Letters in Applied NanoBioScience
Open-Access Journal (ISSN: 2284-6808)

Article
Volume 12, Issue 4, 2023, 144
https://doi.org/10.33263/LIANBS124.144

Purification and Characterization of an Extracellular Thermostable Laccase from *Bacillus cereus* UV25 and its Potential in Enrichment of Fruit Juices

Urvashi Bhankar [1], Vijay Kumar Gupta [1,*]

[1] Department of Biochemistry, Kurukshetra University, Kurukshetra-136119, Haryana, India; urvashi.rimpi@gmail.com (U.B.); vkgupta59@rediffmail.com (V.K.G.);
* Correspondence: vkgupta59@rediffmail.com (V.K.G.);

Scopus Author ID 56972366300
Received: 25.08.2022; Accepted: 23.09.2022; Published: 17.11.2022

Abstract: As there is scarce information on the characterization and application of bacterial laccases in fruit juice processing, this work was envisaged to purify and characterize an extracellular laccase secreted by *Bacillus cereus* UV25 for evaluating its potential in the enrichment of fruit juices. Laccase was produced in submerged fermentation and purified to homogeneity with 14.73-fold and 22.82 % recovery using gel filtration chromatography. Purified laccase was a monomeric protein of 25.3 kDa exhibiting K_m and V_{max} for guaiacol as 10.69 mM and 42.37 IU/L, respectively. It showed optimum activity at 70 °C and pH 4.2. It exhibited significant stability at 70-80 °C, pH 3.8-4.8, and during storage, at 4°C. Laccase activity was enhanced by Mn^{2+}, Mg^{2+}, Cu^{2+}, Na^{+}, Ca^{2+}, Tween 20, Tween 80, and Triton X-100 but inhibited by Fe^{2+}, K^{+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Co^{2+}, EDTA, SDS, sodium azide, cysteine, and β-mercaptoethanol. It was stable at 4°C for four weeks without any activity loss. Physico-chemical analysis of juices obtained from laccase-treated apple and mango fruit pulps showed an improvement in juice characteristics. The properties of this enzyme pose it a potential candidate for biotechnological applications.

Keywords: *Bacillus cereus*; extracellular laccase; purification; characterization; fruit juice enrichment.

1. Introduction

Laccases (Benzenediol: oxygen oxidoreductases; EC 1.10.3.2) are multicopper-containing oxidases capable of oxidizing a wide range of phenolic and non-phenolic compounds with simultaneous reduction of molecular oxygen (as a final electron acceptor) to produce water as a by-product [1]. They are termed "green biocatalyst" as they use only oxygen to oxidize their substrates. Owing to their broad substrate specificity, these enzymes find diverse applications, including paper pulping and bleaching, textile refining, dye decolorization, detoxification of industrial pollutants, biosensors, bioremediation, production of anti-cancerous drugs, food industry, pharmaceutical industry, etc. [2-5]. Laccases have been found in plants, fungi, bacteria, and insects, where they perform different physiological functions. Fungal laccases have been used in most studies because of their high redox potential. Still, their long fermentation period and inability to withstand harsh conditions of temperature, pH and salt prevailing in the industry have hampered their use in several applications [1]. To overcome these challenges, it may be advantageous to use bacterial laccases owing to their shorter duration of production, better stability at high temperatures and pH values, and

suitability for genetic improvement, which are important for their biotechnological application [1,3,6,7].

Several bacterial species from the genus *Bacillus*, *Pseudomonas*, *Alcaligenes*, *Serratia*, *Streptomyces*, etc., are known to produce laccases [8-19]. Most bacterial laccases are either intracellular or spore confined, making their industrial application unfeasible due to the higher cost of purification [8,20]. The bacteria-producing thermostable and salt-tolerant laccases have received considerable scientific attention for suitability in industry. It would be desirable to search for such thermostable and salt-tolerant bacterial strains which could produce extracellular laccase for industrial applications. Extracellular enzymes are preferred for industrial applications due to their lower purification cost. Some biotechnological applications of enzymes, for example, in the food industry, exclusively require these in purified form. There are limited reports on the purification of extracellular laccases from bacterial species [8,9,13,15,21].

Fruit juices are non-fermented beverages obtained by mechanically squeezing or macerating fruits. These are preferred by consumers owing to their nutritional and health-promoting benefits. Apple and mango fruit juices are commonly consumed by people, rich in micro-nutrients, fiber, and antioxidants, as well as their positive effects on human health. During storage, raw fruit juices may become turbid due to polysaccharides (cellulose, hemicellulose, and pectins), phenolics, and proteins leading to reduced shelf-life and consumer acceptability [22]. Phenolic compounds present naturally in fruits provide nutritional and therapeutic benefits [23] besides imparting color and taste to the juices [24]. However, excessive oxidation and polymerization reactions of the phenolic compounds in fruit juices result in unwanted changes in their organoleptic properties affecting the product quality and shelf-life [24]. Enzymatic browning is the most common color change, which varies in magnitude depending on the concentration of polyphenols naturally present in these juices [24]. The polyphenols in fruit juices may form complexes with proteins resulting in turbidity, browning, and an unpleasant taste during storage [23]. Enzymes are considered effective processing aids to improve fruit juices' yield, clarity, and stability [25]. Laccase can be employed to clarify and stabilize fruit juices by removing undesirable phenols responsible for browning and turbidity [26]. Mostly, fungal laccases have been used in fruit juice processing [26]. Exploiting bacterial strains producing extracellular laccases with desired characteristics for industrial application is likely important to meet their commercial needs.

In the present study, an extracellular thermostable laccase has been purified and characterized from moderately salt-tolerant *Bacillus cereus* UV25. Its potential in the enrichment of apple and mango juices has been evaluated.

2. Materials and Methods

2.1. Bacterial strain.

B. cereus UV25 (Accession number: MH916571), used for purification of extracellular laccase, was isolated in our laboratory from soil contaminated with decaying wood using nutrient agar medium containing 0.1% guaiacol. The purified bacterial culture was grown and maintained on nutrient agar slants at 4°C.

2.2. Production of extracellular laccase by B. cereus UV25.

Laccase was produced from *B. cereus* UV25 under optimized submerged fermentation (SmF) conditions using a modified Horikoshi medium comprising 5.0 g peptone, 5.0 g beef extract, 5.0 g yeast extract, 5.0 g KNO_3, 0.2g KH_2PO_4, 0.1g $MgSO_4.7H_2O$ and 1.0 mL guaiacol in one liter of water, pH 7.0. The culture filtrate was collected and centrifuged at 10,000 x g for 20 min at 4℃. The resulting clear supernatant (crude enzyme extract) was used to purify laccase.

2.3. Laccase activity assay.

Laccase activity was determined by following the method of Das *et al.* [27]. The reaction mixture for laccase assay containing 1.9 mL of 0.1 M sodium acetate buffer (pH 4.2), 0.5 mL of 0.1 M guaiacol, and 0.1 mL enzyme was incubated at 37°C for 15 min. Enzyme and substrate controls were run simultaneously. The absorbance of the resulting colored product in test, enzyme control, and substrate control tubes was measured against distilled water at 470 nm in a Spectrophotometer. The absorbance values of enzyme and substrate controls were subtracted from the absorbance of the test. Each enzyme assay was carried out in duplicates. The laccase activity was calculated using an extinction coefficient of 6740 $M^{-1}cm^{-1}$ and expressed in IU/L. One enzyme unit was defined as the amount of enzyme catalyzing the production of one µmol of colored product per min under specified enzyme assay conditions. Protein content in the culture supernatant and pooled fractions at every step of purification was estimated according to Lowry *et al.* [28] using bovine serum albumin (BSA) as a standard protein.

2.4. Purification of laccase.

Laccase was purified at 4℃ by gel filtration chromatography using Sephadex G-25 and Sephadex G-100. A glass column (30 cm x 2.6 cm) was packed with swollen Sephadex G-25 (Pharmacia) and equilibrated with 0.1M sodium acetate buffer (pH 4.0). The crude enzyme extract obtained from *B. cereus* UV25 culture filtrates was loaded onto the top of the gel in the column and eluted with the above buffer at a flow rate of 30 mL/h. Fractions (3.0 mL each) were collected and analyzed for laccase activity using guaiacol as substrate. The most active fractions were pooled, and the pooled fraction was analyzed for enzyme activity and protein content.

The pooled enzyme fraction obtained from Sephadex G-25 was loaded on a column of Sephadex G-100 (82.6 cm x 1.6 cm) and eluted with 0.1M sodium acetate buffer (pH 4.0) at a flow rate of 15 mL/h. Fractions (3 mL each) were collected and analyzed for protein by recording their absorbance at 280 nm in a UV-VIS spectrophotometer. The protein-containing fractions were examined for laccase activity, and the most active fractions were pooled. The pooled fraction was analyzed for laccase activity and protein content (by Lowry's method).

