



Isolation and characterization of an alkali and thermostable laccase from a novel *Alcaligenes faecalis* and its application in decolorization of synthetic dyes

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ABSTRACT

A laccase producing new bacterial strain (*Alcaligenes faecalis* XF1) was isolated from green site of Chandigarh (India) by standard screening method. Nutrient broth medium containing 0.2 mM CuSO₄ was used for the production of laccase. Maximum production (110 U/ml) was achieved after four days of incubation. The extracellular laccase from the medium was purified by simple salt precipitation and ion exchange technique to get 3.8 fold purified protein with 1637.8 U/mg specific activity. Purified laccase (named as LAC1*) revealed its optimum activity at pH 8.0 and 80 °C temperature, and displayed remarkable stability in the range of 70–90 °C and in the pH range (pH 7.0–9.0). The single bands on SDS-PAGE represents the purity of LAC1* with molecular weight of ~71 kDa. The kinetic parameters for 2,6-DMP oxidation was K_m, V_{max} and K_{cat} were 480 μM, 110 U/mL and 1375 s⁻¹. Enzyme activity of the LAC1* was significantly enhanced by Cu²⁺, Mg²⁺, Mn²⁺. SDS and NaCl, and was slightly inhibited in the presence of conventional inhibitors like cysteine, EDTA and sodium azide. Extracellular nature and significant stability of LAC1* under extreme conditions of temperature, pH, heavy metals, halides and detergents confined its suitability for various biotechnological and industrial applications which required these qualities of laccase. So after recognizing all these properties the purified laccase was studied for its application in decolorization of industrial dyes.

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1. Introduction

Laccases (E.C 1.10.3.2) blue copper containing polyphenol oxidases is one of the best known enzymes that catalyse the oxidation of relatively broad range of aromatic compounds, organic pollutants and inorganic substrates along with the reduction of oxygen to water [1,2]. These enzymes are of low substrate specificity and oxidize a broad group of phenols, metallic ions as well as aromatic amine [3–5]. The signature copper binding regions L1, L2, L3 & L4 exist in all laccases which are used to identify laccases [6,7].

Laccases are eco-friendly in nature as these do not require the harmful hydrogen peroxide for the oxidative reaction like other oxidases and peroxidases [8–10]. Many researchers in this field have used laccases to remove phenolic contaminants, polycyclic aromatic hydrocarbons etc. from the environment [11,12]. These enzymes have also prevailed particular interest in bioremediation

applications like textile dye decolourisation [13,14], detoxification of environmental pollutants [15] and biobleaching of pulp in paper industry etc. [16,17]. These applications require suitable laccase for each specific purpose which should be originated from nature and produced in adequate amount.

Most of the laccases are studied from plants and fungi, [18,19] particularly from white rot fungi [20]. Laccases from plants and fungi work better at low temperature and at acidic condition [21,22]. Bacterial laccases have certain significant properties like activity at alkaline pH, high temperature, less dependent on metal ions and less susceptible to inhibitory agents [22–24], in contrast to fungal laccases, that are very sensitive to high temperature and high pH [25,26]. As the biobleaching of pulp and treatment of industrial waste water requires enzymes that can perform activity and stability at high temperature and alkaline pH etc. [27,28]. So, bacterial laccases are more appropriate for these purposes. Thus fungal laccases are mostly unsuitable for the same [29] whereas bacterial laccases are potential alternatives. Analysis on bacterial genome suggests laccase like genes have been widespread in bacteria [30].

Albeit of their significant characteristics, not much is known about their biotechnological and industrial applications. Therefore,

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isolating and characterising a novel bacterial laccase and studying their significant applications will be of great help to enhance their use for industrial processes. To obtain new bacterial laccases with ideal characteristic, six potential laccase producing bacterial strains were isolated from three different sites of Chandigarh and laccase with highest activity was further characterised and studied for its application.

2. Material and methods

2.1. Isolation, screening and identification of laccase producing bacterium

Laccase producing bacterium were screened and isolated from soil samples composed of plant material waste and saw dust collected from the forest area, wood market, and green sites around the Chandigarh, India. All the collected samples (10 g of each soil sample) were sieved and added to 100 ml of nutrient broth medium (Himedia) supplemented with 0.2 mM CuSO_4 and 2 mM guaiacol as inducers and incubated at 37 °C for 48 h to prepare the stock. All the stock samples were successively diluted in the ratio of 10^{-1} to 10^{-7} and 100 μl of each dilution was plated on nutrient agar plates containing 5 mM guaiacol (a potent substrate for laccase) at 37 °C for about 3–4 days of incubation. The bacterium colonies visualized by appearance of coloured zones of substrate oxidation in nutrient agar medium were selected as laccase producing isolates. Following the primary screening procedure further selection was done by quantifying their laccase activity. The isolate showing highest laccase activity was selected for further detailed study.

The selected bacterial strain was identified by morphological, biochemical and physiological analysis according to Bergey's manual of determinative bacteriology [31] and further confirmed by 16S rRNA sequence analysis.

2.2. Time course study of laccase production from the selected strain

To investigate the growth and the optimum incubation time for laccase production, basal broth medium containing (w/v); Peptone 0.5 %, Beef extract 0.3 %, Yeast extract 0.5 %, and NaCl 1 % supplemented with 0.2 mM CuSO_4 , pH 7.5 was used. 200 ml of medium in a 500 ml erlenmeyer flask was sterilized and inoculated with 2 % of overnight culture of the selected strain and grown at 37 °C under shaking at 120 rpm for 144 h. The samples were withdrawn at different time intervals and were studied for growth and laccase activity as per standard protocol.

