An alkaline bacterial laccase for polymerization of natural precursors for hair dye synthesis

Deepak Kumar¹ · Aditya Kumar¹ · Sonica Sondhi¹ · Prince Sharma¹ · Naveen Gupta¹

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Abstract

In the present study, an extracellular alkali stable laccase (Lac DS) from *Bacillus subtilis* DS which has pH optima at 8.5 using *p*-phenylenediamine (PPD) as substrate has been reported. Lac DS retained 70% activity for 4 h at pH 8.5 and 90% activity for 24 h at 55 °C. The enzyme yield was enhanced by optimization of fermentation conditions. A 746-fold increase in yield was observed under optimized conditions using 150 µM MgSO₄, 1.2% yeast extract, 0.35% tryptone, and 150 µM vanillic acid. Lac DS was used to polymerize natural dye precursor catechol, pyrogallol, syringaldehyde, syringic acid, ferulic acid and gallic acid to develop a range of natural hair colors such as black, golden yellow, and reddish brown. The results indicate that alkaline Lac DS is a suitable candidate to develop a user-friendly and commercially applicable hair dyeing process in the area of cosmetic industry.

Keywords Laccase · Hair dye · Synthesis · Polymerization · PPD · Natural precursors

Introduction

Laccases (benzenediol:oxygen oxidoreductases; EC 1.10.3.2) are multicopper oxidases, that catalyze the oxidation of a wide range of compounds with concomitant four-electron reduction of molecular oxygen to H₂O. They catalyze the oxidation of both phenolic and non-phenolic substrates (Imran et al. 2012). Laccases are obtained from different sources such as plants, actinomycetes, fungi (Ascomycetes, Basidiomycetes, and Deuteromycetes), and bacteria (Zille et al. 2005; Shraddha et al. 2011). Due to their ability to oxidize a wide range of substrates, laccases have found application in number of biotechnological processes such as pulp biobleaching, food industry, degradation of textile dyes and xenobiotics bioremediation (Sondhi et al. 2015).

One of the less-explored roles of laccase/s is in the cosmetic industry, where one possible application is in hair

coloring (Jeon et al. 2010; Saito et al. 2012). At present various available hair coloring products in market mostly used synthetic dye such as PPD. However, synthetic dyes have various side effects and cause hair damage as well. Apart from oxidation, laccases are known to catalyze the polymerization of a number of substrates, therefore, they can be used for the synthesis of dyes by polymerization of natural precursors. However, there are some prerequisites to it; the hair coloring reaction has to be carried out at alkaline pH as it helps in the swelling of the hair and enhance the penetration of the dye. If the hair coloring is carried out at neutral or acidic pH, the hair gets apparently colored but it gets easily decolorized while washing. Although a number of laccase/s have been reported so far but very few alkaline laccases are known. Jeon et al. (2010) had reported the use of laccase for the synthesis of colors from natural precursors at acidic pH. Therefore, a laccase is required that can act on natural precursors at alkaline pH. Moreover, a range of colors is required for commercial hair dyeing, where the candidate laccase should be able to act on various precursors synthesizing different colors at alkaline pH.

In this study, we have isolated an extracellular laccase from *Bacillus subtilis* DS which can synthesize a range of colors from natural precursors at alkaline pH in a short reaction period. These natural dyes can be used to develop a process which can substitute the synthetic hair coloring

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✉ Naveen Gupta
ng.puchd@gmail.com

¹ Department of Microbiology, BMS Block 1, South Campus, Panjab University, Sector 25, Chandigarh, India

dyes. For commercial application, high yields of enzyme need to be produced in an economical way. Enzyme production was optimized following conventional approaches as well as statistical methods such as Plackett–Burman (PB) and Response Surface Methodology (RSM) and high yields of the enzyme was obtained. Therefore, laccase from *Bacillus subtilis* DS can be used to develop commercially applicable user-friendly hair dyeing process.