2.5. Testing of enzyme purity and zymography.

Enzyme homogeneity was tested by performing Native-PAGE [29] and SDS-PAGE [30] using a 12% resolving polyacrylamide gel. Protein bands were stained with Coomassie brilliant blue R-250. Zymogram analysis was performed by Native-PAGE under non-denaturing conditions at 4℃ using a slightly modified method of Patel *et al.* [31]. For

zymography, the sample was not heated before loading. After electrophoresis, the gel was equilibrated in 0.1 M sodium acetate buffer (pH 4.0) and then transferred to the same buffer containing 5 mM ABTS and incubated in the dark for 15–30 min with gentle shaking until the appearance of green-colored bands. The appearance of green bands indicated laccase activity due to the oxidation of ABTS.

2.6. Characterization of purified laccase.

Purified laccase was characterized for its molecular weight (MW), the effect of pH, temperature, metal ions and additives, kinetic parameters (K_m and V_{max}), and storage stability.

2.6.1. Determination of molecular weight.

The MW of laccase was determined by gel filtration through Sephadex G-100 and SDS-PAGE. A column of Sephadex G-100 (95 cm × 0.8 cm), pre-equilibrated with sodium acetate buffer (0.1 M, pH 4.0), was loaded with a mixture of gel filtration protein MW markers (Sigma-Aldrich, USA) containing albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa) and aprotinin (6.5 kDa) which were eluted at a flow rate of 15 mL/h. Fractions of 3.0 mL each were collected, and their absorbance was read at 280 nm. A graph was plotted between A_{280} and the fraction number. This graph was used to calculate each protein's elution volume (Ve) (volume of eluent from the point of injection into the column to the center of the elution peak). The purified enzyme was also run through the column under identical conditions to determine its elution volume (Ve). A graph was plotted between V_e/V_o on the x-axis and log MW of the marker proteins on the y-axis to determine the MW of laccase.

In SDS-PAGE, purified laccase and the standard ladder of protein broad range MW markers (205.9, 97.4, 66, 43, 29, 20.1, and 14.3 kDa) were resolved using a 12% gel. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 and then destained to visualize the protein bands. The distance traveled by the tracking dye, as well as protein bands, was noted. The relative mobility (R_m) of each protein band was determined as follows:

$$Relative\ mobility = \frac{Distance\ moved\ by\ the\ protein\ band}{Distance\ moved\ by\ the\ tracking\ dye}$$

A logarithmic plot between MW of the marker proteins versus the corresponding R_m was used to estimate the MW of laccase.

2.6.2. Effect of pH.

The effect of pH on purified laccase was studied in terms of pH optimum and pH stability. The optimum pH of the reaction was determined by carrying out the enzyme assay over the pH range 3.8-12.0 using 0.1M buffers of different pH *viz.* acetate buffer (pH 3.8-6.0), phosphate buffer (pH 6.0-8.0), Tris-HCl (pH 8.0-9.0) and glycine-NaOH (pH 10.0-12.0). Relative activity (%) was calculated concerning the maximum activity taken as 100%. A graph was plotted between pH on the x-axis and relative laccase activity (%) on the y-axis. The pH corresponding to the highest laccase activity was the optimum.

The pH stability of laccase was investigated by pre-incubating an aliquot 0.50 mL of enzyme and 0.50 mL of 0.1M acetate buffer in the pH range 3.8-5.0 in the absence of substrate for 6 h at room temperature followed by measurement of enzyme activity using the standard

assay. A control was run simultaneously in which the enzyme was incubated with distilled water instead of a buffer. The residual enzyme activity at each pH value was calculated as a percent of the control. The pH stability profile of laccase was drawn between pH on the x-axis and residual activity (%) on the y-axis.

2.6.3. Effect of temperature.

The effect of temperature on purified laccase was studied in terms of optimum temperature and thermal stability. The optimum temperature of the reaction was determined by performing the enzyme assay at different temperatures ranging from 30-80ºC. Relative activity (%) was calculated concerning the maximum activity taken as 100%. A graph was plotted between the temperature on the x-axis and relative laccase activity (%) on the y-axis. The temperature corresponding to the highest laccase activity was taken as the optimum temperature.

The thermal stability of purified laccase was investigated by pre-incubating the enzyme at different temperatures ranging from 37–80 ºC for 6 h in the absence of the substrate, followed by determining laccase activity using the standard assay. Residual enzyme activity at each temperature was calculated as the percent of the control in which the enzyme was not pre-incubated. The thermostability profile of the laccase was drawn between the temperature on the x-axis and residual activity (%) on the y-axis.

2.6.4. Effects of metal ions and additives.

The effect of various metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, and Co^{2+}) on purified laccase was investigated by pre-incubating the enzyme (along with assay buffer) with each metal ion at a concentration of 1 mM for 10 min followed by addition of the substrate and incubation at 37 ℃ for 15 min under optimal assay conditions. Relative activity (%) was calculated concerning the control in which no metal ion was added.

Effect of various additives *viz.* EDTA, SDS, cysteine, sodium azide, β-mercaptoethanol, Tween 20, Tween 80, and Triton X 100 on the activity of purified laccase was assessed by pre-incubating the enzyme (along with assay buffer) with the tested compound followed by addition of substrate to the reaction mixture and incubated at 37℃ for 15 min under optimal assay conditions. All the additives were added at a concentration of 1 mM, except Tween 20, Tween 80, and Triton X-100, which were used at 1% (v/v) concentration. Relative activity (%) was determined concerning the control lacking any additive.

2.6.5. Determination of K_m and $V_{max.}$

The activity of purified laccase was determined at different concentrations (2.5 to 40 mM) of the substrate guaiacol. A Lineweaver-Burk plot (1/v *vs* 1/[S]) was then drawn to calculate the K_m and V_{max} of laccase.

2.6.6. Storage stability of purified laccase.

Purified laccase was kept at 4°C in a refrigerator and room temperature for nine weeks. Samples were collected at different intervals to measure enzyme activity using a standard assay mixture. Laccase activity was calculated as the percent of the initial activity at various time intervals during storage.

2.7. Potential of B. cereus UV25 laccase in the enrichment of apple and mango fruit juices.

Ripened apple (*Malus domestica Borkh*.) and mango (*Mangifera indica* L.) fruits were purchased from the local market, washed thoroughly with water, peeled, and macerated using a blender to get a smooth textured homogeneous fruit pulp. Both the pulps were treated with purified *B. cereus* UV25 laccase under pre-optimized conditions of enzyme dose (2.4 IU/gfp for apple and 1.6 IU/gfp for mango), incubation time (60 min) at 37 °C in a rotary shaker at 50 rpm for slight mixing of the contents. The untreated pulp samples were run simultaneously as a control. After the desired incubation period, the enzyme was inactivated by heating the suspension in a boiling water bath for 5 min followed by cooling. The samples were filtered through 2-4 layers of muslin cloth, and the filtrate was centrifuged at 10,000 x *g* for 10 min. The resulting supernatant (juice) was analyzed for physicochemical parameters *viz.* yield, clarity, reducing sugars, total phenols, and total soluble solids (TSS).

Juice yield was determined by measuring the volume of the supernatant obtained after enzymatic treatment of pulp and expressed as % (volume of juice per 100 g of pulp). Percent transmittance (%T), measured at 650 nm against distilled water in a UV-Visible spectrophotometer (Systronics-2203, India), was considered a measure of juice clarity. According to Miller [32], reducing sugars were estimated using 3, 5-dinitrosalicylic acid reagent and quantified from a standard curve of xylose (10-100 µg). According to Lin and Tang [33], total phenols were determined using gallic acid as standard and expressed as mg/100 g pulp as gallic acid equivalents. TSS was measured using Erma Hand refractometer and expressed as ^{o}Brix.

3. Results and Discussion

3.1. Purification of laccase.

Extracellular laccase was purified to homogeneity from the culture filtrate of *B. cereus* UV25 by gel filtration through Sephadex G-25 and Sephadex G-100 columns. The results of laccase purification are summarized in Table 1. Crude laccase, produced from *B. cereus* UV25 under SmF conditions, showed a specific activity of 0.04 IU/ mg protein. After G-25 gel filtration chromatography, three fractions containing most of the laccase activity were combined, and this pooled fraction revealed a specific activity of 0.052 IU/mg protein with 109.13 % recovery and 1.30-fold purification. This enzyme fraction was further purified by Sephadex G-100 column chromatography, which displayed a single peak of enzyme activity and protein in the gel filtration profile (Figure 1). The fractions containing laccase activity were combined, and the pooled sample was examined for laccase activity and protein content. Its specific activity was calculated as 0.589 IU/mg protein with 22.82% recovery and 14.73-fold overall purification.

