



Heterologous expression, purification, and characterization of thermo- and alkali-tolerant laccase-like multicopper oxidase from *Bacillus mojavensis* TH309 and determination of its antibiotic removal potential

Ali Osman Adigüzel¹ · Serpil Könen-Adigüzel² · Sümeyye Cilmeli¹ · Birgül Mazmanlı² · Erdal Yabalak³ · Sevede Üstün-Odabaşı⁴ · Nisa Gül Kaya¹ · Mehmet Ali Mazmanlı⁵

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Abstract

Laccases or laccase-like multicopper oxidases have great potential in bioremediation to oxidase phenolic or non-phenolic substrates. However, their inability to maintain stability in harsh environmental conditions and against non-substrate compounds is one of the main reasons for their limited use. The gene (*mco*) encoding multicopper oxidase from *Bacillus mojavensis* TH309 were cloned into pET14b(+), expressed in *Escherichia coli*, and purified as histidine tagged enzyme (BmLMCO). The molecular weight of the enzyme was about 60 kDa. The enzyme exhibited laccase-like activity toward 2,6-dimethoxyphenol (2,6-DMP), syringaldazine (SGZ), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). The highest enzyme activity was recorded at 80 °C and pH 8. BmLMCO showed a half-life of ~ 305, 99, 50, 46, 36, and 20 min at 40, 50, 60, 70, 80, and 90 °C, respectively. It retained more than 60% of its activity after pre-incubation in the range of pH 5–12 for 60 min. The enzyme activity significantly increased in the presence of 1 mM of Cu²⁺. Moreover, BmLMCO tolerated various chemicals and showed excellent compatibility with organic solvents. The Michaelis constant (K_m) and the maximum velocity (V_{max}) values of BmLMCO were 0.98 mM and 93.45 μ mol/min, respectively, with 2,6-DMP as the substrate. BmLMCO reduced the antibacterial activity of cefprozil, gentamycin, and erythromycin by $72.3 \pm 1.5\%$, $79.6 \pm 6.4\%$, and $19.7 \pm 4.1\%$, respectively. This is the first revealing shows the recombinant production of laccase-like multicopper oxidase from any *B. mojavensis* strains, its biochemical properties, and potential for use in bioremediation.

Keywords Laccase-like multicopper oxidase · Heterologous expression · Thermo-tolerant · Alkali-tolerant · Bioremediation

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✉ Ali Osman Adigüzel
adiguzel.ali.osman@gmail.com

¹ Department of Molecular Biology and Genetics, Faculty of Science, Ondokuz Mayıs University, Samsun, Turkey

² Department of Biology, Faculty of Science, Mersin University, Mersin, Turkey

³ Department of Chemistry Technology, Vocational School of Technical Sciences, Mersin University, Mersin, Turkey

⁴ Department of Environmental Engineering, Ondokuz Mayıs University, Samsun, Turkey

⁵ Department of Environmental Engineering, Mersin University, Mersin, Turkey

Introduction

Laccases and laccase-like multicopper oxidases' (LMCOs) enzymes catalyze the reduction of four electrons reducing oxygen to water by oxidation of one electron from the substrate, and exhibit broad substrate specificity. They can catalyze the oxidation of aromatics, polyphenols, terphenyls, nitriles, and various non-phenolic compounds. The fact that laccases have an unusually wide range of activities allows them to be used effectively in many industrial applications. They are used to clarify fruit juices and wines, bio-discoloration of denim (Sarafpour et al. 2022) and bio-bleaching of pulp, improve yogurt texture, enhance bread half-life, modify lignin, and produce energy from fuel cells. LMCOs or laccases can be incorporated as formulations of

healthcare products, such as hair dye, deodorant, perfume, hand-face cream, mouthwash, and toothpaste, to reduce their toxic effect (Gigli et al. 2022; Mojtavavi et al. 2022). Furthermore, they are effective bio-agents in environmental remediation applications due to their ability to degrade synthetic dyes, plasticizers, plastics, polycyclic aromatic hydrocarbons (PAHs), pesticides, fertilizers, and pharmaceuticals (Dong et al. 2023).

Antibiotics are listed as emerging contaminants that threaten water resources. Even in low concentration ranges, they can be toxic to organisms and cause microorganisms to become antibiotic-resistant. Conventional treatment techniques contain filtration, sedimentation, coagulation, and flocculation, are insufficient for antibiotic removal (Al-sareji et al. 2023). On the other hand, advanced oxidation processes, including ozonation, Fenton oxidation, and photocatalytic oxidation, can cause additional health and environmental problems due to toxic chemicals used or harmful by-products (Ekeoma et al. 2023). It is also costly to adapt these methods to an industrial scale (Al-sareji et al. 2023). Therefore, studies regarding the use of laccases for antibiotic removal as an environmentally friendly and easily applicable alternative have increased recently. Such studies have been performed with laccases from a limited number of sources, mostly fungi such as *Myceliophthora thermophila* (García-Delgado et al. 2018), *Trametes versicolor* (Wen et al. 2019), *Trametes hirsuta* (Navada and Kulal 2019), and *Aspergillus* spp. (Lou et al. 2022). However, their use in antibiotic removal or other bioremediation applications is quite limited due to rapid activity loss in wastewaters, including alkali ingredients and other contaminants, such as heavy metals, inhibitors, and organic solvents (Mandic et al. 2019; Li et al. 2020; Cheng et al. 2021; Chopra and Sondhi 2022). Fungal laccases are also extremely sensitive to temperature and pH changes during application. Moreover, the heterologous expression of genes in fungi by genetic engineering is quite difficult compared to that of bacterial counterparts.

Bacillus species, which are Gram-positive, endospore-forming, string aerobic/facultative aerobic bacteria with rod shapes, are widely distributed in environments (Roberts et al. 1994). They can also survive in extreme habitats, including deserts, hot springs, salterns, and alkaline lakes, due to the longevity of their endospores (Mandic-Mulec et al. 2016). In time, they may develop mechanisms that allow them to adapt to these environments and thus produce active and stable bioactive compounds resistant to relatively harsh conditions (Parrilli et al. 2021). *Bacillus mojavensis* TH309, previously isolated from plastic waste, is a halotolerant endophyte (Adıgüzel 2020). The strain, which has growth ability over broad temperature (30–60 °C) and pH (5–11) ranges, is a potential source of laccase with desired properties for bioremediation applications. Further, laccases from various *Bacillus* species, such as *Bacillus sphaericus* (Claus

and Filip 1997), *Bacillus halodurans* (Ruijsenaars and Hartmans 2004), *Bacillus vallismortis* (Zhang et al. 2013), *Bacillus clausii* (Brander et al. 2014), *Bacillus tequilensis* (Sondhi et al. 2014), *Bacillus amyloliquefaciens* (Wang et al. 2020b), *Bacillus velezensis* (Li et al. 2020), *Bacillus subtilis* (Cheng et al. 2021), *Bacillus licheniformis* (Sun et al. 2023), *Bacillus pumilus* (Liu et al. 2023), and *Bacillus cereus* (Shafana Farveen et al. 2023). However, there are no reports describing the biochemical and structural features of laccase or LMCO from any *B. mojavensis* strains.

LMCOs are a component of the endospore coat in *Bacillus* species (Martins et al. 2002). Its extraction and purification necessitate using techniques that require high cost and extensive effort. In addition, these techniques applied sequentially cause significant losses in the product. Recombinant DNA technology eliminates such problems by expressing the target gene in high amounts with a tag that facilitates purification (Sinirlioglu et al. 2013). Therefore, in the present study, the LMCO gene of *B. mojavensis* TH309 was cloned into a pET14b(+) vector that carries an N-terminal Histidine tag sequence and expressed in *Escherichia coli* BL21 (DE3) under the control of T7 promoter. The expressed enzyme (BmLMCO) was purified and then characterized. In addition, the antibiotic removal potential of BmLMCO was determined by agar well-diffusion experiments (Zhang et al. 2020).