Materials and methods

Chemicals

Substrates viz. syringaldazine (SGZ), guaiacol, 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS); inhibitors viz. sodium azide and β -mercaptoethanol were purchased from Sigma-Aldrich (USA). Other substrates viz. *p*-phenylenediamine (PPD), catechol, tyrosine, and inducers viz. syringaldehyde, syringic acid, ferulic acid, vanillin, vanillic acid, gallic acid, pyrogallol, 2,6-xylydine were purchased from Hi-Media (India). All the chemicals used were of analytical grade.

Isolation of laccase-producing bacteria

Laccase-producing bacteria were isolated on M162 agar medium (Degryse et al. 1978) supplemented with 0.2% yeast extract, 0.2% tryptone, 100 μ M CuSO₄, and 2 mM guaiacol (Sondhi et al. 2015). Soil samples with decaying wood from the forest area of Chandigarh and Himachal Pradesh were enriched and appropriate dilutions were plated. The plates were incubated at 37 °C for 48 h. The colonies showing reddish brown color were shortlisted for further analysis.

Preparation of enzyme and selection of bacterial strain

Shortlisted bacterial cultures were screened for the presence of extracellular laccase. For this, 0.1% of overnight-grown bacterial culture was inoculated in M162 broth containing 0.2% yeast extract, 0.2% tryptone, and 100 μ M CuSO₄. After 48 h of incubation, culture supernatant was obtained by centrifugation at 10,000 rpm for 15 min and was used as an extracellular enzyme. The laccase activity was assayed using SGZ (2 mM) as substrate. The strain exhibiting maximum extracellular laccase activity was selected. Oxidation of SGZ, ABTS, tyrosine and PPD were also assayed, concentration of each substrate was kept 2 mM.

Laccase estimation

Assay procedure

Laccase activity was determined using 2 mM syringaldazine (SGZ) as substrate at 55 °C (40 °C for initial screening) for 2 min in 0.1 M phosphate buffer (pH 6.5). The change in absorbance due to the oxidation of SGZ was monitored at 530 nm ($\epsilon = 64,000 \text{ M}^{-1} \text{ cm}^{-1}$) in a UV–VIS spectrophotometer (Hitachi U-2900⁺). The enzyme unit was expressed in IU ml⁻¹. One unit of enzyme activity was defined as micromoles of substrate converted into product per minute by one ml of the enzyme under standard assay condition.

Effect of temperature and pH on laccase activity and stability

To determine the temperature optima enzyme activity was analyzed at different temperatures (40–70 °C). The thermo-stability of the enzyme was measured over the temperature range of 40–65 °C by incubating the enzyme in thin-wall test tubes for 0–24 h. At different time intervals, aliquots of enzyme were taken and chilled in an ice-water bath. The residual enzyme activity was determined as per standard assay procedure using SGZ as substrate. The optimum pH for laccase activity using SGZ and PPD as substrate was determined by performing the enzyme assay at different pH values ranging from 6.0 to 9.5 [6.0–7.5, (0.1 M phosphate buffer) and 8.0–9.5, (0.1 M Tris–HCl buffer)]. pH stability of LacDS was measured over a pH range of 6.0–9.5 by incubating the enzyme in buffers of various pH for 0–12 h. At different time intervals, aliquots of enzyme were taken and residual enzyme activity was determined at 55 °C using SGZ as substrate.

Identification of laccase-producing organism

The cellular morphology of the bacterial isolate was examined by Gram staining. Physiological and biochemical characterization was done according to Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). Further, the identification of bacteria was carried out using 16S rRNA sequencing wherein the genomic DNA was isolated and 16S rRNA fragment was amplified. The product was sequenced using universal primers and sequence analysis was carried out. Phylogenetic tree was constructed with 1000 bootstrap replicates using MEGA 7 software (Kumar et al. 2016).

Optimization of laccase production by submerged fermentation

Optimization of laccase production by one variable at a time method (OVAT)

The effect of various physiochemical factors on laccase production was determined by varying one factor at a time and keeping the other constant. Different parameters were optimized which included incubation time, pH, temperature, concentrations of inoculum size, CuSO_4 , xylose and MnSO_4 . All the experiments were carried out in triplicates.