2.3. Extraction and purification of laccase

The selected isolate was grown in 100 ml broth medium at 37 °C and 120 rpm for 96 h. The supernatant obtained after filtration and centrifugation (10,000 g for 15 min) of the culture filtrate was subjected to ammonium sulphate precipitation with the range of 0–80 % saturation. After that the dialysis was performed against the phosphate buffer (50 mM, pH 7.5). Then obtained dialysed sample was applied to ion exchange DEAE-Cellulose column (1*15 cm) that was previously equilibrated with same buffer (pH 7.5). The protein samples (5 ml) were eluted by using increasing gradient of NaCl (0–0.5 M) in the same buffer. The eluted active fractions were desalted by dialysis and assayed for protein content. After use re-equilibration of column was carried out with the same buffer.

2.4. Enzyme assay & protein estimation

The laccase activity will be measured by monitoring the oxidation of 2, 6-Dimethoxyphenol (2, 6-DMP) buffered with

100 mM phosphate buffer at 470 nm for 1 min [32]. To calculate the enzyme activity an absorption coefficient of 14,800 M/cm was used. One unit of enzyme activity defined as the amount of enzyme required to oxidise 1 μM of 2, 6-DMP per minute [33]. Protein estimation was done at every step by Lowry method [34] with Bovine Serum Albumin as a standard protein.

2.5. Characterization of purified laccase

2.5.1. Effect of pH on laccase activity and stability

The optimal pH for laccase activity was evaluated by incubating the enzyme substrate mixture with the following buffer systems: acetate buffer (pH 5.0–5.5), sodium potassium phosphate buffer (pH 5.5–8.0) and Tris-HCl buffer (pH 8.0–10.0) at 40 °C and the relative laccase activity was determined as per standard assay method. The relative activities were calculated by considering the maximum activity as 100 %. The stability of the enzyme at different pH was evaluated by pre-incubation of the enzyme solution with different buffers (aforementioned buffer for pH 7.0–9.0) for 10 days at 40 °C temperature and the residual laccase activity was determined in comparison to initial activity as per standard assay method (under optimal conditions).

2.5.2. Effect of temperature on laccase activity and stability

Effect of temperature on laccase activity was determined by performing the enzyme assay at temperature ranging from 30–100 °C under standard conditions (100 mM phosphate buffer, pH 8.0). The relative activities were calculated by considering the maximum activity as 100 %. Effect of temperature on the stability of purified enzyme was studied in the range of 70–90 °C by pre-incubating the laccase enzyme at these temperatures along with 100 mM phosphate buffer (pH 8.0) for 2 h. The residual enzyme activity was determined by spectrophotometric method using 2, 6-DMP as a substrate in same buffer.

2.5.3. Effect of metal ions and chemical reagents on laccase activity

The analyse the effects of metal ions like $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, halide ions (NaCl) and chemical reagents like SDS, L-cysteine, sodium azide (NaN_3), ethylenediaminetetraacetic acid (EDTA) on laccase activity, the enzyme was pre-incubated with different concentration of each of the reagent for 30 min at room temperature prior to enzyme assay and then residual activity was calculated by standard assay method. The enzyme assay without any reagent was taken as control.

2.5.4. Determination of molecular mass

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed as reported by Laemmli (1970) [35] with 5 % stacking gel and 12 % resolving gel using a small size vertical electrophoresis system. Samples were run with pre-stained standard molecular weight marker (12–245 kDA). Protein bands were visualized by Coomassie Brilliant Blue (staining). To detect the laccase activity, Native-PAGE was done which was carried out without boiling the sample and without the use of SDS & mercaptoethanol. The native gel was rinsed with water and subsequently submerged in 100 mM phosphate buffer (pH 8.0) with 5 mM 2, 6-DMP substrate.

2.5.5. Substrate specificity and kinetic study

The substrate specificity of purified laccase from selected strain was tested by using various phenolic and nonphenolic substrates viz. 2,6-DMP, guaiacol, SGZ, tyrosine, ABTS, catechol, resorcinol and pyragallol at different values of pH ranging from 3.0 to 10.0 [pH 3.0–4.0 (0.1 M citrate buffer), pH 4.0–5.0 (0.1 M acetate buffer), pH 5.0–8.0 (0.1 M sodium potassium phosphate buffer) and

pH 8.0–10.0 (0.1 M Tris–HCL buffer). The relative rate of oxidation for each substrate (at their optimum pH) was compared using the enzyme activity with 2, 6–DMP as 100 %.

For exploring the kinetic properties of the purified LAC1*, three standard substrates viz. DMP, guaiacol and ABTS were prepared at concentration of 50–500 μ M and used for standard enzyme assay under optimized conditions. Kinetic parameters i.e. K_m (Michaelis Menten constant), and V_{max} (maximum velocity) were determined from Lineweaver–Burk plot for each substrate. Further k_{cat} was also calculated for each substrate.