Materials and methods

Chemicals and kits

Yeast extract, tryptone, NaCl, ethylenediaminetetraacetic acid (EDTA), centrifugal ultrafiltration tubes, and buffer components were purchased from Merck (Darmstadt, Germany). Solvents, phenylmethylsulfonyl fluoride (PMSF), 1,4-dithiothreitol (DTT), sodium dodecyl sulfate (SDS), β -mercaptoethanol (β ME), 2,6-dimethoxyphenol (2,6-DMP), syringaldazine (SGZ), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), agarose, acrylamide, bis-acrylamide, bromophenol blue, Coomassie Brilliant Blue R-250, and chromatography column were obtained from Sigma-Aldrich (Darmstadt, Germany). Ampicillin was procured from Fisher Bioreagent (Fair Lawn, NJ). Genomic DNA isolation, plasmid extraction, gel extraction, and DNA clean up kits were purchased from Favorgen (Pintung, Taiwan). Ni-NTA Resin was purchased from Thermo Fisher Scientific (Waltham, MA).

Strains, cultivation, and vector

The strain *Bacillus mojavensis* TH309, previously isolated from waste plastic in our laboratory, was routinely cultured

in Luria–Bertani (LB) broth at 45 °C for 24 h under 200 rpm shaking condition (Adıgüzel 2020). LB broth consists of (in g/L): 10 tryptone, 5 yeast extract, and 10 NaCl. *E. coli* DH5 α and *E. coli* BL21 (DE3) were used for the amplification of plasmid and heterologous expression of the *mco* gene, respectively (Liu et al. 2017). The strain *Bacillus subtilis* ATCC6633 was used in well-diffusion experiments (Vehapi and Özçimen 2021). All strains of *E. coli* were grown in LB broth at 37 °C with 175 rpm agitation. Transformants were cultivated in LB broth supplemented with 50 μ g/mL ampicillin under the same conditions. pET-14b (Novagen, Madison, WI, USA) was used for both cloning and expression vector (Carrillo and Borthakur 2022).

Amplification of *mco* gene

The genomic DNA of *B. mojavensis* TH309 was extracted using a FavorPrep™ Tissue Genomic DNA Extraction Mini Kit according to the manufacturer's protocol (Esmkhani and Shams 2022). Then, it was used as a template for amplifying the *mco* gene. The genomic DNA of *B. mojavensis* TH309, extracted using a bacterial DNA isolation kit according to the manufacturer's protocol, was used as a template for amplifying the *mco* gene. Forward (5'-AACGCTCGAGTTATTTATGGGGGTCAATCAC-3') and reverse (5'-AACGCTCGAGATGACACTTGAAAAATTTGC-3') primers with XhoI restriction sites (*italic*) were designed based on sequence deposited with the accession number of NZ_CP051464.1 in NCBI database. The PCR reaction mixture (100 μ L) was prepared with 1 μ L of Q5® High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA), 20 μ L of 5X Q5® Reaction Buffer (5X), 20 μ L of 5X Q5® High GC Enhancer, 2 μ L of genomic DNA (\cong 40 ng), 5 μ L of forward primer, 5 μ L of reverse primer, 2 μ L of dNTP (10 mM), and 45 μ L nuclease-free water. The PCR was performed for 30 cycles in a thermal cycler (Biorad-T100, Hercules, CA) under the following order: 98 °C-30 s, 98 °C-30 s, 55 °C-30 s, 72 °C-47 s, and 72 °C-300 s. The amplicons were recovered from 0.8% (w/v) agarose gel using a gel extraction kit after visualization.

Cloning of the *mco* gene

pET14b(+) isolated using plasmid extraction kit and amplified *mco* gene were digested in reaction mixture containing 20 μ L of DNA, 20 μ L of nuclease-free water, 6 μ L of 10X buffer (10 mM Tris–HCl, 10 mM MgCl₂, 100 mM KCl, 0.1 mg/mL BSA, pH 8.5), and 3 μ L of XhoI restriction enzyme (10 U/ μ L). Digestion was performed at 37 °C for 2 h. Digestion products were extracted from 0.8% (w/v) agarose gel and purified (Mathews et al. 2016). Mixtures of 7 μ L Linearized pET14b(+), 1 μ L digested amplicon, 1 μ L buffer (400 mM Tris–HCl containing 100 mM MgCl₂, 100 mM

DTT, 5 mM ATP; pH 7.8), and 1 μ L T4 DNA ligase (10 U/ μ L) were incubated overnight at 25 °C (Tabor 1989). The ligation mixture (3.5 μ L) was introduced to CaCl₂-competent cells of *E. coli* DH5 α (50 μ L) to heat-shock transformation (Chung and Miller 1993). Transformants were grown on LB Agar supplemented with ampicillin and then screened to detect clones containing the recombinant vector (pET14b(+)-*mco*). Subsequently, pET14b(+)-*mco* sub-cloned to *E. coli* BL21(DE3) using the same transformation method.

Sequencing of *mco* and sequence analysis of BmLMCO

Plasmid pET14b(+)-*mco* was sequenced (BM Labosis, Ankara, Turkey) using T7 forward (5'-TAATACGACTCACTATAGGG-3') and T7 reverse (5'-GCTAGTTAT TGCTCAGCGG-3') primers. The DNA sequences of the *mco* from *B. mojavensis* TH3069 were converted to amino acid sequences to assess sequence similarity with laccases deposited in the NCBI database. Amino acid sequences were aligned using Clustal W (<https://www.genome.jp/tools-bin/clustalw>). Multiple sequence alignment was visualized using Jalview 2.11.2.0 (Waterhouse et al. 2009). The phylogenetic tree of BmLMCO and various laccases was then built by the neighbor-joining method through MEGA X software (Kumar et al. 2022). The secondary and tertiary structures of BmLMCO predicted with “Sequence Annotated by Structure (SAS)” (Noby et al. 2020) PSIPRED (<http://bioinf.cs.ucl.ac.uk/>), and SWISS-MODEL (Waterhouse et al. 2018) web tools, respectively.

Heterologous expression of BmLMCO

E. coli BL21(DE3) carrying pET14b(+)-*mco* was cultured at 37 °C for 18 h in LB broth supplemented with ampicillin at a concentration of 50 μ g/mL. Thereafter, 500 μ L of the culture were inoculated into 250 mL Erlenmeyer flasks containing 50 mL fresh medium. After the flask was incubated at 18 °C and 175 rpm until the OD600 of the culture reaches 0.4, IPTG was added to the culture at a final concentration of 0.3 mM. Then, the culture was incubated for an additional 5 h under the same conditions. At the end of incubation, the cells were retrieved by centrifugation (8000 \times g, 20 min) at +4 °C and disrupted through sonication (Noby et al. 2020). The cell lysate was centrifuged at 8000 \times g for 20 min at +4 °C and the supernatant was used as crude enzyme solution.