Table 1. Purification of extracellular laccase from the culture filtrates of *B. cereus* UV25.

Fraction	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg protein)	Recovery (%)	Purification (fold)
Crude extract	47.40	1.884	0.040	100	1
Sephadex G-25	39.30	2.056	0.052	109.13	1.30
Sephadex G-100	0.73	0.430	0.589	22.82	14.73

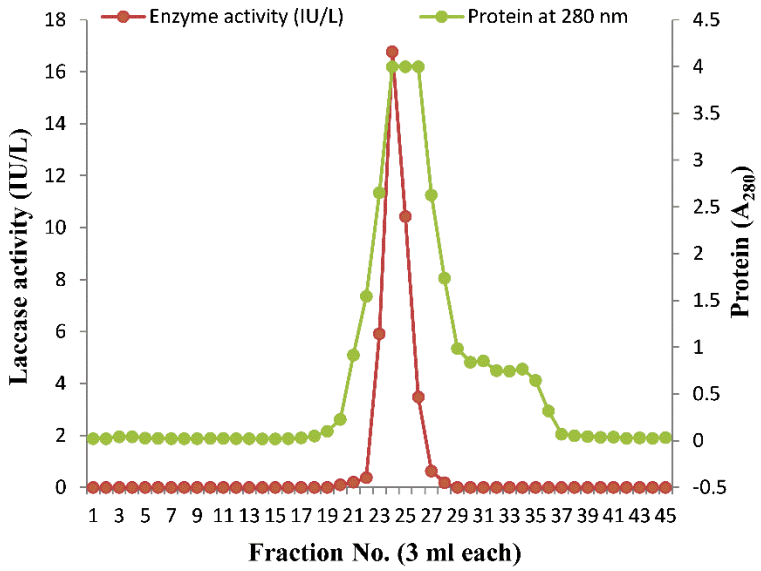

Figure 1. Elution profile of purified laccase from the culture filtrates of *B. cereus* UV25 through a Sephadex G-100 column (82.6 cm x 1.6 cm).

3.2. Testing of enzyme purity.

The purified fraction obtained from Sephadex G-100 chromatography was checked for purity by native-PAGE and SDS-PAGE. The results of the native-PAGE analysis shown in Figure 2a revealed a single protein band (lane P), indicating that it was pure. The activity staining of purified enzyme on native-PAGE using ABTS as substrate showed a single, green-colored band (lane D) which coincided with the protein band of the purified enzyme in lane P, confirming the laccase identity. The crude enzyme (lane C) and Sephadex G-25 pooled fraction (lane G), which were co-electrophoresed along with the purified enzyme, revealed multiple bands rather than a single band indicating the removal of non-enzymatic proteins during purification. On SDS-PAGE, the purified enzyme also revealed a single protein band (Figure 2b, lane P), indicating that it was homogeneous.

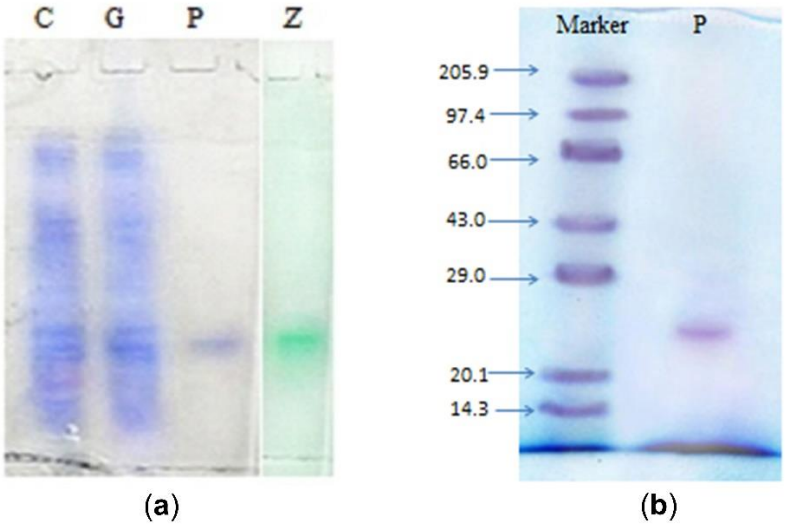

Figure 2. Testing the purity of *B. cereus* UV25 laccase by (a) Native-PAGE (Lanes C, G, P, and Z reveal the profile of crude enzyme, Sephadex G-25 pooled fraction, and Sephadex G-100 pooled fraction (purified laccase), respectively whereas Lane Z shows zymogram of laccase activity) and; (b) SDS-PAGE (Marker and P lanes refer to the profile of standard MW protein markers mixture and purified laccase, respectively).

In the literature, the purification of bacterial laccases has been documented with varying recovery and overall fold purification using different techniques. An extracellular, thermo-alkali-stable laccase was purified 28.46-fold with 13.34% yield from *B. tequilensis* SN4 using 60% acetone precipitation, Sephadex G-150 and DEAE-cellulose [8]. An extracellular thermos-alkali stable laccase was purified 12.16-fold to homogeneity from *Pseudomonas* sp. S2 [12]. A laccase from *A. faecalis* was purified 3.8-fold with 12% recovery using salt fractionation and ion-exchange chromatography [13]. A laccase from *S. psammoticus* was purified 12.1-fold with 22.1% recovery using anion exchange and gel filtration chromatography [14]. The laccase produced from *Bacillus* sp. NU2 was purified to homogeneity by 7.55-fold and 2.38% recovery using ammonium sulfate precipitation, DEAE-Sepharose, and Sephacryl S-200 [21]. Laccases were purified from *B. aquimaris* AKRC02 [10] and *B. vallismortis* fmb-103 [20] by 38.08-fold and 15.2-fold, respectively. Kaushik and Thakur [34] purified laccase from *Bacillus sp*. by 6.6-fold with 4% recovery using salt precipitation and ion-exchange chromatography. A laccase was purified 5.8-fold, and 51.5% recovery from cyanobacteria using ultrafiltration, acetone precipitation, anion exchange, and gel filtration chromatography [35]. A laccase from *Pseudomonas putida* LUA15.1 mutant E4 was purified 60-fold with 8.09% yield using ammonium sulfate precipitation, gel filtration, and ion exchange chromatography [36]. One-step purification of laccase using affinity chromatography with 68.2% yield and 99.8-fold was reported from halophilic *Aquisibacillus elongatus* [37].

It is evident from the published reports that the yield (22.82%) and/or overall purification (14.73-fold) of purified extracellular laccase from *B. cereus* UV25 fell within the range of recovery and fold purification reported from different bacteria. Moreover, laccase in this study was purified using only gel filtration, whereas most researchers have also employed ion-exchange chromatography. The purification of extracellular laccases at a larger scale is easier and may lower the cost of its application in industry.

3.3. Characterization of purified laccase.

3.3.1. Determination of molecular weight.

It was determined through gel filtration chromatography and SDS-PAGE. In gel filtration, elution of purified laccase through a Sephadex G-100 column revealed a single peak of enzyme activity which overlapped with the protein peak. The elution volume of the enzyme peak was noted to calculate the ratio of Ve/Vo, which was extrapolated to the standard curve of marker proteins. The MW of purified laccase, calculated from the standard graph plotted between Ve/Vo versus log MW of the marker proteins, was found to be 25.3 kDa.

In SDS-PAGE, purified *B. cereus* UV25 laccase and co-electrophoresed standard MW protein markers resolved as separate bands (Figure 2b). The MW of purified laccase, calculated from the logarithmic plot between MW of the marker proteins versus the corresponding R_m was found to be 25.3 kDa.

The identical MW of purified laccase from *B. cereus* UV25 estimated from gel filtration chromatography and SDS-PAGE suggested that the enzyme was a monomeric protein consisting of a single polypeptide chain having a molecular mass of 25.3 kDa. This finding agrees with a majority of bacterial laccases, which were monomeric proteins with molecular mass ranging from 20-80 kDa [1].

Purified laccases from various bacterial strains have been reported to differ in their molecular mass. The MW of *B. cereus* UV25 laccase is close to 29.8 kDa of recombinant laccase [38] but lower than that of other laccases, including 32 kDa of *B. tequilensis* SN4 [8], 33 kDa of *Bacillus* sp. A4 [39] and 34 kDa of *B. subtilis* [40], 38 kDa of *B. licheniformis* TPNR6 [9], 43 kDa from *S. psammoticus* [14], 55 kDa of *Bacillus* sp. NU2 [21], 65 kDa from *B. aquimaris* AKRC02 [10], 70 kDa from *Bacillus* sp. [34], and 71 kDa from *A. faecalis* [13]. These variations may be related to either genetic makeup or physiological function.