2.6. Decolorization of dyes by using purified laccase

The purified laccase was studied for its ability to decolorize various synthetic dyes including Malachite green, Crystal violet, Congo red and Indigo carmine to evaluate its use for industrial applications. Decolorization experiments were performed by adding purified laccase (1 U) to each dye solution (concentration of 100 mg/L) prepared in phosphate buffer (0.1 M, pH 7.0), followed by the incubation of the mixture (final volume of 3 mL) at 50°C and 50 rpm for 3 h. The initial concentration of the dye at time zero was taken as a control. The extent of decolorization of each dye was monitored at the λ_{max} of the respective dye. The reaction mixture was incubated for the whole night to observe any positive change in decolorization. The decolorization efficiency of purified laccase was defined by the following expression:

$$\text{Decolorization (\%)} = \frac{A_i - A_f}{A_i} \times 100$$

Where A_i is the initial abs. of specific dye at its respective wavelength

and A_f is the final abs. of specific dye at its respective wavelength.

A negative control was also prepared by adding the heat inactivated enzyme to the dye solution and by incubating as described above. All experiments were performed in triplicate and the means of decolorization percentages were reported. Decolorization studies were then investigated at various pH levels (pH 4.0–10.0) while initial temperature was 50 °C. In addition, the effect of laccase-mediated system on decolourization was also examined by adding 1-Hydroxybenzotriazole (HBT, a non-phenolic laccase mediator) to the reaction mixture described in the previous section. The decolorization percentage was calculated in the same way as described above.

3. RESULTS & DISCUSSION

3.1. Screening, isolation and identification of potential laccase producing bacterium

From three soil samples, six bacterial isolates showing reddish brown coloured zone around their colonies on agar plates were selected and isolated by streak plate method [36]. The coloured zones represents the production of extracellular laccase oxidized the substrate present in the solid medium. The ratio of coloured halo size (HS) around the colony size (CS) was determined and used as an indicator to select suitable isolates for further investigations [37]. An isolate showing largest coloured zone and highest laccase activity was selected and named as LB1 for further study.

On the basis of morphological, biochemical and physiological analysis, the most potential strain (LB1) was identified as, *Alcaligenes faecalis*. The strain showed white and rough colonies with irregular edge on solid agar medium. Gram staining revealed that the studied isolate was a gram negative and long rod shaped, motile bacterium. The identity of the strain was further affirmed by

16S rRNA sequence analysis, and designated as *Alcaligenes faecalis* XF1 under accession number MG009240. Multiple Sequence Alignment was performed by 16S rRNA sequence of 10 type strains relative to the closest related type strain of the other species within the genus *Alcaligenes* available at the NCBI database [38]. Phylogenetic tree of bacteria (Fig. 1) was constructed by neighbour joining method [39] using MEGA software version 6 [40].

3.2. Enzyme Production from the selected strain

Alcaligenes faecalis XF1 strain was studied for its laccase production with respect to time at different phases of growth as shown in Fig. 2. It is observed that the selected strain produces high yield of extracellular laccase when grown in the presence of CuSO_4 under submerged fermentation. In previous studies it was also observed that the presence of Cu^{+2} ions in the medium regulates the laccase production through gene expression induction or through translational or posttranslational regulation [41,42]. So, induction of laccase production from *Alcaligenes faecalis* XF1 in the presence of Cu^{+2} ions may be due to one of the same reason. Growth and laccase production profile of *Alcaligenes faecalis* XF1 indicate that the extracellular laccase production starts after 24 h of incubation and it goes on increasing with increase in growth of the bacteria. The maximum laccase production was achieved after 96 h of incubation with slight decrease in laccase production on further incubation up to 144 h. The decrease in laccase production at this stage of growth may be due to inhibitory effect of metabolites released by the

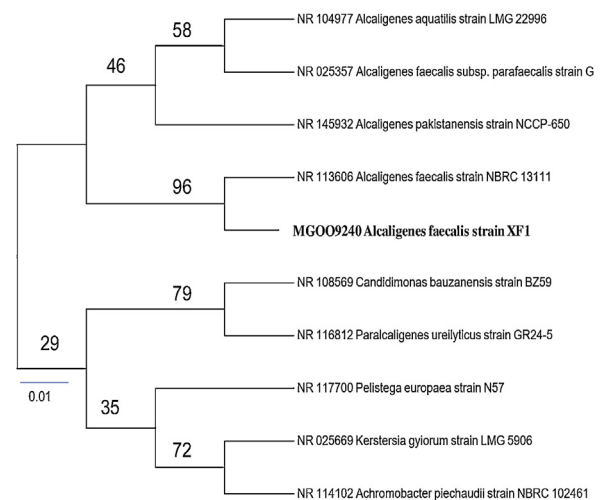


Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparison showing the teiposition of *Alcaligenes faecalis* strain XF1 and other related species of genus.

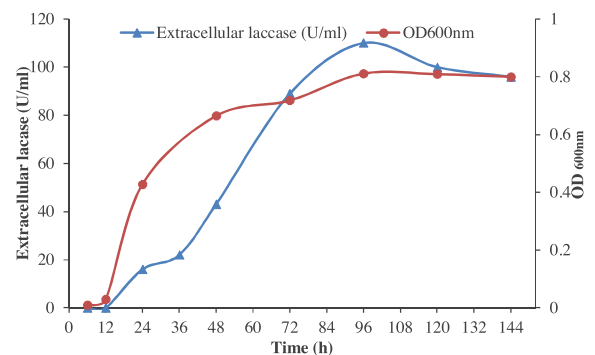


Fig. 2. Growth profile of *Alcaligenes faecalis* XF1 showing optical density (OD) at 600 nm and extracellular laccase production during incubation period of 0–144 hours.