Purification of BmLMCO

BmLMCO was purified using HisPur™ Ni–NTA Resin equilibrated with 50 mM phosphate buffer (pH 7.4) (Cordas

et al. 2022). The crude enzyme solution was loaded onto the resin and incubated at +4 °C for 1 h. Unbound proteins were eluted from the resin by passing the equilibration buffer through the column. The bounded proteins were eluted with 50 mM phosphate buffer (pH 7.4) containing imidazole at a final concentration of 200 mM. Fractions (1 mL) in which enzyme activity was detected were pooled, concentrated, and dialyzed for further studies.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram analysis

Protein solutions were mixed with 2X loading buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 25% glycerol, and 0.01% bromophenol blue) with β ME (5%) for SDS-PAGE analysis. Mixtures were incubated at 97 °C for 7 min and then loaded into the wells of stacking gel (5%). Separation was achieved in 10% polyacrylamide gel using Tris–glycine–SDS buffer under a constant voltage of 120 V using the Mini-Protean Tetra Cell Electrophoresis System (Bio-Rad Laboratories, Hercules, CA) (Laemmli 1970). Proteins were stained by Coomassie Brilliant Blue R-250 according to Zehr et al. (1989). The mixture of purified BmLMCO and β ME-free loading buffer was subjected to electrophoresis without incubation at 97 °C. The gel was used for zymogram analysis after performing the electrophoresis as described above. First, the gel was kept in potassium phosphate buffer containing Triton X-100 at a final concentration of 10 mM for 60 min to remove SDS. Afterward, it was washed with distilled water three times. Finally, the gel was incubated in 100 mM potassium phosphate buffer containing 0.2 mM CuCl_2 and 0.02 mM 2,6-DMP until a band corresponding to the laccase activity appeared (Yang et al. 2012).

Laccase assay and total protein assays

The laccase activity of BmLMCO was measured spectrophotometrically using 2,6-DMP as substrate. Briefly, the reaction was performed in 50 mM potassium phosphate buffer (pH 7.0) containing 2.96 mM 2,6-DMP and 0.27 mM CuCl_2 at 40 °C for 10 min. The activity was calculated based on the increase in absorbance of the reaction mixture at 470 nm (ΔG_{470}) using the following formula [Eq. (1)] (Baltierra-Trejo et al. 2015; Abdelgalil et al. 2022):

$$\text{Laccase activity} \left(\frac{U}{\text{mL}} \right) = \frac{\Delta G_{470} \times V_r \times D_f}{t \times \epsilon \times V_e}, \quad (1)$$

where V_r , D_f , t , ϵ , and V_e are volume of the reaction mixture (mL), dilution factor, reaction time (min), absorption coefficient incorporated with correction factor ($0.035645 \mu\text{M}^{-1} \text{cm}^{-1}$), and volume of enzyme solution (mL), respectively.

Total protein was determined by the Bradford method. Briefly, 1 mL of Bradford solution (10 mg of Coomassie Blue G250, 5 mL of 95% ethanol, 10 mL of 85% ortho-phosphoric acid, and 85 mL of distilled water) were added to 100 μL of protein solution. The mixture was vortexed for 5 s and incubated at room temperature for 5 min. Then, the absorbance of the mixture at 595 nm was determined spectrophotometrically (Thermo Scientific Multiskan GO). Total protein was quantified with the help of a standard curve drawn with different concentrations of BSA solution.

Biochemical characterization of BmLMCO

Lyophilized BmLMCO was used in the characterization studies. Lyophilization was carried out in the Karadeniz Advanced Technology Research and Application Center (KITAM) using Labconco FreeZone 12 Plus (Kansas City, Missouri, USA) for 48 h after the purified enzyme was frozen at -80 °C.

Effects of temperature and pH on the activity of BmLMCO

The influence of temperature on the activity of BmLMCO was investigated by assaying laccase activity in potassium phosphate buffer (pH 7.0) at different temperatures ranging from 20 to 90 °C as described earlier (laccase assay) (Birge et al. 2022). The optimum pH of BmLMCO was determined by assaying the laccase activity at different pHs ranging from 4 to 12 using sodium citrate (pH 4.0–6.0), potassium phosphate (pH 6.0–8.0), and Tris–HCl (pH 8.0–12.0) buffer systems (Olmeda et al. 2021; Cilmeli et al. 2022). The final concentration of BmLMCO in reaction mixtures was 0.25 mg/mL.

Effects of temperature and pH on the stability of BmLMCO

The thermostability of BmLMCO was examined by pre-incubation of the enzyme solution (1 mg/mL, pH 7.0) at 20–90 °C for 480 min following the calculation of residual activity at optimum temperature. The pH stability of BmLMCO was evaluated by pre-incubation of the enzyme at 0.25 mg/mL of final concentration in the above-stated buffer systems over the pH 4.0–12.0 for 60 min at room temperature. The initial laccase activity of the BmLMCO was accepted as 100%.

Effects of metal ions and chemicals on the activity and stability of BmLMCO

The laccase activity of BmLMCO (0.25 mg/mL) was separately assessed in the presence of various metal ions (Ag^{2+} , Ni^{2+} , Fe^{2+} , Co^{2+} , Cu^{2+} , K^+ , Mn^{2+} , Mg^{2+} , Ca^{2+} , and Zn^{2+}) and chemicals (EDTA, BME, Triton X-100, Tween 20,

PMSF, and DTT) at a final concentration of 1 mM under optimum conditions. Relative activities were calculated by comparison with the enzyme activity determined without reagents and presented as percentages (Ghatge et al. 2018). To determine the effect of metal ions and reagents on the stability, relative laccase activity (%) was calculated after BmLMCO (1 mg/mL) was kept with the reagents individually at room temperature for 60 min. The initial laccase activity of the BmLMCO was accepted as 100% (Jeon and Park 2020).

Effects of solvents on activity and stability of BmLMCO

The laccase activity of BmLMCO (0.25 mg/mL) was assayed in the presence of various organic solvents at a final concentration of 10% or 30% to assess their effect on the activity. Reactions were carried out at an optimum temperature and pH of BmLMCO. To investigate the influence of organic solvent on activity, a buffered BmLMCO solution (1 mg/mL) containing organic solvents (10% or 30%) was incubated at room temperature for 60 min before enzymatic assay. Afterward, their activities were detected at optimum pH and temperature. Results were expressed as percentages after calculating by comparing the activity of solvent-free buffered BmLMCO solutions incubated in the same conditions (100%) for the same time (Cilmeli et al. 2022).

Kinetic properties of BmLMCO

The kinetic parameters (K_m and V_{max}) of BmLMCO were calculated via the Lineweaver–Burk plot constructed by testing the activity of the enzyme toward different concentrations of 2,6-DMP (0.2–20 mM). Assays were performed at 80 °C in Tris–HCl buffer system (pH 8.0). Data from triplicate experiments were fitted to the Michaelis–Menten equation by linear regression (Olmeda et al. 2021).

Storage stability of BmLMCO

The laccase activity was assayed after BmLMCO solution (1 mg/mL), prepared in 50 mM potassium phosphate buffer (pH 8), and was kept at -20 and +4 °C for 0.5, 1, 2, 4, 8, 16, 32, and 48 days. The remaining activity was presented as a % relative to the initial activity.

Antibiotic removal potential of BmLMCO

The reaction was carried out in 50 mM potassium phosphate buffer containing 20 µg/mL antibiotic and 20 µg/mL lyophilized BmLMCO at 40 °C for 4 h. BmLMCO was removed by ultrafiltration after the reaction was completed. The enzyme-free reaction mixture was incubated under the same conditions for reaction control. On the other hand, 20 µg/mL

of the antibiotic solution was prepared in the same buffer to control antibiotic stability. All solutions were subjected to an agar well-diffusion experiment after passing through a 10 kDa molecular weight cut-off (MWCO) ultrafiltration membrane and sterilizing by a syringe filter. The agar well-diffusion experiment was done as the following procedure (Mazmancı et al. 2022). First, approximately 5×10^7 *B. subtilis* ATCC6633 cells were spread on Mueller–Hinton Agar (MHA). Wells with a diameter of 8 mm were created in MHA and then filled 150 µL of suspensions. The plate was incubated at 35 °C for 16 h. The diameters of the inhibitory zone (the area where bacteria cannot grow) surrounding the wells were determined. The ability of BmLMCO to reduce the antimicrobial activity of the antibiotic was calculated according to the formula Eq. (2)

$$\begin{aligned} \text{Decrease in antimicrobial activity (\%)} \\ = \left[100 - \frac{(E - 50.24) \times 100}{T_0} \right] \\ - \left[100 - \frac{(T_4 - 50.24) \times 100}{T_0} \right], \end{aligned} \quad (2)$$

where T_0 , E , and T_4 are inhibition zone areas of the antibiotic solution, reaction mixture after enzymatic treatment, and enzyme-free reaction mixture, respectively. The area of the well is calculated as 50.24.

