wastewater since fungal laccases are easily inhibited already by 100 mM chloride ions [29,30]. From process development point of view resistance to the high salt may serve to increase the polarity of solution and facilitate better separation of product in two phase systems where end product is more hydrophobic [31].

Studies of a threshold concentration of organic solvent required to initiate rapid enzyme inactivation have shown that enzymes which can retain the activity in the high organic solvent concentration are very rare [32]. Recent example of *Rhus vernicifera* laccase losing more than 85% of initial activity in 20% ethanol and methanol may further emphasize significance of *BliLacc* resistance to organic solvents inactivation [33].

New conducting materials are being screened for the electronic industry and laccase mediated polymerization of the natural phenols such as gallic acid is proof of concept in this field of application [34]. *BliLacc* shows ability to produce water insoluble polymers from gallic acid, *p*-phenylenediamine, diaminobenzidine and 2-naphthol. Remarkable conversion of 77% of 2-naphthol at 250 mg/L is opening up the possibility for a clean method for wastewater treatment as naphthol is removed as insoluble polymer and metabolites are not present in the water. Formation of insoluble polymers upon oxidation of phenolics has been shown before. *BliLacc*

may be useful for polymers synthesis, i.e. oxidized naphthol may be coupled via S_N2 reaction as demonstrated for some catechols [35].

Various oxidases are being tested for the action on lignin aiming at deriving added value chemicals [36,37]. Heterogeneity of lignin is a major obstacle for detailed study of the enzyme activity and model compounds are used instead. Guaiacylglycerol- β -guaiacyl ether is a phenolic model compound that contains ether bonds which constitutes up to 50% of total bonds in lignin. *BliLacc* seems not to act on beta ether linkage in model compound but instead on phenolic group. Products are formed *via* oxidative coupling after one electron oxidation of phenolic group as shown also for coupling of cinnamic acid derivatives for other laccases and peroxidases [38,39]. Measured mass of the product corresponds well with the mass of predicted dimer (Supporting information, Fig. S2). Additional peak of small intensity was eluted after the main product peak and showed same mass (Supporting information, Fig. S3.1). Most likely it is a structural isomer due to the coupling at different position as described recently for laccase application for the synthesis of pinosresinol starting from eugenol [39].

Good starting point, meaning relatively stable enzyme with good expression yield is necessary in order to continue towards the rational, semi-rational and directed evolution approaches to ultimately convert laccases into high value-added biocatalysts [40]. *BliLacc* fulfils these requirements and may be tailored into a more superb enzyme for specific applications.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by the Serbian Ministry of Education, Science and Technological Development, project grant number 172048.

References

[1] P. Giardina, V. Faraco, C. Pezzella, A. Piscitelli, S. Vanhulle, G. Sannia, Laccases: a never-ending story, *Cellular and Molecular Life Sciences*. 67 (2010) 369-385.