Table 1
Summary of purification of laccase from *Alcaligenes faecalis* XF1.

Purification steps	Total Activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude laccase	11,000	25.5	431	100	1
Salt precipitation	5720	6.31	905.1	52.0	2.1
DEAE-Cellulose	1320	0.8	1637.8	12.0	3.8

Values represent mean \pm SD (n=3).

bacterium itself in the medium. Most of the bacterial laccases are reported as intracellular [43] or spore bound [44], however some extracellular laccase were also obtained like laccase from *Bacillus tequilensis* SN4 [45], *Pseudomonas putida* MTCC 7525 [46]. For industrial applications the extracellular bacterial laccase are highly suitable as their production and purification at high scale is comparatively easier than intracellular and spore bound laccases.

3.3. Purification and characterization of purified laccase

The extracellular laccase obtained after centrifugation of the cultured medium was purified by ammonium sulphate precipitation followed by dialysis and then DEAE- cellulose chromatography. Specific activity, protein content and % yield were monitored after each step of purification and summarized in Table 1. The procedure yielded 0.8 mg of purified enzyme with 3.8 fold purification. The purified laccase from the selected strain was named as LAC1* for convenience.

The purity of the protein was further confirmed by SDS-PAGE and native- PAGE analysis. SDS-PAGE analysis of the purified enzyme showed protein band of ~71kDa correspond to LAC1* as revealed by activity staining of native-PAGE (Fig. 3). It is nearly close to the molecular mass of laccase recently obtained from *Aquisalibacillus elongates*

(75 kDa) [43] but higher than the molecular mass of laccase from *Pseudomonas putida* MTCC 7525 (39.5 kDa) [46], *Bacillus* sp. HR03 (65 kDa) [47]. Single band on native- PAGE, concluded that LAC1* is a monoglycoprotein. The appearance of colored band on the gel might be due to the oxidation of substrate (2, 6-DMP) by the migrating protein (laccase) during native-PAGE.

3.4. Biochemical characterization of LAC1*

3.4.1. Effect of pH on LAC1* activity and stability

As shown in Fig. 4 (a), purified LAC1* was active over a wide range of pH 5.0–10.0 with highest activity at pH 8.0 (optimum pH). By contrast the LAC1* was completely inactive at low values pH

2.0–4.0 and there was a prominent decrease in activity at pH below 7.0 and it retain only 51 % at pH 6.0 and further 37 % at pH 5.0 of its initial activity. The enzyme could retain up to 80 % activity even at pH 9.0 but activity somewhat reduced to 52 % at pH 10.0. Higher activity at a range of pH 7.0–9.0 implies the alkaline nature of the purified LAC1*. However, optimum pH value varies for bacterial laccases extracted from different sources like laccase from *Pseudomonas putida* MTCC 7525, pH 8.0 [46], recombinant laccase lac 15, pH 7.5 [57]. Further it may also vary for different substrates like spore laccase of *B. licheniformis* LS04 strain showed optimum pH 4.2, pH 6.6 and pH 7.4 for ABTS, syringaldazine and 2, 6-Dimethoxyphenol substrates respectively [44].

LAC1* was highly stable at a pH range of 7.0–9.0, retained about 100 % and 47 % of the original activity after incubation at pH 7.0 and pH 9.0 respectively after 10 days incubation at 40 °C as shown in Fig. 4(b). The alkaline resistant activity and stability of LAC1* is comparable to the previously studied bacterial laccases [48,49], but different from fungal laccases, that lost their activities at alkaline pH and comparatively efficient in acidic conditions [26,50].

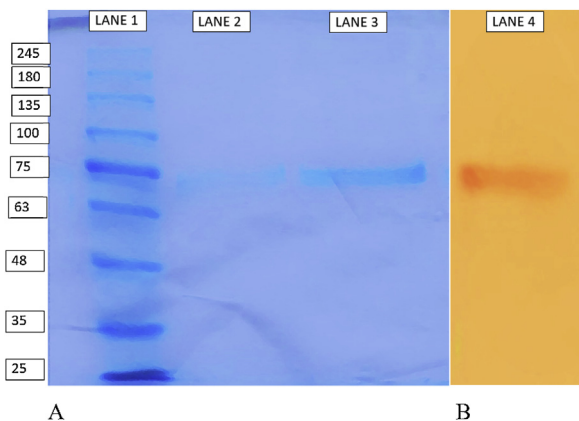
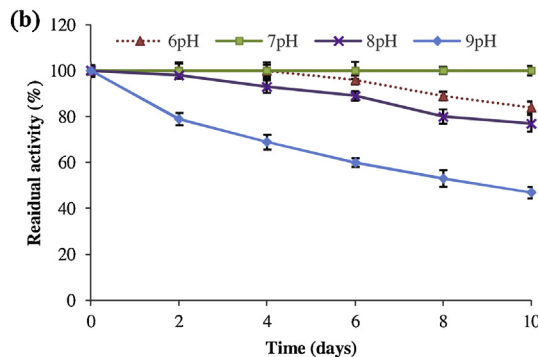
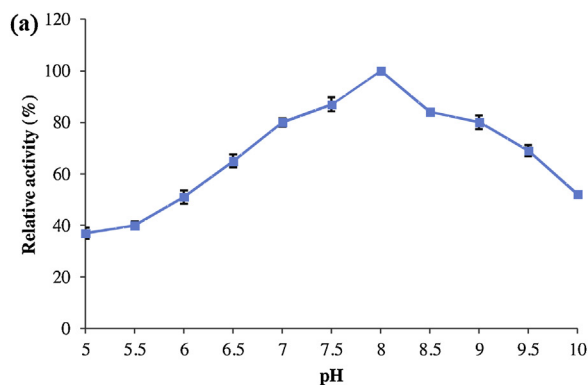