Statistical analysis

Standard deviations and standard errors of results from each experiment in triplicate were calculated using Microsoft Excel Software. The statistical meaningfulness of the results was assessed by a one-way ANOVA test using XL Toolbox NG (<https://www.xltoolbox.net/>) tool integrated with the Microsoft Excel Software.

Results and discussion

The gene *mco* was successfully amplified from the genomic DNA of *B. mojavensis* TH309 using the PCR technique. Electrophoretic analysis showed that the PCR product was about 1500 bp in length, as predicted. The *mco* gene and pet14b(+), digested with XhoI restriction enzyme, hybridized by applying the ligation process. Transformation, selection, and subcloning were performed as described in Materials and methods. Afterward, the plasmid was extracted from the selected colony grown overnight at 37 °C in the presence of ampicillin. Orientation of the gene was checked and approved by sequencing and restriction digestion with HindIII and XhoI. Thus, the recombinant plasmid was named pet14b(+)-*mco*. Genomic DNA, PCR product, plasmids,

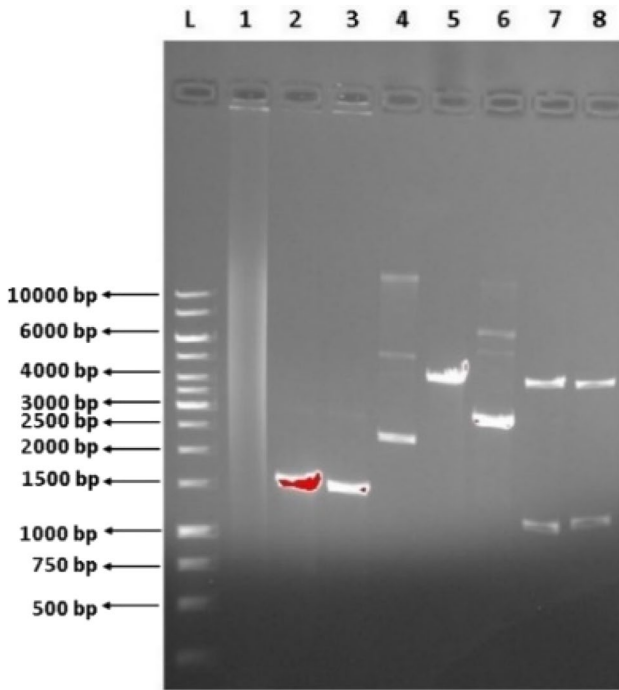


Fig. 1 Agarose gel (0.8%) DNA electrophoresis blot showing the different molecular structures used during this work: (L) ladder; (1) genomic DNA of *B. mojavensis* TH309; (2) amplified PCR products after amplification of *mco*; (3) PCR product digested by XhoI restriction enzyme; (4) pET14b(+); (5) pET14b(+) digested by XhoI restriction enzyme; (6) pET14b(+) after ligation (pET14b(+)-*mco*); (7) pET14b(+)-*mco* digested by HindIII restriction enzyme; (8) pET14b(+)-*mco* digested by XhoI restriction enzyme

and digested DNA fragments, which were obtained as a result of the processes applied during cloning, are shown in Fig. 1.

Sequences analysis showed that *mco* gene contains a 1584 bp length open reading frame (ORF) and encodes a

protein of 527 amino acid residues. Analysis with Signal P 6.0 (<https://services.healthtech.dtu.dk/service.php?SignalP>) elicited that the protein did not contain any signal peptide sequences. BLASTp analysis of the protein revealed that 78.72%, 74.67%, 73.72%, 73.53%, 61.93% 53.51%, and 51.04% identity with those of laccases from *Bacillus subtilis* (NCBI, AEK80414.1), *Lysinibacillus fusiformis* (NCBI, AYW03784.1), *Bacillus vallismortis* (NCBI, AGR50961.1), *Pseudomonas stutzeri* (NCBI, AND62506.1), *Bacillus amyloliquefaciens* (NCBI, QHT73050.1), *Bacillus pumilus* (NCBI, QKI37671.1), and *Bacillus licheniformis* (NCBI, QGX86460.1), respectively. Multiple sequence alignment revealed that BmLMCO, like other aligned laccases, has four conserved histidine-rich sequence motifs (105–107 HGH, 153–155 HDH, 428–433 HPIHLH, 503–510 HF CHSLEH) associated with the binding of coppers to the enzyme's T1, T2, and T3 centers (Solano et al 2001) (Fig. 2). Moreover, the phylogenetic neighbor-joining tree generated from the multiple sequence alignment is shown in Fig. 3. It showed that BmLMCO is in a different evolutionary branch from fungal laccases as well as some bacterial counterparts, including *Sinorhizobium meliloti*, *Pseudomonas stutzeri*, *Acetobacter ascendens*, and *Paenibacillus glucanolyticus*.

The secondary structure prediction of BmLMCO revealed that the enzyme consists of a high number of β -sheets ($\cong 27$) and three α -helix (Supplementary material 1). It was observed that 3% and 30% of the amino acid residues of BmLMCO are located in the alpha helix and the beta layers, respectively. PSIPRED analysis also showed that BmLMCO includes three domains with boundary locations at 181th and 336th amino acids. The three-dimensional structure of BmLMCO was plotted using a template (crystal structure of the CotA native enzyme) deposited in PDB with 4989.2.A code through SWISS-MODEL (Fig. 4). BmLMCO shared an identity 76.17% with the template and had a 0.79 of

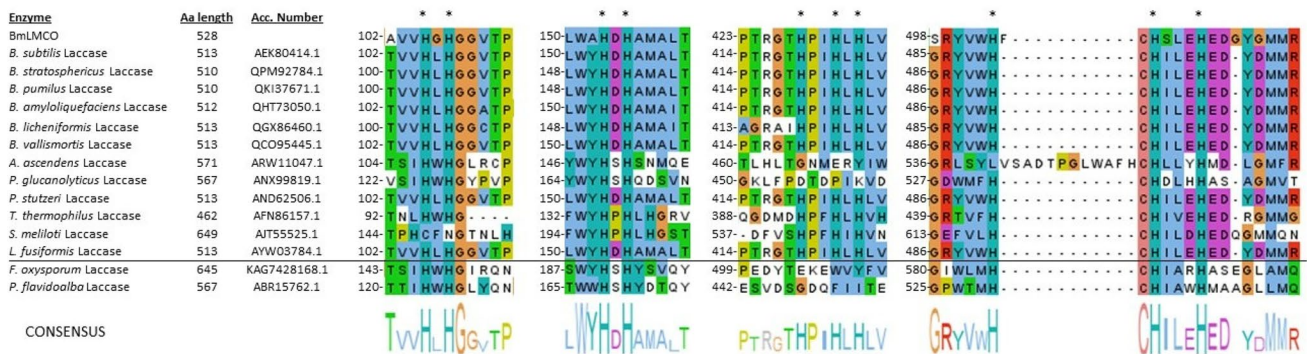


Fig. 2 Alignment of copper-binding motifs in BmLMCO and laccases from *Bacillus subtilis*, *Bacillus stratosphericus*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus vallismortis*, *Acetobacter ascendens*, *Paenibacillus glucanolyticus*, *Pseudomonas stutzeri*, *Thermus thermophilus*, *Sinorhizo-*

bium meliloti, *Lysinibacillus fusiformis*, *Fusarium oxysporum* f. sp. raphani, *Phanerochaete flavidoalba*. Acc. Number imply the accession number of the enzymes in NCBI database. The asterisk symbol (*) shows histidine residues associated with copper ions. The black line separates prokaryotic laccases from eukaryotic