- [2] S. Callejón, R. Sendra, S. Ferrer, I. Pardo, Cloning and characterization of a new laccase from *Lactobacillus plantarum* J16 CECT 8944 catalyzing biogenic amines degradation, *Appl. Microbiol. Biotechnol.* (2015) 1-12.
- [3] L.O. Martins, C.M. Soares, M.M. Pereira, M. Teixeira, T. Costa, G.H. Jones, A.O. Henriques, Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat, *J. Biol. Chem.* 277 (2002) 18849-18859.
- [4] A. Ulcnik, I. Kralj Cigic, F. Pohleven, Degradation of lindane and endosulfan by fungi, fungal and bacterial laccases, *World J. Microbiol. Biotechnol.* 29 (2013) 2239-2247.
- [5] Y. Liu, X. Hua, Degradation of acenaphthylene and anthracene by chemically modified laccase from *Trametes versicolor*, *RSC Adv.* 4 (2014) 31120-31122.
- [6] J. Ihssen, R. Reiss, R. Luchsinger, L. Thony-Meyer, M. Richter, Biochemical properties and yields of diverse bacterial laccase-like multicopper oxidases expressed in *Escherichia coli*, *Sci. Rep.* 5 (2015) 10465.
- [7] F. Hollmann, I.W.C.E. Arends, K. Buehler, A. Schallmeyer, B. Buhler, Enzyme-mediated oxidations for the chemist, *Green Chem.* 13 (2011) 226-265.
- [8] C. Asta, D. Schmidt, J. Conrad, B. Forster-Fromme, T. Tolasch, U. Beifuss, The first enzymatic Achmatowicz reaction: selective laccase-catalyzed synthesis of 6-hydroxy-(2H)-pyran-3(6H)-ones and (2H)-pyran-2,5(6H)-diones, *RSC Adv.* 3 (2013) 19259-19263.
- [9] E. Ricklefs, N. Winkler, K. Koschorreck, V.B. Urlacher, Expanding the laccase-toolbox: a laccase from *Corynebacterium glutamicum* with phenol coupling and cuprous oxidase activity, *J. Biotechnol.* 191 (2014) 46-53.
- [10] M. Nagai, M. Kawata, H. Watanabe, M. Ogawa, K. Saito, T. Takesawa, K. Kanda, T. Sato, Important role of fungal intracellular laccase for melanin synthesis: purification and characterization of an intracellular laccase from *Lentinula edodes* fruit bodies, *Microbiology.* 149 (2003) 2455-2462.
- [11] M.C. Machczynski, E. Vijgenboom, B. Samyn, G.W. Canters, Characterization of SLAC: A small laccase from *Streptomyces coelicolor* with unprecedented activity, *Protein Science : A Publication of the Protein Society.* 13 (2004) 2388-2397.

- [12] U.B. Ericsson, B.M. Hallberg, G.T. Detitta, N. Dekker, P. Nordlund, Thermofluor-based high-throughput stability optimization of proteins for structural studies, *Anal. Biochem.* 357 (2006) 289-298.
- [13] M. Rachinger, S. Volland, F. Meinhardt, R. Daniel, H. Liesegang, First Insights into the Completely Annotated Genome Sequence of *Bacillus licheniformis* Strain 9945A, *Genome Announc.* 1 (2013) 10.1128/genomeA.00525-13.
- [14] N. Božić, J. Ruiz, J. López-Santín, Z. Vujčić, Production and properties of the highly efficient raw starch digesting α -amylase from a *Bacillus licheniformis* ATCC 9945a, *Biochem. Eng. J.* 53 (2011) 203-209.
- [15] G. Kedia, D. Hill, R. Hill, I. Radecka, Production of poly-gamma-glutamic acid by *Bacillus subtilis* and *Bacillus licheniformis* with different growth media, *J. Nanosci Nanotechnol.* 10 (2010) 5926-5934.
- [16] Y.H. Ko, R.A. Gross, Effects of glucose and glycerol on gamma-poly(glutamic acid) formation by *Bacillus licheniformis* ATCC 9945a, *Biotechnol. Bioeng.* 57 (1998) 430-437.
- [17] Z. Fang, P. Zhou, F. Chang, Q. Yin, W. Fang, J. Yuan, X. Zhang, Y. Xiao, Structure-based rational design to enhance the solubility and thermostability of a bacterial laccase Lac15, *PLoS One.* 9 (2014) e102423.
- [18] N. Loncar, N. Bozic, J. Lopez-Santin, Z. Vujcic, *Bacillus amyloliquefaciens* laccase--from soil bacteria to recombinant enzyme for wastewater decolorization, *Bioresour. Technol.* 147 (2013) 177-183.
- [19] L. Lu, T.N. Wang, T.F. Xu, J.Y. Wang, C.L. Wang, M. Zhao, Cloning and expression of thermo-alkali-stable laccase of *Bacillus licheniformis* in *Pichia pastoris* and its characterization, *Bioresour. Technol.* 134 (2013) 81-86.
- [20] P. Durao, Z. Chen, A.T. Fernandes, P. Hildebrandt, D.H. Murgida, S. Todorovic, M.M. Pereira, E.P. Melo, L.O. Martins, Copper incorporation into recombinant CotA laccase from *Bacillus subtilis*: characterization of fully copper loaded enzymes, *J. Biol. Inorg. Chem.* 13 (2008) 183-193.
- [21] M. Gunne, D. Al-Sultani, V.B. Urlacher, Enhancement of copper content and specific activity of CotA laccase from *Bacillus licheniformis* by coexpression with CopZ copper chaperone in *E. coli*, *J. Biotechnol.* 168 (2013) 252-255.