Fig. 3. SDS-PAGE (12%w/v)gel(A)and Native-PAGE of the purified laccase from *Alcaligenes faecalis* XF1(B). Lane 1, represent the protein staining bands of molecular markers; Lane 2 and 3, represent the protein staining bands of purified laccase; Lane 4, represents the activity staining band of purified enzyme with 2,6-Dimethoxyphenol.

Fig. 4. Effect of pH on activity (a) and stability (b) of the purified laccase from *Alcaligenes faecalis* XF1 at 40 °C. (a) Laccase activity was measured at different pH (pH 5.0–10.0) with 2,6-Dimethoxyphenol; (b) Residual activity was measured after pre-incubation of the enzyme solution at pH 7.0, pH 8.0 and pH 9.0 for 10 days.

3.4.2. Effect of temperature on LAC1* activity and stability

The present enzyme showed maximum activity over a wide range of temperature (60–100 °C), with an optimum temperature of 80 °C (Fig. 5a). LAC1* could retained about 96 % & 72 % of initial activity even at 90 and 100 °C respectively. It is completely stable at 60 °C (Fig. 5b) and retained about more than 80 % activity at 70 °C even after 24 h of incubation (data not shown), alike of the some discovered bacterial laccases, which showed remarkable thermostability above 70 °C, such as laccase from *Bacillus licheniformis* LS04 [44] and *Bacillus tequilensis* SN4 [32], but more thermostable in comparison to other bacterial laccases [46,51]. The significant activity and stability over a wide range of temperature and pH shows that the purified laccase from *Alcaligenes faecalis* XF1 is applicable for diverse industrial and biotechnological processes which takes place under harsh conditions.

3.4.3. Substrate specificity and kinetic study of purified LAC1*

Among the diverse substrates used to figure out the oxidation ability of LAC1*, it is able to oxidise all types of phenolic like guaiacol, syringaldehyde, 2, 6-DMP, catechol as well as non-phenolic substrate such as ABTS, but did not show any activity towards tyrosine, resorcinol and pyragallol etc. The optimum pH and the relative rate of oxidation for different substrates were calculated and compared at their respective pH optima and are tabulated in Table 2. The laccase produced by *Alcaligenes faecalis* was more sensitive towards 2, 6-DMP & ABTS than guaiacol as a substrate, that is may be due to laccase inactivation by reaction by-product formed after guaiacol oxidation [44,52,53]. Though, better oxidation of 2, 6-DMP, & other substrates at high temperature has

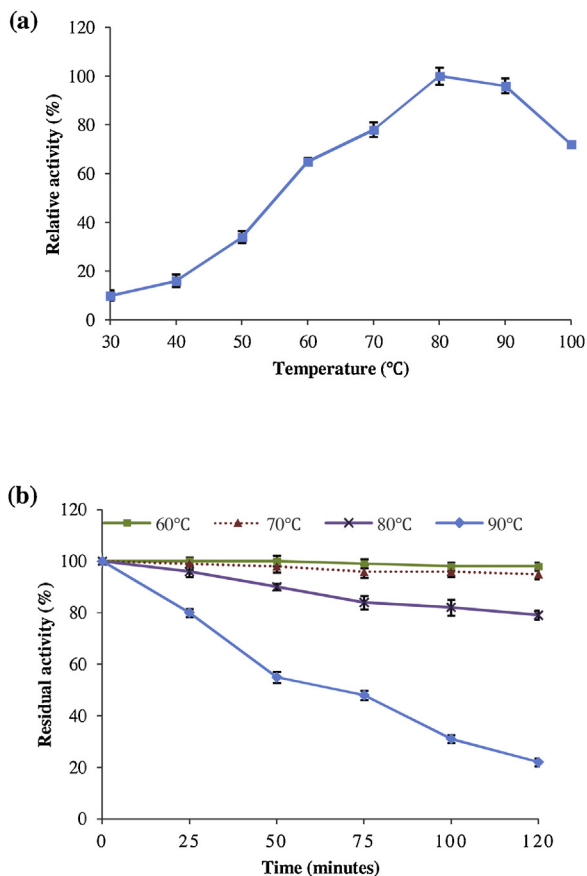


Fig. 5. Effect of temperature on activity (a) and stability (b) of the purified laccase from *Alcaligenes faecalis* XF1 at pH 8.0. (a) Laccase activity was measured at different temperatures (30–100 °C); (b) Residual activity was measured after pre-incubation of laccase at 60–90 °C for 0–120 min.

Table 2

Substrate profile of purified laccase from *Alcaligenes faecalis* XF1.