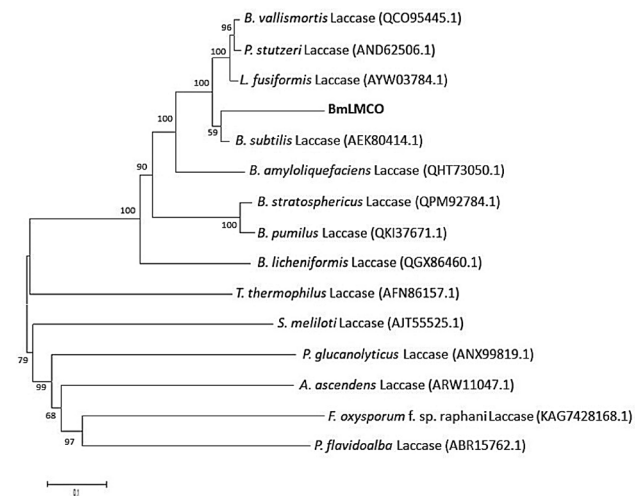


Fig. 3 The phylogenetic neighbor-joining tree of BmLMCO and some other laccases. The numbers at the nodes represent the bootstrap confidence-level percentage of 1000 copies

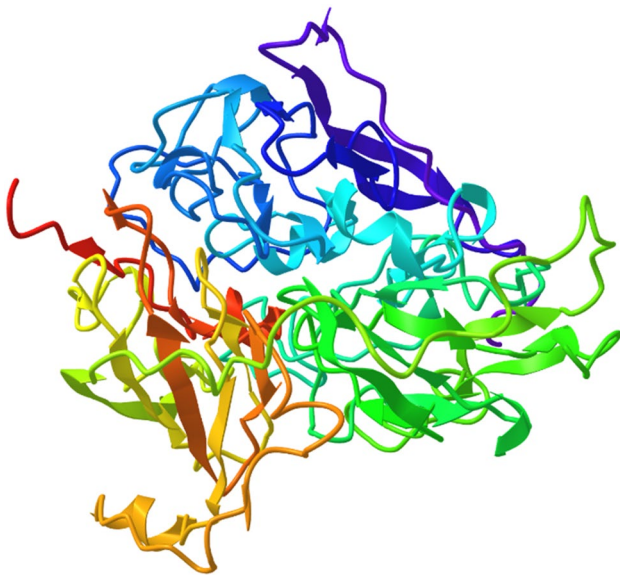


Fig. 4 Three-dimensional structure of BmLMCO plotted by SWISS-MODEL

GMQE value. Constructed structure of BmLMCO was highly compatible with its predicted secondary structure. Furthermore, it clearly showed that the enzyme has three cupredoxin domain with a β -barrel folding motif formed by the binding of antiparallel/parallel β -strands with β -turn and relatively large loops. Considering that laccases (except for a few archaeal and bacterial laccases) and ascorbate oxidases (found in plants and fungi) contain three cupredoxin domains, unlike ferroxidases, nitrite oxidases, and ceruloplasmin found in MCO, structural analysis shows that the enzyme is a laccase-like multicopper oxidase (Nakamura

and Go 2005). According to ProtParam analysis, the computed isoelectric point (pI) of BmLMCO was 6.80. The half-life of BmLMCO was estimated to be 30 h, >20 h, and >10 h in mammalian, yeast, and *E. coli* cells, respectively. The aliphatic index and grand average of hydropathicity (GRAVY) values were 72.63 and -0.468, respectively, which indicated that the enzyme was thermally stable and hydrophilic.

First, lysates of *E. coli* BL21(DE3), *E. coli* BL21(DE3) with pET14b(+), and *E. coli* BL21(DE3) with pET14B(+)-mco cells grown in the absence and presence of IPTG were analyzed by SDS-PAGE (Fig. 4a). A distinct protein band with a molecular weight of about 65 kDa in lysates of *E. coli* BL21(DE3) cells harboring pET14b(+)-mco, unlike lysates of *E. coli* BL21(DE3) cells with and without pet14b(+) indicated that BmLMCO was expressed heterologously. Furthermore, laccase activity was only observed in the lysate of *E. coli* cells harboring pET14b(+)-mco.

Crude enzyme solution with 121.71 ± 6.71 U/mg of specific laccase activity subjected to Ni-NTA chromatography and thus BmLMCO was purified 3.25 ± 0.09 -fold with 32.86 ± 0.91 of the final recovery yield. BmLMCO was confirmed to have a molecular weight of ≈ 65 kDa by SDS-page analysis performed after purification (Fig. 5a). This value was similar to that of laccases and LMCOs from *Lactobacillus plantarum* (Callejón et al. 2016), *B. licheniformis* (Koschorreck et al. 2008; Chopra and Sondhi 2022), *B. subtilis* (Cheng et al. 2021), *Bacillus aquimaris* (Kumar et al. 2022), and *B. pumilus* (Reiss et al. 2011; Yan et al. 2022). Zymogram analysis resulted in the appearance of an

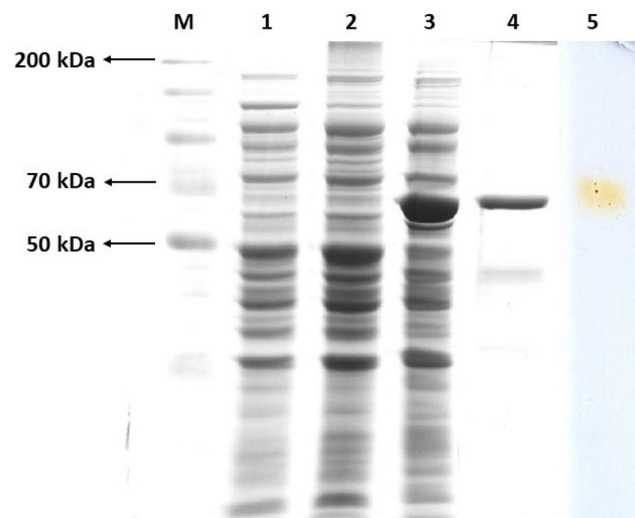


Fig. 5 SDS-PAGE showing the protein cell lysate of the normal and recombinant bacteria. (M) Molecular weight protein ladder; (1) *E. coli* BL21(DE3) protein cell lysate; (2) *E. coli* BL21(DE3)—pET14b(+) protein cell lysate; (3) *E. coli* BL21(DE3) pET14b(+)-mco protein cell lysate; (4) SDS-PAGE of the purified BmLMCO protein. (5) Zymography of BmLMCO after purification revealed with 2,6-DMP

orange band resulting from the laccase activity of BmLMCO (Fig. 5b).