- [22] N. Mollania, K. Khajeh, B. Ranjbar, F. Rashno, N. Akbari, M. Fathi-Roudsari, An efficient in vitro refolding of recombinant bacterial laccase in *Escherichia coli*, *Enzyme Microb. Technol.* 52 (2013) 325-330.
- [23] N. Nasoohi, K. Khajeh, M. Mohammadian, B. Ranjbar, Enhancement of catalysis and functional expression of a bacterial laccase by single amino acid replacement, *Int. J. Biol. Macromol.* 60 (2013) 56-61.
- [24] N. Božić, J. Puertas, N. Lončar, C.S. Duran, J. López-Santín, Z. Vujčić, The DsbA signal peptide-mediated secretion of a highly efficient raw-starch-digesting, recombinant α -amylase from *Bacillus licheniformis* ATCC 9945a, *Process Biochemistry.* 48 (2013) 438-442.
- [25] B. Reinhammar, Y. Oda, Spectroscopic and catalytic properties of rhus vernicifera laccase depleted in type 2 copper, *J. Inorg. Biochem.* 11 (1979) 115-127.
- [26] A.A. Telke, G.S. Ghodake, D.C. Kalyani, R.S. Dhanve, S.P. Govindwar, Biochemical characteristics of a textile dye degrading extracellular laccase from a *Bacillus* sp. ADR, *Bioresour. Technol.* 102 (2011) 1752-1756.
- [27] L. Lu, M. Zhao, T.N. Wang, L.Y. Zhao, M.H. Du, T.L. Li, D.B. Li, Characterization and dye decolorization ability of an alkaline resistant and organic solvents tolerant laccase from *Bacillus licheniformis* LS04, *Bioresour. Technol.* 115 (2012) 35-40.
- [28] A.A. Telke, D.C. Kalyani, U.U. Jadhav, G.K. Parshetti, S.P. Govindwar, Purification and characterization of an extracellular laccase from a *Pseudomonas* sp. LBC1 and its application for the removal of bisphenol A, *J Molec Catal B.* 61 (2009) 252-260.
- [29] N. Jimenez-Juarez, R. Roman-Miranda, A. Baeza, A. Sánchez-Amat, R. Vazquez-Duhalt, B. Valderrama, Alkali and halide-resistant catalysis by the multipotent oxidase from *Marinomonas mediterranea*, *J. Biotechnol.* 117 (2005) 73-82.
- [30] N. Lončar, N. Gligorijević, N. Božić, Z. Vujčić, Congo red degrading laccases from *Bacillus amyloliquefaciens* strains isolated from salt spring in Serbia, *Int. Biodeterior. Biodegrad.* 91 (2014) 18-23.
- [31] P. Tufvesson, J. Lima-Ramos, N.A. Haque, K.V. Gernaey, J.M. Woodley, Advances in the Process Development of Biocatalytic Processes, *Org. Process Res. Dev.* 17 (2013) 1233-1238.
- [32] V.V. Mozhaev, Y.L. Khmelnitsky, M.V. Sergeeva, A.B. Belova, N.L. Klyachko, A.V. Levashov, K. Martinek, Catalytic activity and denaturation of enzymes in water/organic cosolvent mixtures, *European Journal of Biochemistry.* 184 (1989) 597-602.