3.3.2. Determination of optimum pH.

The activity of an enzyme is greatly influenced by pH, which can either change the ionization state of active site amino acids or alter the enzyme conformational, thereby affecting its activity and stability. The activity of purified *B. cereus* UV25 laccase was measured using guaiacol as substrate. Its pH-activity profile showed the optimum pH of the enzyme at 4.2 since the activity was highest at this pH (Figure 3a). This observation is in line with earlier reports which described the optimum pH range for working of bacterial laccases as 3.0-9.0 [1]. The acidic pH optimum in this study is similar to that reported for laccase purified from *A. faecalis* NYSO [41], *B. licheniformis* TPNR1 [9], *B. licheniformis* VNQ [18], cyanobacteria [42] and *Bacillus sp.* [41]. The optimum acidic pH of laccase was at variance with several bacterial laccases, which have been shown to exhibit optimum activity at an alkaline pH range of 8.0-9.0 [12-14,21,42]. The optimum pH has been reported to vary for different substrates, which might be due to the difference in the redox potential of Type I copper of enzyme and the substrate [8].

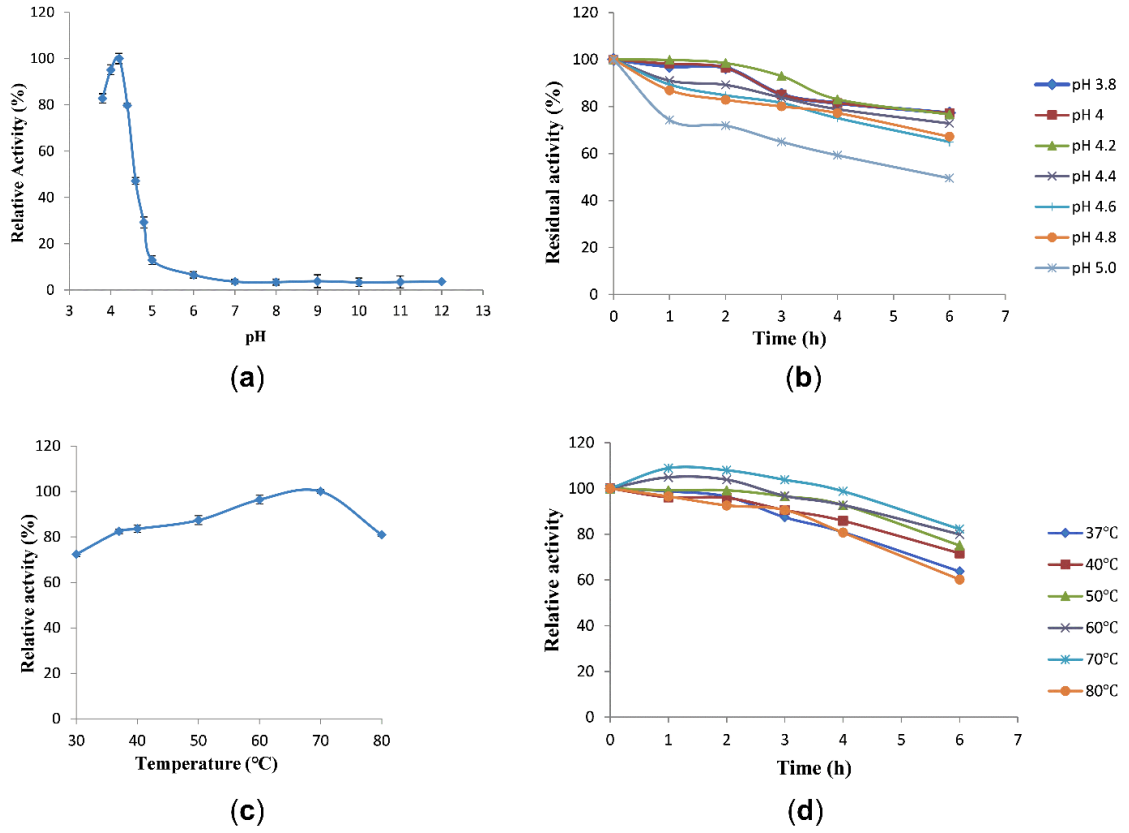

Figure 3. Effect of pH and temperature on extracellular laccase purified from *B. cereus* UV25 laccase: (a) Optimum pH; (b) pH stability; (c) Optimum temperature; and (d) Thermal stability.

3.3.3. Determination of pH stability.

The pH stability profile of purified laccase over a range of pH 3.8-5.0 revealed the highest enzyme stability at pH 4.0-4.2 upon pre-incubation for 2 h, followed by a decline on incubation for longer periods. The stability at a particular pH decreased with an increase in pre-incubation time such that nearly 83.11% and 76.62% activity was recorded on incubation at pH 4.2, whereas 77.27% and 67.23% activity was recovered after pre-incubation at pH 4.8 for 4 h and 6 h, respectively (Figure 3b). So, the enzyme was stable at acidic pH values. Generally, fungal laccases are stable at acidic and neutral pH, but bacterial laccases exhibit stability at alkaline pH [43]. In previous reports, the pH stability of laccase has been found to vary amongst different bacterial strains. The pH stability of the laccase in this study was similar to the partially purified laccase from *A. faecalis* NYSO, which was stable at pH 4.0-5.0 [41]. Some laccases demonstrated stability over a broad pH range [20,37] or at alkaline pH values [13, 44,45].

3.3.4. Determination of optimum temperature.

The activity profile of purified laccase at various temperatures revealed an increase in enzyme activity with a rise in temperature up to 70°C followed by a decline at higher temperatures (Figure 3c). The enzyme showed 80.98 % relative activity at 80°C, indicating that it could catalyze its reaction at temperatures as high as 70°C. The optimum temperature for purified *B. cereus* UV25 extracellular laccase was recorded at 70ºC.

Bacterial laccases have been found to work over a broad temperature range of 30-85 °C [1]. The optimum temperature of *B. cereus* UV25 laccase was similar to that reported for laccases from *B. licheniformis* [11] and *Bacillus* sp. SS4 [44] but higher than 40 °C of *Bacillus* sp. A4 [39], 50 °C of *B. subtilis* [40], and *B. licheniformis* TPNR1 [9], 55 °C of *A. faecalis* NYSO [41] and 60 °C of *B. subtilis* X1 [46], and *Bacillus* sp. NU2 [21]. In contrast, a higher optimum temperature for laccase has been recorded at 80 °C [9,10] and 85 °C [8].

3.3.5. Thermo-stability.

Thermo-stability profile of purified laccase from *B. cereus* UV25 revealed significant stability over a temperature range of 37-80℃ with a maximum of 70 ℃ (Figure 3d). Thermal stability was dependent on the pre-incubation time. The residual enzyme activity was 98.76% and 82.24% after 4 h and 6 h pre-incubation at 70℃, whereas the corresponding activity at 80 ℃ was 80.67% and 60.2%, respectively. The enzyme retained >90% and >80% of initial activity upon pre-incubation for 2 h and 4 h, respectively, over all the tested temperatures. At longer incubations, residual activity declined gradually. After 6 h of incubation, the residual activity was recorded as 75.08%, 79.93%, 82.24%, and 60.2% at 50, 60, 70, and 80 ℃, respectively. Enzyme stability at high temperatures is one of the characteristics desirable for its industrial applications. Significant stability of *B. cereus* UV25 laccase at high temperatures over a broad range of temperatures would be advantageous for its use in industry.

The thermal stability of bacterial laccases has been found to vary in the literature. The thermo-stability of *B. cereus* UV25 laccase was following Singh *et al.* [44], who reported that *Bacillus* sp. SS4 laccase exhibited the best working at 70–80 ℃ retaining 90% activity at 70℃ after 5 h and 88% at 80 ℃ after 3 h. The *Bacillus* sp. laccase showed a half-life of 23 min at 75℃ [34]. The half-life of *B. subtilis* X1 exceeded 6 h at 70 °C and was approximately 2.5 h

at 80 °C [46]. The *B. tequilensis* SN4 laccase retained more than 80% activity at 70 ℃ and was completely stable at 65 ℃ for 24 h [8]. Recombinant laccase from *B. pumilus* retained 50.3% and 24.6% of original activity after 2 h of incubation at 70 ℃ and 80 ℃, respectively [43]. The laccase from *B. vallismortis* spores was found to retain more than 50% of the original activity after 10 h at 70 ℃ [20]. The *B. licheniformis* TPNR 1 laccase exhibited a half-life of 4 h at 70 ℃ but retained 79% of original activity after 3 h at 60 ℃ [9]. The *B. licheniformis* laccase displayed maximum stability at 50 ℃ and retained more than 85% of original activity at 60 ℃ after 10 h [11]. The relative activity of *Bacillus* sp. A4 laccase was observed at 81%, 65%, and 51% at 60, 70, and 80 °C, respectively [39]. The *A. elongatus* laccase retained more than 80% of its original activity after 6 h incubation at 25–75 °C [37]. Purified *A. faecalis* laccase exhibited stability in the range of 70-90 °C [10]. Some researchers have reported optimum stability of laccase at 45 ℃ for 8 h [10] and 50 ℃ for 90 min [14]. Afreen *et al.* [35] showed optimum stability of laccase at 30-40 ℃ and retained 85% and 54% activity at 50 and 60 ℃ for 30 min, respectively.