The relative laccase activity of BmLMCO was above 80% in the temperature range of 50–70 °C, and still above 73% at 90 °C and 40 °C. The relative activity reached its optimum level at 80 °C (Fig. 6a). However, its activity significantly dropped off when the reaction temperature was decreased to 20 °C and 30 °C. The same temperature optima has been reported for laccases from *Geobacillus* sp. JS12 (Jeon and Park 2020), *B. velezensis* (Li et al. 2020), and *Enterococcus faecium* A2 (Birge et al. 2022). The temperature optima of BmLMCO was higher than those of thermostable/thermotolerant laccases from *Thioalkalivibrio* sp. ALRh (Ausec et al. 2015), *Meiothermus ruber* (Kalyani et al. 2016), *Klebsiella pneumoniae* (Liu et al. 2017), *Caldalkalibacillus thermarum* (Ghatge et al. 2018), *Bacillus* sp. PC-3 (Sharma et al. 2019), *Achromobacter xylosoxidans* (Unuofin et al. 2019), *Anoxybacillus ayderensis* (Wang et al. 2020a, b), *Geobacillus* sp. ID17 (Atalah and Blamey 2022), *Methylobacterium extorquens* (Ainiwaer et al. 2022), and *B. licheniformis* VNQ (Sharma et al. 2022). On the other hand, the laccase obtained from *Thermus thermophilus* (Stevens et al. 2020),

B. vallismortis fmb-103 (Zhang et al. 2013), *Bacillus tequilensis* (Sondhi et al. 2014), and *Anoxybacillus* sp. UARK-01 (Al-kahem Al-balawi et al. 2017) showed optimal activity at above 80 °C.

BmLMCO exhibited oxidizing activity against 2,6-DMP at all pH values (4–12) (Fig. 6b). Relative activity was over 70% between pH 7 and 12, and maximum activity was measured at pH 8.0 in both potassium phosphate and tris-HCl buffers. As with many bacterial laccases, the relative activity was found to be over 50% in low pH environments (pH 5–7) (Mandic et al. 2019; Cheng et al. 2021; Birge et al. 2022). Our study findings showed that BmLMCO could be used effectively in biotechnological applications in alkaline, neutral, and moderately acidic conditions.

The thermal stability of BmLMCO assessed by measuring the residual enzyme activity against 2–4 DMP after pre-incubation at temperatures ranging from 20 to 90 °C for 480 min is shown in Fig. 7a. BmLMCO was pretty stable up to 40 °C. It exhibited moderate stability at 50–70 °C. The calculated half-life of BmLMCO was 98.66 ($R^2:0.9268$), 49.90 ($R^2:0.9103$), and 45.97 ($R^2:0.8522$) at 50, 60, and 70 °C, respectively. However, its activity dropped to below

Fig. 6 **a** The effect of temperature on BmLMCO activity. **b** The effect of pH on BmLMCO activity. pHs of reaction mixtures were adjusted using sodium citrate (pH 4–6, Δ), potassium phosphate (pH 6–9, \circ), and Tris-HCl (pH 9–12, \diamond) buffers. The measured maximum laccase activity was accepted as 100%

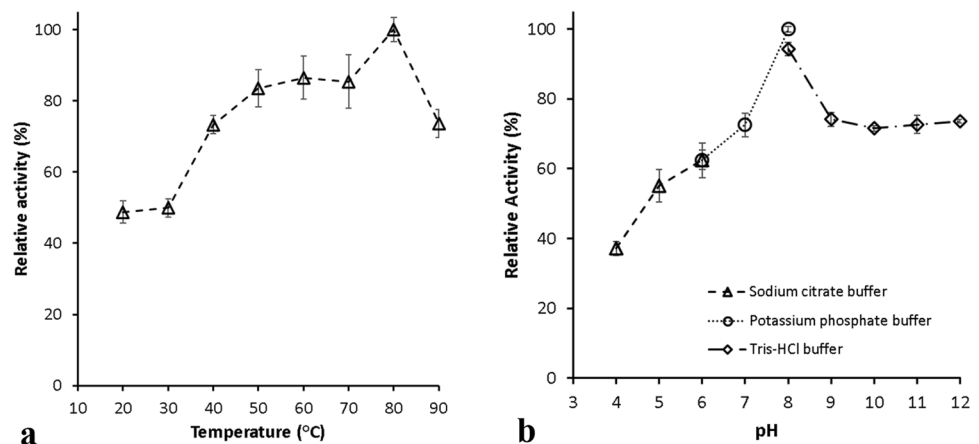
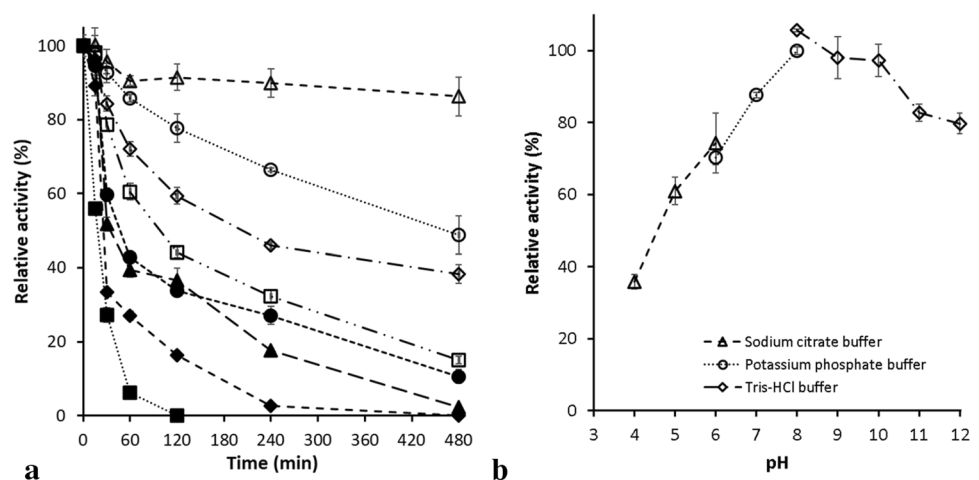


Fig. 7 **a** The effect of temperature on BmLMCO stability at 20 °C (Δ), 30 °C (\circ), 40 °C (\diamond), 50 °C (\square), 60 °C (\blacktriangle), 70 °C (\bullet), 80 °C (\blacklozenge), and 90 °C (\blacksquare). **b** The effect of pH on BmLMCO stability. Sodium citrate (pH 4–6, Δ), potassium phosphate (pH 6–9, \circ), and Tris-HCl (pH 9–12, \diamond) buffers were used in experiments. Initial laccase activity of BmLMCO assessed as 100%



50% after incubation at 80 and 90 °C for 30 min. It is well known that fungal laccases have lower thermal stability than their bacterial counterparts. On the other hand, it has been reported that some bacterial laccases can maintain their stability at high temperatures. It was reported that the half-life of laccase from *B. amyloliquefaciens* was 87 min at 70 °C and 42 min at 80 °C, respectively (Wang et al. 2020b). Lončar et al. (2016) reported that a thermostable and organic solvent-tolerant laccase from *B. licheniformis* ATCC 9945a lost half of its activity after pre-incubation at 60 °C for 100 min and at 70 °C for 59 min. In another study, it was reported that the temperature value at which the Pp4816 laccase from *Pediococcus pentosaceus* could maintain half of its activity after 5 min of pre-incubation (T_{50}) was 83.7 °C (Olmeda et al. 2021). The thermo-tolerant property of BmLMCO may be due to the tight packing of the native protein around the copper centers (Fan et al. 2015).

Results plotted in Fig. 7b showed that BmLMCO was stable over a broad pH range (pH 5–12). Even more, the activity of BmLMCO increased by 5% from that of the initial after pre-incubated at pH 8 for 1 h. The relative activity of BmLMCO, pre-incubated at pH 10.0 in Tris HCl buffer, was 97.18%. Moreover, in the same buffer, it retained more than 79% of its activity against 2,4-DMP after pre-incubation at pH 12.0. The alkaline tolerant capacity of BmLMCO was superior to that of many laccases or LMCOs from bacteria (Chauhan and Jha 2018; Ainiwaer et al. 2022).