- [33] Y. Wan, R. Lu, L. Xiao, Y. Du, T. Miyakoshi, C. Chen, C.J. Knill, J.F. Kennedy, Effects of organic solvents on the activity of free and immobilised laccase from *Rhus vernicifera*, *Int. J. Biol. Macromol.* 47 (2010) 488-495.
- [34] J. López, J.M. Hernández-Alcántara, P. Roquero, C. Montiel, K. Shirai, M. Gimeno, E. Bárzana, *Trametes versicolor* laccase oxidation of gallic acid toward a polyconjugated semiconducting material, *J Molec Catal B.* 97 (2013) 100-105.
- [35] H.T. Abdel-Mohsen, J. Conrad, U. Beifuss, Laccase-catalyzed synthesis of catechol thioethers by reaction of catechols with thiols using air as an oxidant, *Green Chem.* 16 (2014) 90.
- [36] T.D. Bugg, R. Rahmanpour, Enzymatic conversion of lignin into renewable chemicals, *Curr. Opin. Chem. Biol.* 29 (2015) 10-17.
- [37] R. Chandra, P. Chowdhary, Properties of bacterial laccases and their application in bioremediation of industrial wastes, *Environ. Sci. Process. Impacts.* 17 (2015) 326-342.
- [38] R.T. Nishimura, C.H. Giammanco, D.A. Vosburg, Green, Enzymatic Syntheses of Divanillin and Diapocynin for the Organic, Biochemistry, or Advanced General Chemistry Laboratory, *J. Chem. Educ.* 87 (2010) 526-527.
- [39] E. Ricklefs, M. Girhard, K. Koschorreck, M.S. Smit, V.B. Urlacher, Two-Step One-Pot Synthesis of Pinoresinol from Eugenol in an Enzymatic Cascade, *ChemCatChem.* 7 (2015) 1857-1864.
- [40] D.M. Mate, M. Alcalde, Laccase engineering: from rational design to directed evolution, *Biotechnol. Adv.* 33 (2015) 25-40.

FIGURE CAPTIONS

Fig. 1. (a) SDS PAGE analysis. Lane 1: molecular markers molecular weights (kDa); lane 2: molecular markers; lane 3: purified *BliLacc*. (b) UV Vis spectrum of 100 μ M *BliLacc*. Arrows indicate shoulder at 330 nm that corresponds to Type 3 copper and peak at 606 nm that corresponds to Type 1 copper.

Fig. 2. (a) Effect of pH on activity of *BliLacc* towards syringaldazine, ABTS and 2,6-dimethoxyphenol. (b) Effect of temperature on activity of *BliLacc* towards ABTS as measured by stop assay.

Fig. 3. (a) Effect of pH on apparent melting temperature of *BliLacc*. (b) Effect of 10% solvents on apparent melting temperature of *BliLacc* at pH 7 and (c) effect of 10% and 50% solvents on *BliLacc* activity towards SGZ.

Fig. 4. Thermal stability of *BliLacc* at 60°C and 70°C.