It is evident from the above reports that the thermal stability of *B. cereus* UV25 laccase was higher than the enzymes purified from several bacterial species. The present findings on the effect of temperature revealed that the purified enzyme was active at higher temperatures (70-80 °C) and demonstrated considerable thermal stability at these temperatures, implying it to be a thermostable enzyme. This observation is pertinent for the industrial application of the enzyme.

3.3.6. Determination K_m and V_{max}.

The K_m and V_{max} of the enzyme, as calculated from the double reciprocal plot, were found to be 10.69 mM and 42.37 IU/L, respectively, for guaiacol as substrate. In the literature, K_m and V_{max} values for laccase have been reported to vary depending on the source bacterial strain as well as the substrate used for enzyme assay. In this study, the K_m value for guaiacol was higher, and V_{max} was lower than those reported earlier for purified bacterial laccases indicating a lower affinity of the enzyme [8,13,40].

3.3.7. Effect of metal ions on laccase activity.

Metal ions are known to influence the activity of enzymes, including laccase, and may act as either activators or inhibitors of activity. These may affect the amino acid residues at the active site and the exterior surface of an enzyme, inducing alterations in its charge and distorting the structure. In the present study, the effects of metal ions *viz.* Ca^{2+}, Hg^{2+}, K^{+}, Na^{+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, and Zn^{2+} were examined on the activity of purified laccase from *B. cereus* UV25. It was observed that the addition of Ca^{2+}, Na^{+}, Mn^{2+}, Mg^{2+}, and Cu^{2+} in the enzyme assay stimulated the laccase activity by 15.87%, 33.08%, 55.32%, 45.08%, and 71.16%, respectively whereas the presence of Fe^{2+}, K^{+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Co^{2+} significantly reduced the enzyme activity by 71.84%, 69.40%, 46.51%, 37.70%, 27.70%, and 22.48%, respectively as compared with the control in which no metal ion was added in the assay (Table 2).

Table 2. Effect of metal ions on the activity of purified laccase.

Metal ions (1 mM)	Relative activity (%)	% Stimulation (+) or Inhibition (-)
Control	100	-
Ca^{2+}	115.87 ± 0.63	+ 15.87
Cu^{2+}	171.16 ± 1.08	+ 71.16
Na^{+}	133.08 ± 1.20	+ 33.08

Metal ions (1 mM)	Relative activity (%)	% Stimulation (+) or Inhibition (-)
Mn^{2+}	155.32 ± 0.85	+ 55.32
Mg^{2+}	145.08 ± 1.13	+ 45.08
Fe^{2+}	28.16 ± 0.63	- 71.84
Co^{2+}	77.52 ± 1.01	- 22.48
Cd^{2+}	62.30 ± 1.10	- 37.70
K^{+}	30.60 ± 0.69	- 69.40
Zn^{2+}	53.49 ± 0.82	- 46.51
Hg^{2+}	72.30 ± 1.20	- 27.70

The values represent mean ± S.D. The control was devoid of metal ions.

Among these metal ions, Cu^{2+} ions were the most effective activator, which could be due to the filling of type I copper binding site by these ions, thereby indicating their involvement in enzyme catalysis [47]. Activation of the enzyme by Na^{+} might be due to the salt tolerance characteristic of the bacterial strain. The decline in laccase activity by Fe^{2+} ions could be explained by their possible attachment to the copper types II and type III, thereby interfering with internal electron transfer reactions of the enzyme [8]. The inhibition by Hg^{2+} ions indicated their key role in laccase activity. These ions might alter the enzyme conformation by their ability to bind sulfhydryl groups, thereby suppressing the enzyme activity.

Variations have been observed by researchers concerning the effect of metal ions on bacterial laccase activity. In agreement with the results of the present study, the stimulatory effect of $Cu^{2+,}$ Na^{+}, Mn^{2+}, $Mg^{2+,}$ and Ca^{2+} has been reported on the activity of bacterial laccase from [8, 12,13,45]. Activation of laccase activity by Cu^{2+}, Mn^{2+} and Zn^{2+} [35] and by Fe^{2+}, $Cu^{2+,}$ Na^{+}, Zn^{2+} and Mg^{2+} [14] has also been documented. It was found that laccase activity was enhanced by $Cu^{2+,}$ Zn^{2+}, Ni^{2+}, Mn^{2+}, Mg^{2+} and Cd^{2+} but inhibited by Fe^{2+} and Hg^{2+} [41]. Similar to the present findings, laccase activity was inhibited by Zn^{2+}, Cd^{2+}, and Hg^{2+} [8, 45] and by Fe^{2+} and Co^{2+} [43]. The inhibitory effect of Ni^{2+} and Fe^{2+} has also been recorded previously [8]. In contrast to the present findings, laccase from some bacteria was activated by Co^{2+} [8,12,41,45].

3.3.8. Effects of additives on laccase activity.

The interaction of additives with extracellular laccase is particularly important for a better understanding of the biotechnological processes. In the current work, it was noticed that EDTA, sodium azide, L-cysteine, and β-mercaptoethanol at 1 mM concentration were potent inhibitors of laccase suppressing the enzyme activity by 88.23%, 75.24%, 72.88%, and 14.18% (Table 3). Similarly, EDTA, sodium azide, L-cysteine, and β-mercaptoethanol were found to inhibit the activity of laccase from *B. tequilensis* SN4 [8] and *B. pumilus* [43]. Abdelgalil *et al.* [41] also observed inhibition of laccase activity by β-mercaptoethanol, cysteine-HCl, thioglycolic acid, sodium azide, and dithiothreitol but EDTA showed activation. Other researchers have also reported the inhibition of bacterial laccase by EDTA, sodium azide, and dithiothreitol, although their concentrations might differ [11,39,42]. Singh *et al.* [45] showed inhibition of laccase by azide, cysteine, thioglycolic acid, and diethyldithiocarbamate.

The significant inhibitory effect of EDTA might be due to the chelation of type I copper ions indicating the involvement of these ions in the catalytic reaction of laccase [34]. It was also likely that EDTA attached to type II copper ions facilitated their collection, causing further inhibition of laccase activity [48]. Sodium azide-mediated inhibition of laccase could be due to the binding of azide with the trinuclear copper center, thereby interfering with the electron transfer in type II and type III copper binding sites [39]. Cysteine is a competitive inhibitor of phenol oxidases, and hence it could bind to the active site of laccase, forming an enzyme-

cysteine complex and causing a decrease in enzyme activity [49]. β-mercaptoethanol is a sulfhydryl compound that could reduce the oxidized substrate back to its reduced form with the help of their –SH groups resulting in the inhibition of laccase activity [8].

The addition of non-ionic surfactants such as Tween 80, Tween 20, and Triton X-100 at a concentration of 1% (v/v) significantly enhanced the enzyme activity by 45.07%, 25.78%, and 33.80%, respectively. The stimulatory effect of surfactants was probably due to enzyme stabilization resulting from their interaction with laccase. An improvement in the stability of laccase by its interaction with Triton X-100 has also been shown earlier [48]. The binding of laccase to Triton X-100 also mitigated the inactivating effect of free radicals and polymerization products. The non-ionic surfactants probably stimulated laccase activity in the same manner. In this study, SDS, an anionic surfactant, was found to inhibit enzyme activity by 82.71%, which is in line with the earlier reports on bacterial laccases [13,20,39,41]. However, Sondhi *et al.* [8] observed stimulation of laccase activity by ionic surfactants at lower concentrations but inhibition at higher concentrations.

Table 3. Effect of various additives on the activity of purified laccase.

Additives	Relative activity (%)	% Stimulation (+) or Inhibition (-)
Control	100	
EDTA	11.77 ± 0.57	- 88.23
Sodium azide	24.76 ± 0.50	- 75.24
Cysteine	27.12 ± 1.14	-72.88
β-mercaptoethanol	85.82 ± 0.97	- 14.18
Tween 20	125.78 ± 0.88	+ 25.78
Tween 80	145.07 ± 0.63	+ 45.07
Triton X 100)	133.80 ± 1.08	+33.80
SDS	17.28 ± 0.69	- 82.71

All the additives were used at a concentration of 1 mM, except Tween 20, Tween 80, and Triton X-100, which were 1% (v/v). The values represent mean ± S.D.