Statistical optimization of laccase production

On the basis of results obtained from one factor at a time studies and data available in literature, 19 different parameters viz. A: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; B: $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; C: $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; D: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; E: $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$; F: $\text{Al}_2\text{SO}_4 \cdot 16\text{H}_2\text{O}$; G: $\text{NiSO}_4 \cdot \text{H}_2\text{O}$; H: yeast extract; J: xylose; K: 2,6,xylydine; L: catechol; M: ferulic acid; N: tryptone; O: vanillic acid; P: vanillin; Q: methanol; R: $\text{CaSO}_4 \cdot 7\text{H}_2\text{O}$; S: $(\text{NH}_4)\text{SO}_4$; T: ethanol were selected and PB design was employed to choose significant parameters amongst them.

Each factor was investigated at two levels, high (+) and low (−). A design of total 20 experiments was generated using the software Design Expert 8.0.7.1. The effect of individual factors on enzyme activity was calculated according to the following equation:

$$E_i = \frac{\sum P_{i+} - \sum P_{i-}}{N} \quad (1)$$

In Eq. 1, E_i is the effect of parameter i under study, P_{i+} and P_{i-} are responses (enzyme activity) of trials at which the parameter was at its high and low level, respectively, and N is the total number of trials. From the Pareto chart, the factors showing maximum positive effects were selected.

Central composite design (CCD)

The variables which showed positive effect (yeast extract, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, tryptone, and vanillic acid) on the production of laccase were optimized using CCD. The minimum and maximum ranges of the selected variables, i.e., yeast extract (0.4–1.6 g), MgSO_4 (50–200 μM) $\cdot 7\text{H}_2\text{O}$, tryptone (0.2–0.5 g), and vanillic acid (50–250 μM), were used in 30 combinations (Table 2). All the other parameters were used at their optimum level as obtained from OVAT results. The RSM model was further validated for predicted versus actual responses.

Partial purification

The cell-free supernatant was precipitated using fractional (60–80%) ammonium sulfate precipitation. The precipitates were dialyzed and the protein thus obtained was used for further experiments.

Synthesis of color from natural precursors using Lac DS

Natural precursors such as catechol, gallic acid, syringaldehyde, syringic acid, ferulic acid and pyrogallol were used for the color development (Jeon et al. 2010); 2 mM solution of each precursor was prepared in 100 mM Tris-HCl pH 8.5 and made to react with $10 \pm 0.5 \text{ IU ml}^{-1}$ of Lac DS for 30 min and color development was observed. Appropriate substrate control (Substrate + Denatured Enzyme + Buffer), and enzyme control (Enzyme + Buffer) were used.

Results

Isolation and screening of extracellular laccase-producing bacteria

Thirty-nine bacterial isolates were examined for extracellular laccase activity. Only two isolates exhibited extracellular laccase activity, maximum enzyme yield was obtained from bacterial Isolate 1. Laccase from this strain oxidized laccase-specific substrates such as SGZ, ABTS and dyes used in hair coloring like PPD, but no activity was found with tyrosine. Laccase activity was inhibited by laccase inhibitors, β -mercaptoethanol and sodium azide (Sondhi et al. 2014).

Effect of temperature and pH on laccase activity and stability

Laccase from Isolate 1 was found to be active in the temperature range of 45–70 °C, maximum being at 55 °C (Fig. 1a). Enzyme retained 90% of its activity even after 24 h at its optimum temperature (Fig. 1b). The optimum pH of the enzyme was found to be 6.5 using SGZ as substrate. Enzyme oxidized hair dye PPD optimally at pH 8.0–9.5 (Fig. 1c). Moreover, this enzyme was highly alkali stable as it retained 60–70% of initial activity after 4 h incubation at pH 8.5–9.0 (Fig. 1d). Isolate 1 was selected and designated as Isolate DS.

Identification of the isolate

On the basis of the morphological and biochemical analysis, Isolate 1 was found to be similar to the Genus *Bacillus*. 16S rRNA sequence (Accession no MF359736) analysis was carried out and a phylogenetic tree of this sequence

Fig. 1 Effect of temperature and pH on laccase enzyme activity **a** temperature optima, **b** temperature stability, **c** pH optima, **d** pH stability

was constructed by maximum likelihood method with 1000 bootstrap replicates using MEGA7 (Fig. 2). The result of this phylogenetic analysis inferred that this isolate is most closely related to *Bacillus subtilis*. Therefore, Isolate DS was identified as a strain of *Bacillus subtilis* and named *Bacillus subtilis* DS and strain culture was deposited at MTCC, Chandigarh, with accession no 12618.