Substrate	λ_{\max} (nm)	Optimum pH	Relative activity (%)
2, 6-DMP	470	8	100
ABTS	420	5.5	98.95
SGZ	530	7.5	95.10
Guaiacol	465	8	85.25
Catechol	392	7	24.59
Pyragallol	450	–	ND
Tyrosine	278	–	ND
Resorcinol	340	–	ND

also been reported for laccase from *Bacillus tequilensis* SN4 (90 °C) [32], CotA from *Bacillus subtilis* (75 °C) [54], in contrast to LAC21, which could not oxidise ABTS & guaiacol [22] and *Bacillus* sp. ADR laccase, not able to oxidise ABTS & syringaldehyde [55]. The ability of LAC1* to oxidise ABTS and SGZ, but not to tyrosine, indicates a true laccase property which is in agreement with the previous studies [45]. Hence, LAC1* studied to be nonspecific to their substrates, but is able to oxidise a broad range of aromatic compounds and hence, it can be used in textile dye bleaching and detoxification of contaminated soil and water.

Kinetic parameters (K_m & V_{\max}) of LAC1* for oxidation of different substrates were obtained from their Lineweaver–Burke plots, $1/V$ versus $1/[S]$ [for 2, 6-DMP (Fig. 6)] and summarized in Table 3. The observed data indicates the efficient oxidation ability of LAC1* for all types of mentioned substrates. The calculated K_m and V_{\max} for 2, 6-DMP, ABTS and guaiacol were 480 μM , 500 μM , 1000.56 μM and 110 U/ml, 99.59 U/ml, 67.5 U/ml respectively. The lower K_m and higher V_{\max} value of LAC1* for 2, 6-DMP demonstrates its higher affinity and reaction velocity towards DMP as compared to ABTS and guaiacol. The k_{cat} value of LAC1* for 2, 6-DMP was 1375 s^{-1} , which was much higher than that of the thermostable laccase from *Bacillus tequilensis* SN4 (k_{cat} 73.15 s^{-1}) [32] and *Bacillus* sp.HR03 (k_{cat} 3.0 s^{-1}) [23] indicating higher catalytic efficiency of LAC1*. However, previous reports also believed that the oxidation efficiency and substrate affinity of laccases are significantly depends upon the nature, substitution of the phenolic ring and the type of the substrate used [47,53]

3.4.4. Effect of metal ions and inhibitors on the LAC1* activity

The influence of chemical inhibitors on LAC1* activity was analysed with 2, 6-DMP as the assay substrate and the investigated results are summarized in Table 4. The observed data represent the significant resistance to most of the tested known laccase inhibitors at lower concentration. However, the influence goes on increasing with increasing concentration of inhibitors, as the

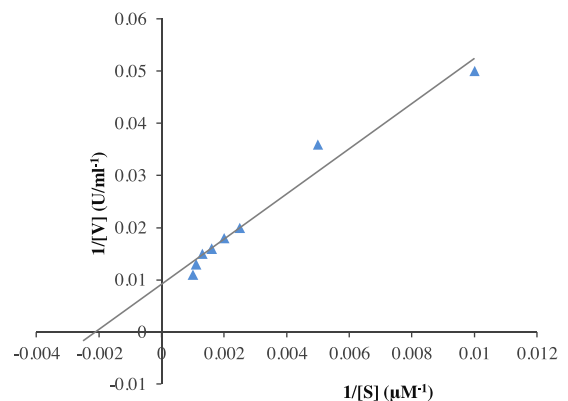


Fig. 6. Lineweaver-Burk plot for 2,6-Dimethoxyphenol oxidation for purified laccase under optimized conditions (pH 8.0, 80 °C).

Table 3
Kinetic properties of purified laccase for different substrates.

Substrate	K_m (μ M)	V_{max} (U/ml)	k_{cat} (s^{-1})
2, 6-DMP	480	110	1375
ABTS	500	99.59	1243.75
Guaiacol	1000.56	67.5	843.75

Table 4
Effect of inhibitors on the activity of laccase from *Alcaligenes faecalis* XF1.

Inhibitors	Concentration (mM)	Relative activity (%)
Control	–	100
Cysteine	0.1	48.70 \pm 7.64
	0.5	2.79 \pm 2.48
	1.0	0
NaN ₃	0.1	76.4 \pm 3.8
	0.5	13.68 \pm 7.7
	1.0	0
EDTA	1.0	87.8 \pm 6.1
	5.0	36.9 \pm 3.5
	10.0	6.2 \pm 9.1
	20.0	0
SDS	1	103.05 \pm 4.28
	5	43.04 \pm 2.24
	10	0

presence of sodium azide, cysteine and EDTA at the concentration of 1 mM, 1 mM and 20 mM respectively causes the complete inhibition of LAC1*. Complete inhibition of LAC1* at higher concentration is correspond to most of the bacterial laccases reported previously [19,50]. Effect of SDS (surfactant) on LAC1* showed that 1 mM SDS effectively enhanced the activity of LAC1* to about 103.51 % and retained about 43.50 % of the initial activity even in the presence of 5 mM SDS. The results obtained in this study were also supported by Lu et al. [56], and Feng et al. [57], and that is inhibitory effect of chemical inhibitors on laccase is may be due to the binding nature of the inhibitors to the copper sites of laccase and thus interfering with their catalytic activity [58].