3.3.9. Storage stability of purified laccase.

The storage stability of purified laccase was assessed at 4 ºC and room temperature for 9 weeks at weekly intervals. Purified laccase was found to be completely stable at 4 °C for four weeks, but a gradual decline in activity was recorded on further storage. After nine weeks of storage at 4°C, the enzyme retained 69.87% starting activity (Table 4). At room temperature, the residual laccase activity remained unchanged during storage for three weeks, but after that, the activity showed a gradual reduction. The residual activity after nine weeks of storage was observed as 50.61%. The substantial storage stability of *B. cereus* UV25 laccase would likely be useful for its application.

Table 4. Storage stability of purified laccase at 4 ºC and room temperature.

Storage time (weeks)	Relative activity (%)	
	4 °C	Room temperature
0	100	100
1	99.56	98.07
2	98.15	97.80
3	97.51	94.78
4	96.19	90.09
5	89.49	82.40
6	82.24	70.89
7	79.08	64.76
8	74.20	56.24
9	69.87	50.61

3.4. Potential of B. cereus UV25 laccase in the enrichment of apple and mango fruit juices.

Before juice extraction, treatment of apple pulp with *B. cereus* UV25 laccase at the optimal dose (2.4 IU/gfp) for 60 min enhanced the yield, clarity, reducing sugars and TSS by 9.52%, 27.27%, 58.89%, and 31.13 respectively while treatment of mango pulp with the optimal enzyme dose (1.6 IU/gfp) improved the above parameters by 11.86%, 27.25%, 43.12%, and 15.08%, respectively as compared to the control. On the other hand, polyphenolic contents of apple and mango juices were reduced by 11.11% and 12.82%, respectively, after treatment of the fruit pulps with laccase (Figure 4a,b).

The quantity of phenolic compounds in raw fruit juices affects the juice quality in terms of changes in color, aroma, and taste during processing and storage. Laccase has immense potential to enhance fruit juice color and flavor stabilization by removing undesirable phenols [26]. The decline in apple and mango juices' polyphenolic content after treatment of fruit pulps with B. cereus UV25 laccase was attributed to the enzyme-catalyzed oxidation of phenols, which enhanced juice clarity. Several workers have recorded a decline in the phenol content of juices after adding laccase. The use of laccase was found to be more effective in enhancing color and flavor stability compared to conventional treatments [50]. Ribeiro *et al.* [51] documented a substantial decline in fruit juices' phenolic content and enhanced color stability on treatment with laccase. Laccase treatment is usually followed by ultrafiltration. Ghosh and Ghosh [52] reported 26% and 36.3% drop in phenolic content along with a decrease in turbidity after treatment of apple juice with fungal laccase for 12 and 24 h, respectively. An improvement in apple juice's clarity and sensory quality characteristics has been reported on treatment with 1% of purified fungal laccase [36]. Nadaroglu and Tasgin [53] pointed out that treating raw fruit material with a high concentration of phenolic compounds with laccase increased the juice output and reduced its phenolic content. They reported a 36.1% and 29.5% reduction in phenolic compounds of apple and grape juices by using purified laccase from *Lactarius volemus*. Artik *et al.* [54] observed a 70% decrease in phenolic content along with an increase in the clarity of sour cherry juice after oxidation with laccase for 6 h at 50 °C, followed by filtration after 1 h. Neifer *et al.* [55] recorded a 50% decline in total phenols and a 30% increase in pomegranate juice clarity and stability by combined laccase treatment (5 U/mL) and ultrafiltration.

In contrast, an increase in polyphenolic content was noticed in pomegranate juice [56] after laccase treatment. A fungal laccase immobilized on low-cost coconut fibers has also been used for apple juice clarification [57]. The magnitude of the decline in phenolic content may vary with the number of phenols in juices, enzyme characteristics, dose, and starting material (fruit pulp or juice) used for enzymatic treatment.

An increase in the juice yield upon enzymatic treatment of apple and mango fruit pulps observed in this study might be due to the removal of phenols attached to other pulp constituents of the fruit wall resulting in more juice release. The apple juice yield and clarity are also reported to increase after the fruit pulp treatment with bacterial xylanase [58,59]. Sharma *et al.* [60] used fungal xylanase to enhance the clarity of orange and mosambi juices. The observed increase in TSS along with reducing sugars following treatment of apple and mango fruit pulps with laccase might be to the greater degree of tissue breakdown in enzyme-treated juice, releasing sugars added to soluble solids in juices. An increase in soluble solids in enzyme-treated juices was also recorded by other researchers [56].

The results showing an improvement in physicochemical characteristics of apple and mango fruit juices after treatment of their pulps with *B. cereus* UV25 laccase implied the potential of this bacterial enzyme in juice enrichment. However, this application requires detailed investigations.

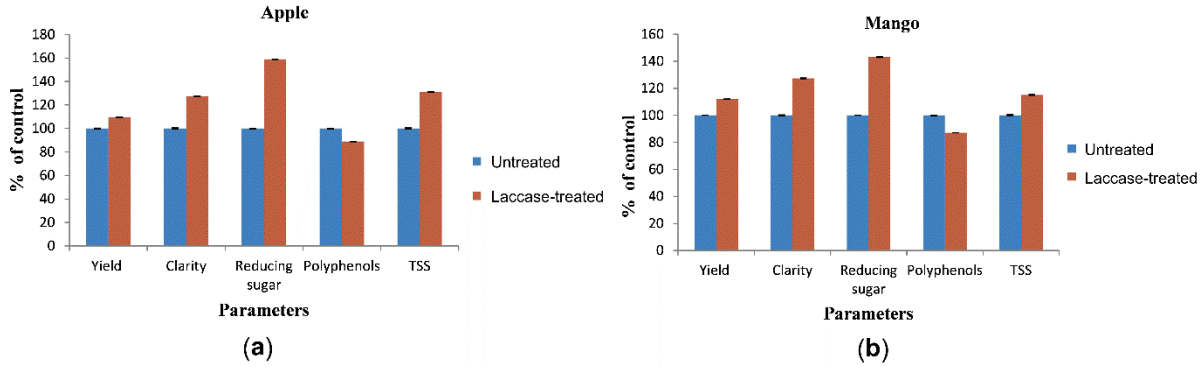

Figure 4. Physico-chemical properties of juices obtained from apple (a) and mango (b) fruit pulps treated with purified *B. cereus* UV25 laccase.

4. Conclusions

An extracellular, thermostable, and moderately salt-tolerant laccase secreted in the culture filtrates by *Bacillus cereus* UV25 has been purified to homogeneity employing only gel filtration chromatography. It is a monomeric protein of molecular weight 25.3 kDa. Considerable stability of this enzyme at high temperatures, acidic pH, and during storage, as well as modulation of its activity by metal ions, non-ionic surfactants, chelating agents, and sulfhydryl compounds, can be exploited in biotechnological applications. Its capability in enriching juices obtained from laccase-treated apple and mango fruit pulps has been demonstrated, but it needs further investigation.

Funding

This research received no external funding except the CSIR fellowship contingent grant to Urvashi Bhankar.

Acknowledgments

Ms. Urvashi Bhankar is obliged to Kurukshetra University, Kurukshetra, for awarding a University Research Fellowship and to the Council of Scientific and Industrial Research (CSIR), New Delhi, India, for providing financial assistance as Junior Research Fellowship (award letter no. 09/105(0291)/2019-EMR-1).

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Chauhan, P.S.; Goradia, B.; Saxena, A. Bacterial laccase: recent update on production, properties and industrial applications. *3 Biotech* **2017**, *7*, 323-343, https://doi.org/10.1007/s13205-017-0955-7.
2. Singh, D.; Gupta, N. Microbial laccase: a robust enzyme and its industrial applications. *Biologia* **2020**, *75*, 118-1193, https://doi.org/10.2478/s11756-019-00414-9.