Fig. 1

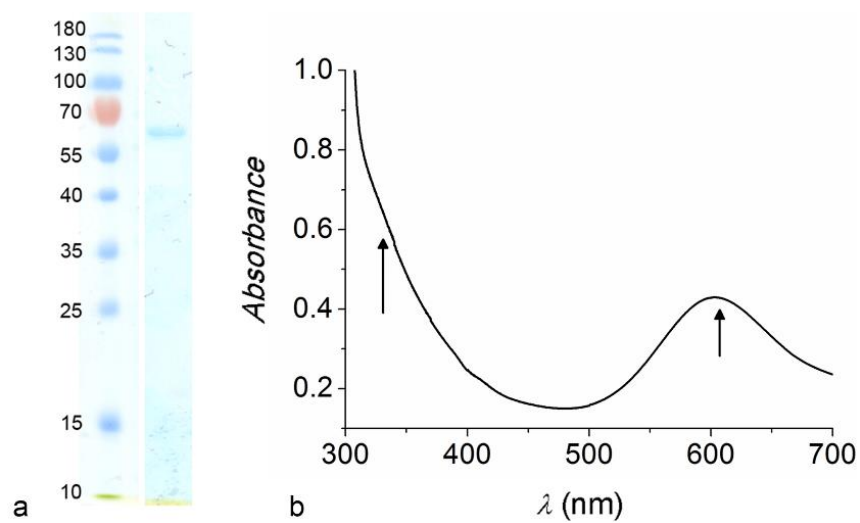


Fig. 2

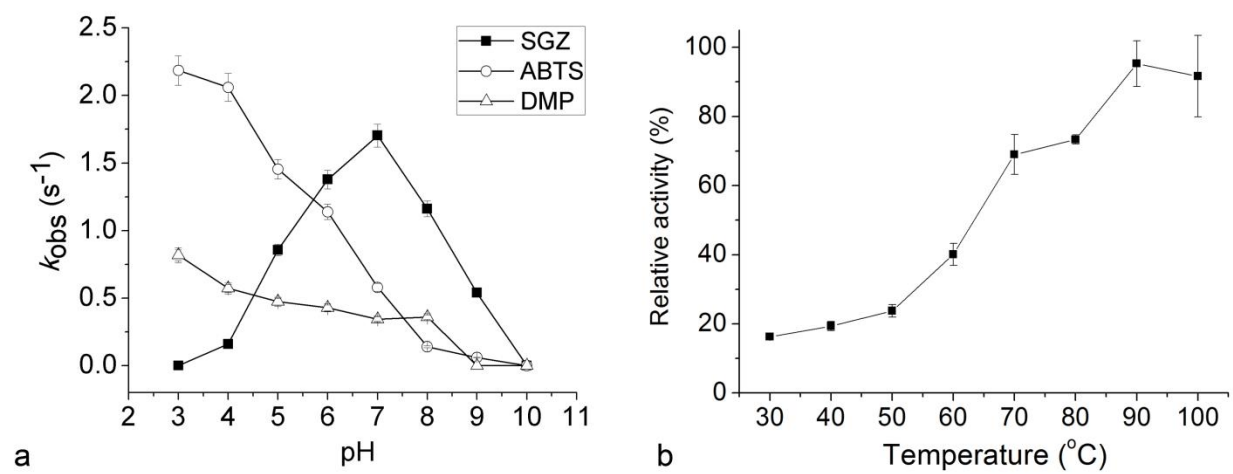


Fig. 3

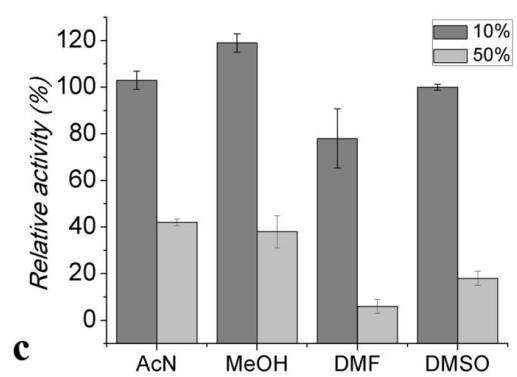
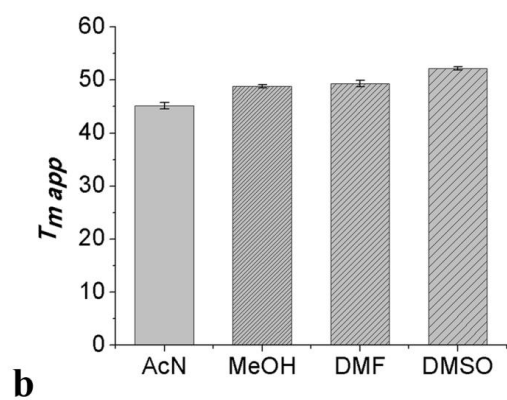
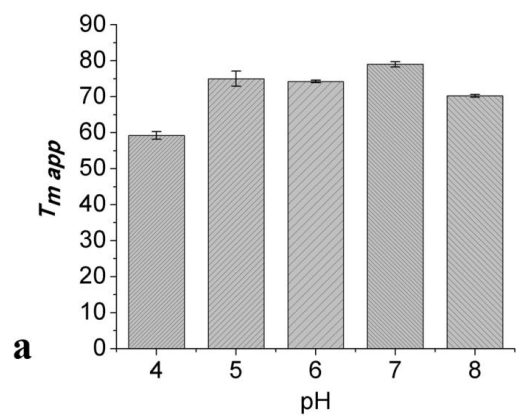
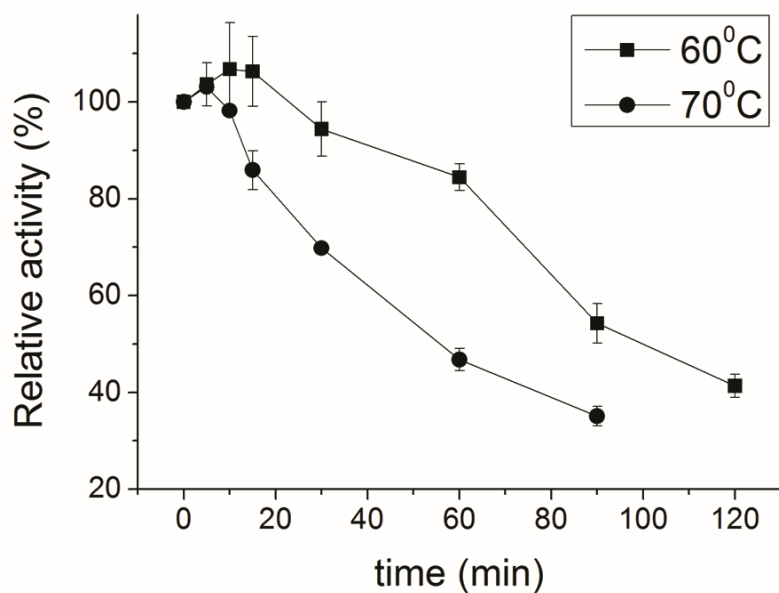


Fig. 4

**Table 1** Effect of inhibitors on *BliLacc* activity

	Concentration (mM)	k_{obs} (s^{-1})	Relative activity (%)
control		1.65 ± 0.05	100
NaN3	10	0.15 ± 0.01	9
	1	0.68 ± 0.01	40
EDTA	10	2.11 ± 0.04	124
	1	2.17 ± 0.05	127
NaCl	100	1.78 ± 0.01	105
	300	1.77 ± 0.09	104
DTT	10	0.00	0
	1	0.00	0
L-Cys	10	0.00	0
	1	0.00	0

Table 2 Substrate specificity of *BliLacc* expressed as observed turnover number (k_{obs}) and relative activity.

Substrate (1 mM)	λ (nm)	ϵ (mM ⁻¹ cm ⁻¹)	k_{obs} (s ⁻¹)	Relative activity (%)
Syringaldazine	525	6.50	1.60 ± 0.03	100
<i>o</i> -Dianisidine	460	11.30	1.43 ± 0.06	90
ABTS	414	36.60	1.16 ± 0.05 ^a	73
2,6-DMP	468	49.60	1.10 ± 0.04	69
L-DOPA	475	3.70	0.56 ± 0.004	35
Catechol	420	3.45	0.44 ± 0.09	28
<i>o</i> -Phenylenediamine	420	31.30	0.07 ± 0.004	4
Hydroquinone	320	11.00	0.02 ± 0.002	1
Gallic acid	520	N.A.	0.72 ± 0.10 ^b	45
<i>p</i> -Phenylenediamine	485	N.A.	1.71 ± 0.19 ^b	107

^a Measured at pH 3.0.^b Calculated as mAbs/s per μM of *BliLacc*.