Fig. 2 Phylogenetic analysis based on 16S rRNA sequence

OVAT method

Classical one variable at a time method was used for the optimization of enzyme production; a 27-fold increase in enzyme yield was achieved under following optimized conditions: incubation time-96 h, pH-7.0, temperature-37 °C, inoculum size-1%, CuSO₄-50 μM, xylose-0.4%, MnSO₄-150 μM (Table 1).

Table 1 Enhancement of laccase production from different parameters using OVAT

	Unit	Range	Optimum	Activity (U ml ⁻¹)
Initial unop- timized Medium ^a	–			1.1
Incubation time	h	24–120	96	2.4 ± 0.16
pH		6–10	7	4.72 ± 0.03
Temperature	°C	20–42 °C	37 °C	6.67 ± 0.07
Inoculum size	Percentage	0.1–5.0%	1%	7.41 ± 0.23
CuSO ₄ ·5H ₂ O	μM	0.0–5.0	0.05	11.3 ± 0.10
Xylose	Percentage	0.0–1.6%	0.4	27.77 ± 0.15
MnSO ₄	Mm	0.05–0.300	0.15	29.46 ± 0.14

^a0.2% yeast extract, 0.2% tryptone, 100 μM CuSO₄, 10X M162

Screening of important components using PB-design

The effect of 19 different parameters on the production of laccase estimated with an enzyme yield ranging from 1.0 ± 0.1 to 39.68 ± 0.04 IU ml⁻¹ was observed (Supplementary Table 1). Influence of various parameters on laccase production is represented in Pareto chart Fig. 3. Out of 19 factors, 10 (FeSO₄·7H₂O, CuSO₄·5H₂O, MgSO₄·7H₂O, CoSO₄·7H₂O, Al₂SO₄·16H₂O, Yeast Extract, xylose, ferulic acid, tryptone, vanillic acid, methanol) showed positive effect, 4 factors viz. yeast extract, MgSO₄·7H₂O, tryptone, and vanillic acid showed maximum influence on enzyme production. Therefore, to obtain the maximum enzyme production, optimal concentration of yeast extract, MgSO₄·7H₂O, tryptone, and vanillic acid were investigated with RSM.

Optimization by CCD

On the basis of PB design, yeast extract, MgSO₄·7H₂O, tryptone, and vanillic acid were found to be most influential components, which enhanced the yield. A CCD was formulated to find the optimal concentration of these parameters and the response values are represented in Table 2. By applying multiple regression analysis on the experimental

Fig. 3 Pareto-chart showing significant factors (yellow color) and non-significant (blue color)