3. Akram, F.; Ashraf, S.; Haq, I.U.; Shah, F.I.; Akeel, A. Eminent industrial and biotechnological applications from bacterial source: a current overview. *App. Biochem. Biotechnol.* **2022**, *194*, 2336-2356, https://doi.org/10.1007/s12010-021-03781-9.
4. Kaur, R.; Salwan, R.; Sharma, V. Structural properties, genome distribution of laccases from Streptomyces and their applications. *Process Biochem.* **2022**, *118*, 133-144, https://doi.org/10.1016/j.procbio.2022.04.015.
5. Lei, L.; Yang, X.; Song, Y.; Huang, H.; Li, Y. Current research progress on laccase-like nanomaterials. *New J. Chem.* **2022**, *46*, 3541-3550, https://doi.org/10.1039/D1NJ05658A.
6. Brugnari, T.; Braga, D.M.; dos Santos, C.S.A.; Torres, B.H.C.; Modkovski, T.A.; Haminiuk, C.W.I.; Maciel, G.M. Laccases as green and versatile biocatalysts: from lab to enzyme market-an overview. *Bioresour. Bioprocess.* **2021**, *8*, https://doi.org/10.1186/s40643-021-00484-1.
7. Sharma, N.; Leung, I.K.H. Novel Thermophilic bacterial laccase for the degradation of aromatic organic pollutants. *Front. Chem.* **2021**, *9*, https://doi.org/10.3389/fchem.2021.711345.
8. Sondhi, S.; Sharma, P.; Saini, S.; Puri, N.; Gupta, N. Purification and characterization of an extracellular, thermos-alkali-stable, metal tolerant laccase from *Bacillus tequilensis* SN4. *PLOS One* **2014**, *9*, https://doi.org/10.1371/journal.pone.0096951.
9. Sharma, V.; Upadhyay, L.S.B.; Vasanth, D. Extracellular, thermostable laccase-like enzymes from *Bacillus licheniformis* strains: production, purification and characterization. *Appl. Biochem. Microbiol*. **2020**, *56*, 420-432, https://doi.org/10.1134/S0003683820040146.
10. Kumar, A.; Singh, A.K.; Bilal, M.; Chandra, R. Sustainable production of thermostable laccase from agro-residues waste by *Bacillus aquimaris* AKRC02. *Catal. Lett.* **2022**, *152*, 1784-1800, https://doi.org/10.1007/s10562-021-03753-y.
11. Lu, L.; Wang, T.N.; Xu, T.F.; Wang, J.Y.; Wang, C.L.; Zhao, M. Cloning and expression of thermo alkali-stable laccase of *Bacillus licheniformis* in *Pichia pastoris* and its characterization. *Bioresour. Technol*. **2013**, *134*, 81-86, https://doi.org/10.1016/j.biortech.2013.02.015.
12. Chauhan, P.S.; Jha, B. Pilot scale production of extracellular thermos-alkali-stable laccase from *Pseudomonas* sp. S2 using agro waste and its application in organophosphorous pesticides degradation. *J. Chem. Technol. Biotechnol.* **2018**, *93*, 1022-1030, https://doi.org/10.1002/jctb.5454.
13. Mehandia, S.; Sharma, S.C.; Arya, S.K. Isolation and characterization of an alkali and thermostable laccase from a novel *Alcaligenes faecalis* and its application in decolorization of synthetic dyes. *Biotechnol. Rep.* **2020**, *25*, https://doi.org/10.1016/j.btre.2019.e00413.
14. Niladevi, K.N.; Jacob, N.; Prema, P. Evidence for a halotolerant-alkaline laccase in *Streptomyces psammoticus*: Purification and characterization. *Process Biochem.* **2008**, *43*, 654-660, https://doi.org/10.1016/j.procbio.2008.02.002.
15. Ali, N.S.; Huang, F.; Qin, W.; Yang, T.C. Identification and characterization of a new *Serratia proteamaculans* strain that naturally produces significant amount of extracellular laccase. *Front. Microbiol.* **2022**, *13*, https://doi.org/10.3389/fmicb.2022.878360.
16. Haq, I.; Kalamdhad, A.S.; Pandey, A. Genotoxicity evaluation of paper industry wastewater prior and post-treatment with laccase producing *Pseudomonas putida* MTCC 7525. *J. Clean. Prod.* **2022**, *342*, https://doi.org/10.1016/j.jclepro.2022.130981.
17. Xiong D.; Wen, J.; Lu, G.; Li, T.; Long, M. Isolation, purification, and characterization of a laccase-degrading Aflatoxin B1 from *Bacillus amyloliquefaciens* B10. *Toxins* **2022**, *14*, https://doi.org/10.3390/toxins14040250.
18. Sharma, V.; Pugazhenthi, G.; Vasanth, D. Production and characterization of a novel thermostable laccase from *Bacillus licheniformis* VNQ and its application in synthesis of bioactive 1,4-naphthaquinones. *J. Biosci. Bioeng*. **2022**, *133*, 8-16, https://doi.org/10.1016/j.jbiosc.2021.09.008.
19. Khaled, J.M.; Alyahya, S.A.; Govindan, R.; Chelliah, C.K.; Maruthupandy, M.; Alharbi, N.S.; Kadaikunnan, S.; Issac, R.; Murugan, S.; Li, W-J. Laccase producing bacteria influenced the high decolorization of textile azo dyes with advanced study. *Environ. Res.* **2022**, *207*, https://doi.org/10.1016/j.envres.2021.112211.
20. Zhang, C.; Zhang, S.; Diao, H.; Zhao, H.; Zhu, X.; Lu, F.; Lu, Z. Purification and characterization of a temperature and pH-stable laccase from the spores of *Bacillus vallismortis* fmb-103 and its application in the degradation of malachite green. *J. Agric. Food Chem.* **2013**, *61*, 5468-5473, https://doi.org/10.1021/jf4010498.
21. Edoamodu, C.E.; Nwodo, U.U. Decolourization of synthetic dyes by laccase from *Bacillus* sp. NU2. *Biotechnol. Biotechnol. Equip.* **2022**, *36*, 94-105, https://doi.org/10.1080/13102818.2022.2053341.
22. Kumar, L.; Nagar, S.; Mittal, A.; Garg, N.; Gupta, V.K. Immobilization of xylanase purified from *Bacillus pumilus* VLK-1 and its application in enrichment of orange and grape juices. *J. Food Sci. Technol.* **2014**, *51*, 1737-1749, https://doi.org/10.1007/s13197-014-1268-z.
23. Backes, E.; Kato, C.G.; Correa, R.C.G.; Moreira, R.F.P.M.; Peralta, R.A.; Barros, L.; Ferreira, I.C.F.R.; Zanin, G.M.; Bracht, A.; Peralta, R.M. Laccases in food industry: current status, bottlenecks and perspectives. *Trends Food Sci. Technol.* **2021**, *115*, 445-460, https://doi.org/10.1016/j.tifs.2021.06.052.
24. Upadhyay, P.; Srivastava, R.; Agrawal, P.K. Bioprospecting and biotechnological applications of fungal laccase. *3 Biotech* **2016**, *6*, 15-26, https://doi.org/10.1007/s13205-015-0316-3.