BmLMCO activity was tested in the presence of metal ions at a final concentration of 1 mM (Fig. 8). Results showed that Ag^{2+} and Cu^{2+} enhanced the activity of BmLMCO by 1.73% and 28.53%, respectively. However, the metal ions Fe^{2+} , Co^{2+} , K^+ , Mg^{2+} , and Zn^{2+} inhibited

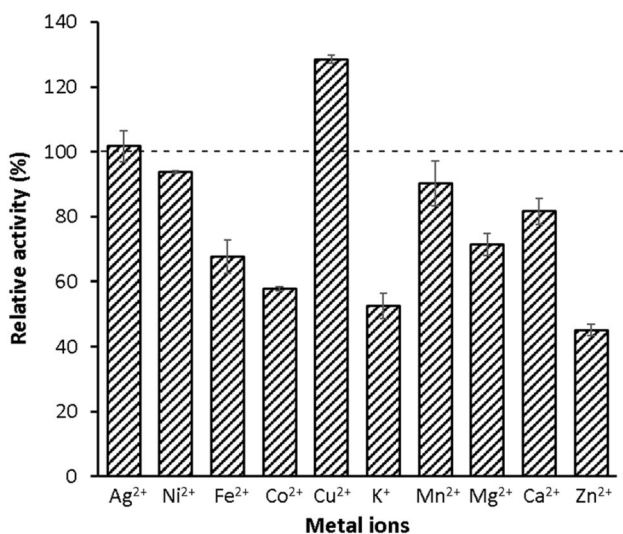


Fig. 8 The effect of metal ions (1 mM) on BmLMCO activity. The activity of BmLMCO in the absence of metal ions was evaluated as 100%

the enzyme activity significantly. Among metal ions, Ni^{2+} , Mn^{2+} , and Ca^{2+} had little influence on BmLMCO activity. The effect of metal ions on the stability of BmLMCO was also tested (data was not shown). Pre-incubation of BmLMCO with Cu^{2+} increased its catalytic activity toward 2,4-DMP. On the other hand, the enzyme showed residual activity ranging from 78.28% to 99.32% after pre-incubation with Ag^{2+} , Ni^{2+} , Fe^{2+} , Co^{2+} , and Ca^{2+} for 60 min at room temperature. It is well known that copper accelerates the activity of many laccases (Sondhi et al. 2014; Chauhan and Jha 2018). However, the effects of other metal ions on the activities of laccases cannot be generalized. Chauhan and Jha (2018) reported that 1 mM Na^+ , K^+ , Pb^{2+} , Ca^{2+} , Cu^{2+} , and Co^{2+} promoted the activity of laccase from *Pseudomonas* sp. S2. In another study, Zhang et al. (2022) found that K^+ , Co^{2+} , Ni^{2+} , and Zn^{2+} stimulated the activity of laccase obtained from *Psychrobacter* sp. NJ228.

The effect of various chemicals on the activity of BmLMCO is depicted in Fig. 9. Accordingly, no noticeable reduction in BmLMCO activity was seen in the presence of DTT, tween 20, triton X-100, and SDS. Adding PMSF, β ME, and EDTA to the reaction mixture decreased BmLMCO activity by 20.07%, 35.00%, and 22.58%, respectively. Pre-incubation of the enzyme with DTT and SDS for 60 min increased the activity by 9.15% and 6.13%, respectively (data were not shown). The remaining enzyme activity was still higher than 88% after pre-incubation with the other chemicals except for, β ME. Similarly, SDS-activated laccases from *B. licheniformis* (Lu et al. 2013), *B. tequilensis*

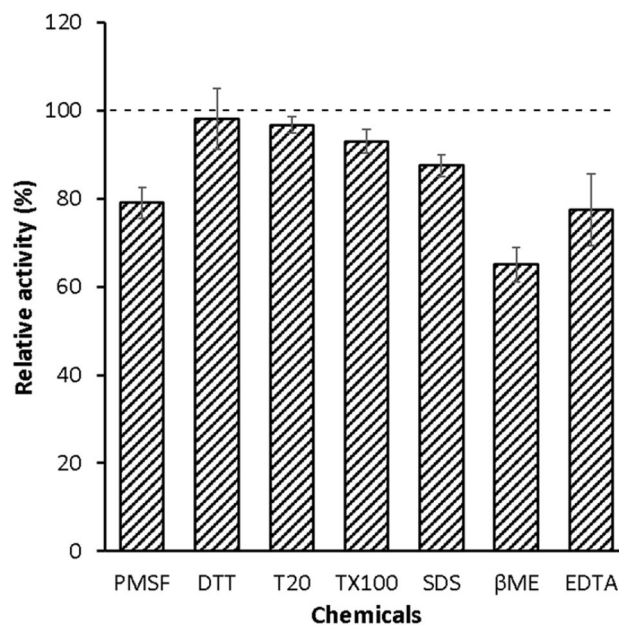


Fig. 9 The effect of chemicals (1 mM) on BmLMCO activity. The activity of BmLMCO in the absence of chemicals was evaluated as 100%

SN4 (Sondhi et al. 2014), and *Alcaligenes faecalis* (Mehandia et al. 2020) have also been reported previously. Results also showed that BmLMCO was more stable than that of many previously reported laccases or LMCOs (Neelkant et al. 2020; Wang et al. 2020b; Olmeda et al. 2021).

The influences of various organic solvents on BmLMCO activity are illustrated in Fig. 10. The presence of acetone, chloroform, or isopropanol at a final concentration of 30% in the reaction mixture caused an increase in the activity

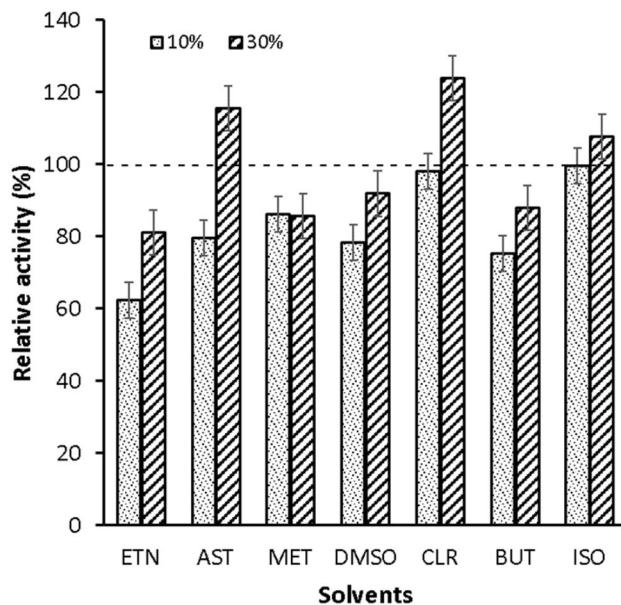


Fig. 10 The effect of various solvent (10% and 30%) on BmLMCO activity. The activity of BmLMCO in the absence of solvents was assessed as 100%