Halides as well as metal ions are also known to interact with the active site of enzymes and alter their stability [28]. Therefore the effect of different metal ions on LAC1* were studied and compiled in the form of Table 5. According to results, the presence of 1 mM of Cu²⁺, Mg²⁺ and Mn²⁺ significantly enhanced the activity of LAC1* to about 112.24 %, 109.68 % and 105.91 % respectively. The activity was gradually increases as Cu²⁺ ion concentration further

Table 5
Effect of metal ions and halide on the activity of purified laccase from *Alcaligenes faecalis* XF1.

Metal ions	Conc. (mM)	Relative activity (%)
Control	–	100
Cu ²⁺	1	112.24 \pm 1.33
	3	135.39 \pm 0.96
	5	152.46 \pm 1.61
	10	109.68 \pm 1.45
Mg ²⁺	1	80.71 \pm 2.63
	3	74.56 \pm 2.58
	5	99.43 \pm 3.25
Zn ²⁺	1	73.03 \pm 1.32
	3	55.75 \pm 2.41
	5	105.91 \pm 1.36
Mn ²⁺	1	79.30 \pm 1.17
	3	64.84 \pm 2.50
	5	94.76 \pm 1.49
	10	80.39 \pm 2.06
Ca ²⁺	1	76.99 \pm 3.45
	3	110.92 \pm 1.66
	5	101.18 \pm 2.14
	10	56.76 \pm 1.88
NaCl	100	110.92 \pm 1.66
	500	101.18 \pm 2.14
	1000	56.76 \pm 1.88

increased, and enhanced up to 152.46 % of the initial value in the presence of 5 mM CuSO₄. Whereas presence of Mg²⁺ and Mn²⁺ at higher concentration (5 mM) effectively reduced the LAC1* activity to 74.56 % and 64.84 % respectively. The phenomena observed in this study is supported by Fang et al., [22] and Sondhi et al., [32]. According to their observations, some low concentration of metal ions are favourable for the laccase activity, yet the enzyme was significantly activated by Cu²⁺ ions. The significant effect of Cu²⁺ on laccase activity may be due to filling of type II Copper binding site by Cu ions [56]. Other tested metal ions i.e. Zn²⁺ and Ca²⁺ (at conc. of 1 mM) shows none of any significant or inhibitory effect on LAC1* activity. The obtained data showed that the LAC1* activity was effectively enhanced to about 110.92 % of the original value by 100 mM of NaCl and furthermore retained its activity to original level, even at the 500 mM of NaCl. Similarly, a bacterial laccase (Lac15) from the marine microbial metagenome retained the maximum activity in the presence of 1000 mM NaCl [57]. Thus, purified LAC1* showed high tolerance ability towards chloride ions and most of the metal ions makes it a potential candidate for waste water treatment, released by different textile and paper-pulp industries, which usually contains high amount of salt concentration [43,57].

3.5. Dye decolorization

Decolorization of dyes is the most promising biotechnological application of bacterial laccases [59]. Thus four synthetic dyes with indigo (Indigo carmine), anthraquinonic (Malachite green and azo (Crystal violet and Congo red) structures were used to analyze the decolorization ability of LAC1* (Fig. 7). The decolorization process was studied in the presence and absence of HBT as a redox mediator. As expected, the purified LAC1* decolorized all the tested dyes with varied efficiency of 31–70 % under preliminary conditions. No color change was observed in the negative controls. As shown in (Fig. 7a), the rate of decolorization was observed in the order of Indigo carmine > Malachite green > Crystal violet > Congo red in the respective percentage of 70 %, 48 %, 36 % and 31 % within 3 h of incubation. Whereas no further increase in decolorization was observed till 24 h of incubation under standard conditions (data not shown). About 68 % and 47 % of decolorization were observed for Indigo carmine and Malachite green, in contrast to Crystal violet and Cresol red which represents more resistance to decolorization in the absence of mediator and showed only 34 % (Crystal violet) and 30 % (Congo red) of decolorization in initial 2 h of incubation. In a recent study, a spore laccase from strain LS04 showed only 15.76 % of Reactive blue 19 and 16 % of Indigo carmine decolorization after 2 h incubation at 40 °C, pH 6.6, which is improved to about 32 % of reactive blue 19 and more than 50 % of reactive black 5 after 6 h of incubation (under same conditions) [44]. In an another study, culture supernatant (extracellular laccase) of *Pseudomonas putida* showed about 36–94 % decolorization of dyes (Brilliant green, Bromophenol blue, Crystal violet and Congo red) within 12–24 h at room temperature [46]. So from the above references it is evident that the rate of decolorization of synthetic dyes is widely depending on the sources of laccases, type of dyes, chemical nature of applied dyes, and other physical factors such as pH and temperature. Due to complex structure, azo dyes are recognised as recalcitrants for laccases thus results in slow oxidation whereas anthraquinones dyes are easily oxidised by most of the laccases [57].

Further to study the effect of pH on the rate of decolorization, the decolorization of different synthetic dye were observed at the pH range of pH 4.0–10.0 and represented in (Fig. 7b).