25. Okpara, M.O. Microbial enzymes and their applications in food industry: A mini-review. *Adv. Enzyme Res.* **2022**, *10*, 23-47, https://doi.org/10.4236/aer.2022.101002.
26. Osma, J.F.; Toca-Herrera, J.L.; Rodriguez-Couto, S. Uses of laccases in food industry. *Enzyme Res.* **2010**, *2010*, https://doi.org/10.4061/2010/918761.
27. Das, N.; Sengupta, S.; Mukherjee, M. Importance of laccase in vegetative growth of *Pleurotus florida*. *Appl. Environ. Microbiol.* **1997**, *63*, 4120-4122, https://doi.org/10.1128/aem.63.10.4120-4122.1997.
28. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with folin-phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265-275, https://doi.org/10.1016/S0021-9258(19)52451-6.
29. Davis, B.J. Disc electrophoresis – II. Method and application to human serum proteins. *Ann. NY Acad. Sci.* **1964**, *121*, 404-427, https://doi.org/10.1111/j.1749-6632.1964.tb14213.x.
30. Laemmli, U.K. Cleavage of structural proteins during the assembly of head bacteriophage T4. *Nature* **1970**, *22*, 680-685, https://doi.org/10.1038/227680a0.
31. Patel, H.; Gupte, S.; Gahlout, M.; Gupte, A. Purification and characterization of an extracellular laccase from solid-state culture of *Pleurotus ostreatus* HP-1. *3 Biotech.* **2014**, *4*, 77-84, https://doi.org/10.1007/s13205-013-0129-1.
32. Miller, L.G. Use of dinitrosalycilic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426-428, https://doi.org/10.1021/ac60147a030.
33. Lin, J-Y.; Tang, C-Y. Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects of mouse splenocyte proliferation. *Food Chem.* **2007**, *101*, 140-147, https://doi.org/10.1016/j.foodchem.2006.01.014.
34. Kaushik, G.; Thakur, I.S. Purification, characterization and usage of thermotolerant laccase from *Bacillus* sp. for biodegradation of synthetic dyes. *Appl. Biochem. Microbiol.* **2013**, *49*, 352-359, https://doi.org/10.1134/S0003683813040169.
35. Afreen, S.; Shamsi, T.N.; Baig, M.A.; Ahmad, N.; Fatima, S.; Qureshi, M.I.; Hassan, M.I.; Fatma, T. A novel multicopper oxidase (laccase) from cyanobacteria: Purification, characterization with potential in the decolorization of anthraquinonic dye. *PLOS One* **2017**, *12*, https://doi.org/10.1371/journal.pone.0175144.
36. Verma, A.; Shirkot, P.; Dhiman, K.; Rana, N.; Joshi, V.K. Bacterial laccase mediated inhibition of enzymatic browning in apple juice and its sensory evaluation. *Int. J. Curr. Microbiol. App. Sci.* **2018**, *7*, 3371-3381, https://doi.org/10.20546/ijcmas.2018.701.399.
37. Rezaei, S.; Shahverdi, A.R.; Faramarzi, M.A. Isolation, one-step affinity purification, and characterization of a polyextremotolerant laccase from the halophilic bacterium *Aquisalibacillus elongatus* and its application in the delignification of sugar beet pulp. *Bioresour. Technol.* **2017**, *230*, 67-75, https://doi.org/10.1016/j.biortech.2017.01.036.
38. Wang, H.; Huang, L.; Li, Y.; Ma, J.; Wang, S.; Zhang, Y.; Ge, X.; Wang, N.; Lu, F.; Liu, Y. Characterization and application of a novel laccase derived from *Bacillus amyloliquefaciens*. *Int. J. Biol. Macromol.* **2020**, *150*, 982-990, https://doi.org/10.1016/j.ijbiomac.2019.11.117.
39. Guo, H.; Lin, C.; Wang, S.; Jiang, D.; Zheng, B.; Liu, Y.; Qin, W. Characterization of a Novel laccase-producing *Bacillus* sp. A4 and its application in *Miscanthus* degradation. *BioResources.* **2017**, *12*, 4776-4794.
40. Hajipour, O.; Dogan, N.M.; Dincer, S.; Norizadehazehkand, M. Cloning, expression, and characterization of novel laccase enzyme from native *Bacillus subtilis* strain OH67. *Mol. Biol.* **2020**, *54*, 611-617, https://doi.org/10.31857/S0026898420040060.
41. Abdelgalil, S.A.; Attia, A.R.; Reyed, R.M.; Soliman, N.A. Partial purification and biochemical characterization of a new highly acidic NYSO laccase from Alcaligenes faecalis. *J. Genetic Eng. Biotechnol.* **2020**, *18*, https://doi.org/10.1186/s43141-020-00088-w.
42. Chopra, N.K.; Sondhi, S. Cloning, expression and characterization of laccase from *Bacillus licheniformis* NS2324 in *E. coli* application in dye decolorization. *Int. J. Biol. Macromol.* **2022**, *206*, 1003-1011, https://doi.org/10.1016/j.ijbiomac.2022.03.104.
43. Li, T.; Chu, X.; Yuan, Z.; Yao, Z.; Li, J.; Lu, F.; Liu, Y. Biochemical and structural properties of a high-temperature-active laccase from *Bacillus pumilus* and its application in the decolorization of food dyes. *Foods* **2022**, *11*, https://doi.org/10.3390/foods11101387.
44. Singh, G.; Singh, S.; Kaur, K.; Arya, S.K.; Sharma, P. Thermo and halo tolerant laccase from *Bacillus* sp. SS4: evaluation for its industrial usefulness. *J. Gen. Appl. Microbiol.* **2019**, *65*, 26-33, https://doi.org/10.2323/jgam.2018.04.002.
45. Singh, G.; Capalash, N.; Goel, R.; Sharma, P. A pH-stable laccase from alkali-tolerant β-proteobacterium JB: Purification, characterization and indigo carmine degradation. *Enz. Microb. Technol.* **2007**, *41*, 794-79, https://doi.org/10.1016/j.enzmictec.2007.07.001.
46. Guan, Z.B.; Zhang, N.; Song, C.M.; Zhou, W.; Zhou, L.X.; Zhao, H.; Xu, C.W.; Cai, Y.J.; Liao, X.R. Molecular cloning, characterization, and dye-decolorizing ability of a temperature and pH-stable laccase from *Bacillus subtilis* X1. *Biotechnol. Appl. Biochem.* **2014**, *172*, 1147-1157, https://doi.org/10.1007/s12010-013-0614-3.

47. Shraddha; Shekhar, R.; Sehgal, S.; Kamthania, M.; Kumar, A. Laccase: microbial sources, production, purification and potential biotechnological applications. *Enz. Res.* **2011**, *2011*, https://doi.org/10.4061/2011/217861.
48. Shafiei, M.; Afzali, F.; Karkhane, A.A.; Ebrahimi, S.M.; Haghbeen, K.; Aminzadeh, S. *Cohnella sp.* A01 laccase: thermostable, detergent resistant, anti-environment and industrial pollutants enzyme. *Heliyon* **2019**, *5*, https://doi.org/10.1016/j.heliyon.2019.e02543.
49. Altunkaya, A.; Gokmen, V. Effect of various inhibitors on enzymatic browning, antioxidant activity and total phenol content of fresh lettuce (*Lectuca sativa*). *Food Chem.* **2008**, *107*, 1173-1179, https://doi.org/10.1016/j.foodchem.2007.09.046.
50. Minussi, R.C.; Pastore, G.M.; Durán, N. Potential applications of laccase in the food industry. *Trends in Food Science & Technology* **2002**, *13*, 205-216, https://doi.org/10.1016/S0924-2244(02)00155-3.
51. Ribeiro, D.S.; Henrique, S.M.B.; Oliveira, L.S.; Macedo, G.A.; Fleuri, L.F. Enzymes in juice processing: a review. *Int. J. Food Sci. Technol.* **2010**, *45*, 635-641, https://doi.org/10.1111/j.1365-2621.2010.02177.x.
52. Ghosh, P.; Ghosh, U. Statistical optimisation of laccase production by isolated strain *Aspergillus flavus* PUF5 utilizing ribbed gourd peels as the substrate and enzyme application on apple juice clarification. *Indian Chem. Eng.* **2020**, *62*, 427-438, https://doi.org/10.1080/00194506.2019.1684211.
53. Nadaroglu, H.; Tasgin, E. Purification and characterization of laccase from *Lactarius volemus* and its application in removal of phenolic compounds from fruit juice. *J. Food Agric. Environ.* **2013**, *11*, 109-114..
54. Artik, N.; Karhan, M.; Aydar, G. Effects of polyphenoloxidase (LACCASE) application on clarity stability of sour cherry juice. *J. Food Technol.* **2004**, *2*, 237-243.
55. Neifar, M.; Elleouze-Ghorbel, R.; Kamoun, A.; Baklouti, S.; Mokni, A.; Jaouani, A.; Ellouze-Chaabouni, S. Effective clarification of pomegranate juice using laccase treatment optimized by response surface methodology followed by ultrafiltration. *J. Food Process Eng.* **2011**, *34*, 1199-1219, https://doi.org/10.1111/j.1745-4530.2009.00523.x.
56. Rinaldi, M.; Caligiani, A.; Borgese, R.; Palla, G.; Barbanti, D.; Massini, R. The effect of fruit processing and enzymatic treatments on pomegranate juice composition, antioxidant activity and polyphenols content. *LWT - Food Sci. Technol.* **2013**, *53*, 355-359, https://doi.org/10.1016/j.lwt.2013.02.015.
57. Bezerra, T.M.S.; Bassan, J.C.; Santos, V.T.O.; Ferraz, A.; Monti, R. Covalent immobilization of laccase in green coconut fiber and use in clarification of apple juice. *Process Biochem*. **2015**, *50*, 417-423, https://doi.org/10.1016/j.procbio.2014.12.009.
58. Nagar, S.; Mittal, A.; Gupta, V.K. Enzymatic clarification of fruit juices (apple, pineapple and tomato) using purified *Bacillus pumilus* SV-85S xylanase. *Biotechnol. Bioproc. Eng.* **2012**, *17*, 1165-1175, https://doi.org/10.1007/s12257-012-0375-9.
59. Bajaj, B.K.; Manhas, K. Production and characterization of xylanase from *Bacillus lichemiformis* P11 (C) with potential for fruit juice and bakery industry. Biocatal. *Agric. Biotechnol.* **2012**, *1*, 330-337, http://doi.org/10.1016/j.bcab.2012.07.003.
60. Sharma, S.; Sharma, V.; Nargotra, P.; Bajaj, B.K. Bioprocess development for production of a process-apt xylanase with multifaceted application potential for a range of industrial processes. *SN Appl. Sci.* **2020**, *2*, http://doi.org/10.1007/s42452-020-2541-6.