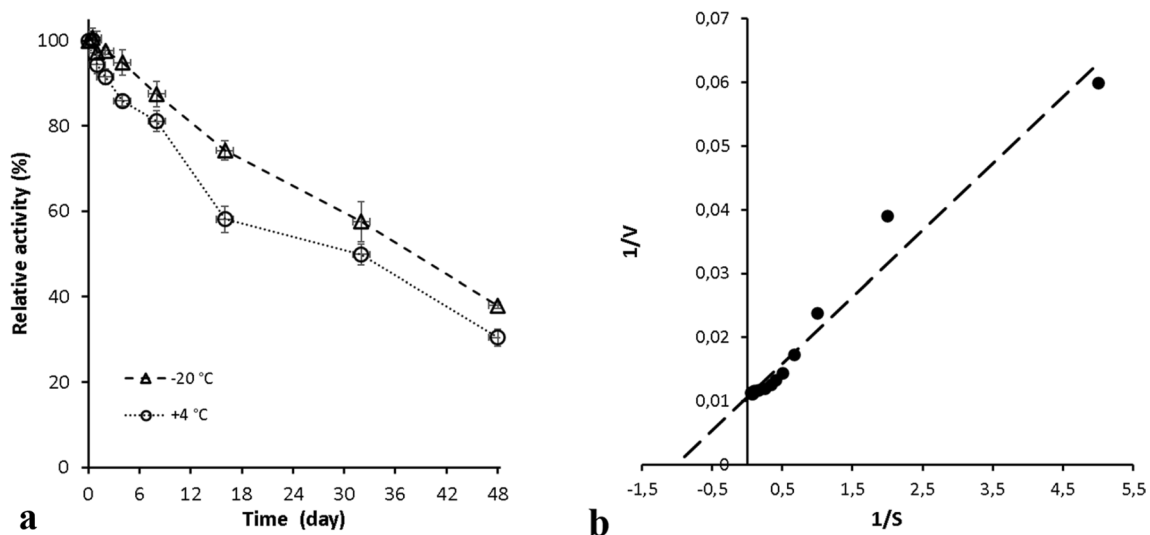


Fig. 11 **a** Storage stability of BmLMCO at $-20\text{ }^{\circ}\text{C}$ and $+4\text{ }^{\circ}\text{C}$. The initial 2,4-DMP oxidizing activity of the enzyme was assumed to be 100%. **b** The Lineweaver–Burk plot of BmLMCO activity toward 2,4-DMP under optimum conditions

up to 123.83%. A moderate increase in laccase activity was also detected by pre-incubation of BmLMCO with chloroform at a final concentration of 10% (data was not shown). On the other hand, ethanol affected adversely the activity and stability. In addition, BmLMCO was pretty much compatible with other tested solvents.

The results of the storage stability study are illustrated in Fig. 11a. The relative activity of lyophilized BmLMCO was 57.53% and 49.86% after the enzyme was stored for 32 days at $-20\text{ }^{\circ}\text{C}$ and $+4\text{ }^{\circ}\text{C}$, respectively. In addition, BmLMCO maintained 37.98% and 30.47% of its activity at the end of the 48-day storage period at $-20\text{ }^{\circ}\text{C}$ and $+4\text{ }^{\circ}\text{C}$, respectively. Xu et al. (2015) observed a more than about 60% reduction in the activity of laccase from *T. versicolor* after 10 days of storage at $+4\text{ }^{\circ}\text{C}$. Qiu et al. (2021) reported an activity loss in laccase from *Aspergillus oryzae* was over 80% after a storage period of 15 days at $+4\text{ }^{\circ}\text{C}$.

K_m and V_{max} values of BmLMCO were 0.98 mM and 93.45 $\mu\text{mol}/\text{min}$, respectively, with 2,6-DMP as the substrate (Fig. 11b). The K_m value of BmLMCO was lower than those of laccases from *B. tequilensis* SN4 (Sondhi et al. 2014), *Streptomyces viridochromogenes* (Trubitsina et al. 2015), *Thioalkalivibrio* sp. ALRh (Ausec et al. 2015), *Lactobacillus plantarum* (Callejón et al. 2016), *Paenibacillus glucanolyticus* (Mathews et al. 2016), *K. pneumoniae* (Liu et al. 2017), *Geobacter metallireducens* (Berini et al. 2018), and *P. pentosaceus* (Olmeda et al. 2021), while higher than those of laccases from *M. ruber* (Kalyani et al. 2016), *Aquisalibacillus elongatus* (Rezaei et al. 2017), *A. faecalis* (Mehandia et al. 2020), and *B.*

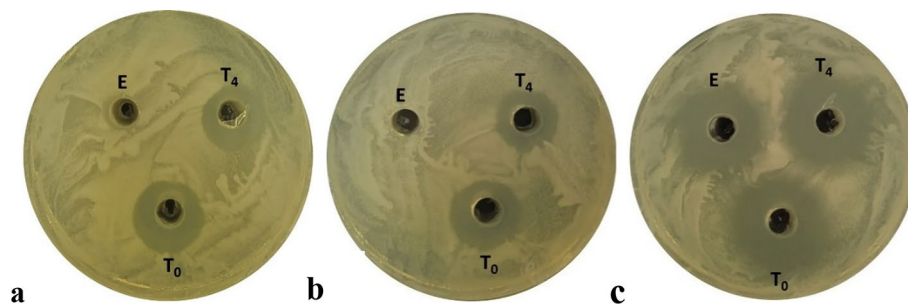


Fig. 12 a Antimicrobial activity of cefprozil solution (T_0), the enzyme-free reaction mixture (T_4), and the reaction mixture after enzymatic treatment. **b** Antimicrobial activity of gentamicin solution (T_0), the enzyme-free reaction mixture (T_4), and reaction mixture

after enzymatic treatment. **c** Antimicrobial activity of erythromycin solution (T_0), the enzyme-free reaction mixture (T_4), and reaction mixture after enzymatic treatment

licheniformis VNQ (Sharma et al. 2022) for the same substrate.

Antibiotic removal potential of BmLMCO

The potential for the use of BmLMCO in antibiotic removal was evaluated based on the loss in antimicrobial activity of antibiotics. Cefprozil, gentamicin, and erythromycin, classified as cephalosporin, aminoglycoside, and macrolide antibiotics, were used and treated with BmLMCO in a potassium phosphate buffer system at 40 °C for 4 h. A decrease in their antimicrobial effect was observed with well-diffusion experiment is shown in Fig. 12(d-f). BmLMCO caused the $72.3 \pm 1.5\%$, $79.6 \pm 6.4\%$, and $19.7 \pm 4.1\%$ decreases in the antimicrobial activity of cefprozil, gentamicin, and erythromycin, respectively. The excellent effect of BmLMCO on gentamicin and cefprozil is most likely due to the opening of the beta-lactam ring, which causes the antimicrobial effect in antibiotics as a result of catalytic activity. Their chemical structures are shown in Supplementary material 2. To sum up, results showed that BmLMCO can be used for removal of antibiotics.

Conclusion

The *mco* gene from *B. mojavensis* is cloned and expressed in *E. coli*, successfully. The enzyme called BmLMCO showed excellent activity and moderate stability at elevated temperatures. It was active and stable over a broad pH range. BmLMCO tolerated various metal ions, chemicals, and solvents considerably. In addition, it diminished the antimicrobial activity of cefprozil, gentamicin, and erythromycin. BmLMCO, with the aforementioned remarkable properties, is a promising candidate to meet the needs of biotechnological applications like the degradation of emerging pollutants.

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Author contributions AOA: project administration, validation, data analysis, visualization, writing, and methodology. SK-A: writing, investigation, and validation. SC: formal analysis. BM: methodology, writing, and curation. EY: data analysis, writing, and validation. SÜ-O: methodology, investigation, and validation. NGK: formal analysis. MAM: supervision, writing, and curation.

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Availability of data and materials Data will be made available on reasonable request.

Declarations

Conflict of interest No potential conflict of interest was reported to the authors. In addition, the authors have no financial interests to disclose.

Ethical approval This study does not involve the need for ethical approval.

Consent to participate Not applicable.

Consent to publish Not applicable.

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