Table 2 Central composite design with predicted and actual responses

Run	Factor 1 A:yeast extract G	Factor 2 B:vanillic acid Micromole	Factor 3 C:tryptone G	Factor 4 D:MgSO ₄ Micromole	Response 1 Laccase activity IU ml ⁻¹	Predicted value	Residual
1	0.80	200.00	0.50	100.00	731.87	732.77	− 0.90
2	1.20	150.00	0.65	150.00	746.09	741.11	4.98
3	0.80	100.00	0.50	100.00	766.25	765.11	1.14
4	0.80	200.00	0.20	200.00	740.31	750.43	− 10.12
5	1.20	150.00	0.35	50.00	733.43	734.96	− 1.53
6	1.60	200.00	0.50	100.00	715.00	722.73	− 7.73
7	1.60	100.00	0.50	200.00	731.09	716.68	14.41
8	1.60	200.00	0.20	100.00	739.21	750.22	− 11.01
9	1.20	50.00	0.35	150.00	734.06	763.55	− 29.49
10	1.60	200.00	0.50	200.00	734.84	737.28	− 2.44
11	1.60	200.00	0.20	200.00	789.30	775.13	14.17
12	0.80	200.00	0.50	200.00	746.47	760.64	− 14.17
13	1.20	250.00	0.35	150.00	779.37	759.11	20.26
14	0.80	100.00	0.50	200.00	733.75	728.83	4.92
15	1.60	100.00	0.20	100.00	815.95	786.46	29.49
16	1.20	150.00	0.35	150.00	815.95	795.84	20.11
17	1.20	150.00	0.35	150.00	809.91	795.84	14.07
18	0.80	100.00	0.20	200.00	734.37	711.32	23.05
19	1.20	150.00	0.35	150.00	820.16	795.84	24.32
20	1.20	150.00	0.35	150.00	799.34	795.84	3.50
21	1.20	150.00	0.35	150.00	735.46	795.84	− 60.38
22	1.60	100.00	0.20	200.00	742.03	747.22	− 5.19
23	0.80	100.00	0.20	100.00	733.59	737.24	− 3.65
24	1.20	150.00	0.35	150.00	794.24	795.84	− 1.60
25	1.20	150.00	0.05	150.00	736.87	751.08	− 14.21
26	2.00	150.00	0.35	150.00	738.12	751.37	− 13.25
27	1.60	100.00	0.50	100.00	770.31	766.28	4.03
28	1.20	150.00	0.35	250.00	715.90	723.60	− 7.70
29	0.40	150.00	0.35	150.00	729.53	725.50	4.03
30	0.80	200.00	0.20	100.00	713.10	712.20	0.90

Values represent mean $\pm$ standard deviation ($n = 3$)

data, a predictive quadratic polynomial equation was constructed to describe the correlation between enzyme yield and the four significant parameters as follows:

$$\begin{aligned}
 (R)^1 = & +795.84 + 6.47 * A - 1.11 * B - 2.49 * C - 2.84 * D \\
 & - 2.80 * AB - 12.01 * AC - 3.33 * AD - 1.82 * \\
 & BC + 16.04 * BD - 2.59 * CD - 14.35 * A^2 \\
 & - 8.63 * B^2 - 12.44 * C^2 - 16.64 * D^2
 \end{aligned} \quad (2)$$

Where, R is the response (laccase yield, IU ml⁻¹), A , B , C and D are the concentrations of yeast extract, vanillic acid, tryptone and MgSO₄, respectively. The analysis of variance for the response surface quadrate model is summarized in

Table 3. The $p < 0.0348$ indicated that all the linear, interactive, and squared terms had a significant influence on enzyme activity. The p value for the lack of fit was 0.8820, indicating that this quadratic model adequately fit into the data. The determination coefficient R^2 (0.7132) indicated that the predicted and experimental values had perfect coherence with each other. The value of adjusted R^2 (0.4456) suggested that the variation in the enzyme activity was attributed to the independent variables and only 0.43% of the total variation could not be explained by the model. The spherical curves in Fig. 4 indicated that the interactions between yeast extract, vanillic acid, tryptone and MgSO₄ were significant. According to Fig. 4, the relative significance of the impact of the parameters on enzyme yield was in the following order: yeast extract > vanillic acid > tryptone > MgSO₄, which

Table 3 ANOVA for response surface

Source	Sum of squares	df	Mean square	F value	p value Prob > F	
Analysis of variance table						
Model	22,377.49	14	1598.39	2.66	0.0348	Significant
A-yeast extract	1003.63	1	1003.63	1.67	0.2154	
B-vanillic acid	29.53	1	29.53	0.049	0.8274	
C-tryptone	149.20	1	149.20	0.25	0.6252	
D-MgSO ₄	193.69	1	193.69	0.32	0.5783	
AB	125.55	1	125.55	0.21	0.6539	
AC	2309.28	1	2309.28	3.85	0.0686	
AD	177.56	1	177.56	0.30	0.5944	
BC	53.29	1	53.29	0.089	0.7697	
BD	4115.22	1	4115.22	6.86	0.0193	
CD	107.33	1	107.33	0.18	0.6783	
A ²	5649.31	1	5649.31	9.42	0.0078	
B ²	2042.30	1	2042.30	3.40	0.0848	
C ²	4243.11	1	4243.11	7.07	0.0178	
D ²	7596.02	1	7596.02	12.66	0.0029	
Residual	8997.48	15	599.83			
Lack of fit	4143.09	10	414.31	0.43	0.8820	Not significant
Pure error	4854.39	5	970.88			
Cor total	31,374.97	29				