As observed from the data, the decolorization rate of four studied synthetic dyes i.e. indigo carmine, malachite green, crystal violet and congo red was 70 %, 48 %, 33 % and 31 % at pH 7.0 now

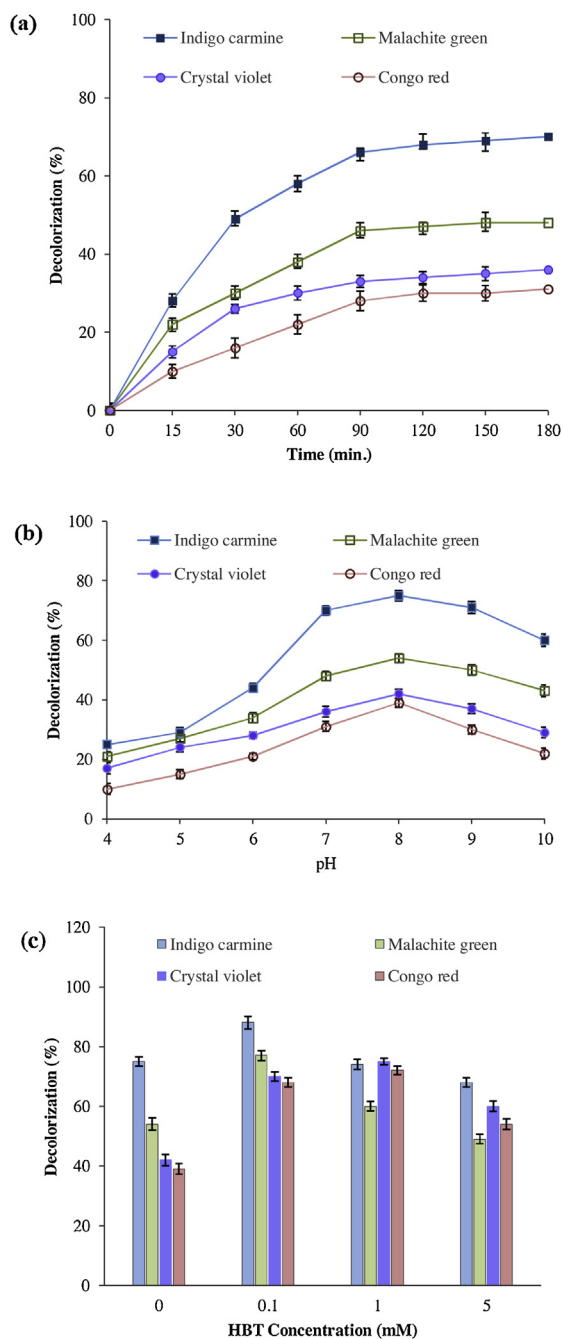


Fig. 7. Decolorization of four different synthetic dyes (conc. of 100 mg/L) by purified laccase (1 U/ml) from *Alcaligenes faecalis Xf1* at 50 °C. (a) Decolorization profile of different synthetic dyes during 180 min of incubation at pH 7.0; (b) Decolorization of different synthetic dyes after 180 min of incubation at different initial pH values (pH 4.0–10.0); (c) Decolorization of synthetic dyes by laccase-HBT system after 120 min of incubation at initial concentration of 0, 0.1, 1, and 5 mM of HBT as a mediator (pH 8.0).

enhanced to 75 %, 54 %, 42 % and 31 %, respectively at pH 8.0, which may be due to the alkaline nature of the purified LAC1*, previously described in this study. Some recent studies also provide the same information that the maximum decolorization of dyes was taken place at the optimum pH of the respective enzyme used for the study [56,60]. Further the change of pH from alkaline to acidic range significantly decrease the rate of decolorization for all type of dyes as shown in graph. The study of Zeng et al. [61], also revealed that the four synthetic dyes were efficiently decolorized by crude

laccase from a *Trametes trogii* strain at the optimum pH of 4.0 and 5.0, with a sharp decline in decolorization rate at lower and higher pH values.

Furthermore the decolorization efficiency of purified LAC1* for four different synthetic dyes were also studied in the presence of HBT (a non-phenolic redox mediator) and the result were shown in (Fig. 7c). The presence of 0.1 mM HBT in the decolorization enzyme mixture significantly increased the percentage of decolorization for all the dyes. The decolorization was greatly enhanced from 75 %, 54 %, 42 % and 39%–88%, 77 %, 70 %, and 68 % for indigo carmine, malachite green, crystal violet and congo red respectively, under optimum pH of 8.0 and within 2 h of incubation. But as the concentration of mediator increased from 0.1 mM to 5 mM, the decolorization percentage were declined to 68 %, 49 %, 60 %, 54 % of the tested dye under same conditions. The significant effect of laccase mediator in dye decolorization have also been reviewed in many studies [24,56,57] which relates it to the lower redox potential of the laccases. So, the present study shows the positive and negative impact of laccase mediated system in the decolorisation of dyes.

4. Conclusion

In the present study, an extracellular laccase producing bacterial strain was isolated and identified as *Alcaligenes faecalis Xf1*. The purified laccase from the isolated strain showed its maximum activity at the optimum pH of 8.0 and optimum temperature of 80 °C. It retained a significant level of activity after a long period of incubation at the extreme conditions of pH and temperature that explains the alkaline and thermostable nature of the purified laccase. Which makes the purified laccase suitable for various industrial processes. Further the purified laccase showed a considerable resistance towards metal ions and halide ions which shows their possible application in decolorization of industrial effluent and waste water treatment. Furthermore the decolorization ability of purified laccase confirmed its wide applications at industrial level.

Declaration of Competing Interest

The authors with listed names declare no conflict of interest to disclose.

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