was in accordance with the results. Therefore, this model could be used for the prediction of laccase yield from *B. subtilis* DS. The maximum enzyme yield of 820.0 ± 1.5 IU ml⁻¹ was obtained when 150 µM MgSO₄, 1.2% yeast extract, 0.35% tryptone, and 150 µM vanillic acid were added to the medium.

Validation of the experimental model

The validation of the statistical model and regression equation was conducted by the cultivation of *B. subtilis* DS in the medium containing 150 µM MgSO₄, 1.2% yeast extract, 0.35% tryptone, and 150 µM vanillic acid. Under this optimized condition, the observed experimental value was 820.16 ± 1.5 IU ml⁻¹. The close relationship between the predicted (795.84 U ml⁻¹) and experimental response value from the validation experiment revealed the validity and acceptability of the statistical model for the optimization. The enzyme yield obtained under optimized conditions was 746-fold higher than under the unoptimized condition.

Synthesis of dye from natural precursors

Different natural precursors were used for the synthesis of dyes using Lac DS at pH 8.5. Different colors were obtained using different precursors within 30 min of the reaction. Black color similar to that of PPD was developed using pyrogallol and catechol, golden yellow color was developed

from ferulic acid and gallic acid, and reddish brown from syringaldehyde and syringic acid (Fig. 5). These wide range of colors can be used to replace synthetic dye like PPD for hair dyeing.

Discussion

In recent years, laccase has found applications in number of industrial processes. One of the less explored applications is in the cosmetic industry. As laccases can efficiently polymerize and oxidize phenolic compounds they can be used for the development of processes such as synthesis of user-friendly hair dyes from natural precursors. For the application of enzyme in hair dyeing, one of the pre-requisite is that it must be active and stable in an alkaline range, which helps in penetration of the dye into the hair. To the best of our knowledge, the only reports on the use of laccase for hair coloring are from Saito et al. (2012) and Jeon et al. (2010) using fungal laccases at acidic pH.

The aim of the present study was to isolate a bacterial laccase which could remain active and stable at alkaline pH and has application in the synthesis of hair dyes. Most of the bacterial laccases are either intracellular or a component of spore coat protein (Sharma et al. 2007). Therefore, attempts were made to isolate bacteria producing extracellular laccase, *Bacillus subtilis* DS was selected on the basis of its

Fig. 4 Three dimensional surface plots showing the effect of interaction of **a** yeast extract and vanillic acid, **b** yeast extract and tryptone, **c** yeast extract and MgSO₄, **d** tryptone and vanillic acid, **e** MgSO₄ and vanillic acid, **f** tryptone and MgSO₄ on laccase production from *B. subtilis* DS

Fig. 5 Results of chromogenic reaction of Lac DS on natural precursors. Lane; A: Test (Substrate + Enzyme + Buffer), B: Substrate control (Substrate + Denatured Enzyme + Buffer), C: Enzyme control (Enzyme + Buffer), D: Blank (Buffer). Row; 1: Catechol, 2: Syringaldehyde, 3: Syringic acid, 4: Ferulic acid, 5: Gallic acid, 6: Pyrogallol, 7: PPD

ability to produce extracellular laccase and oxidize dye PPD at alkaline pH 8.0–9.5.

Enzymes such as tyrosinase have an overlapping range with some of the substrates of laccase (Sondhi et al. 2015). Lac DS was a true laccase as it could oxidize laccase-specific substrates SGZ and ABTS but not tyrosine. Moreover, laccase activity is known to be inhibited by sodium azide and β -mercaptoethanol (Gunne and Urlacher 2012; Sondhi et al. 2014), inhibition of Lac DS activity by these inhibitors further indicated that it is a true laccase.

To the best of our knowledge, no other laccase has been reported which could oxidize hair dyes such as PPD at such a high pH. For the application of Lac DS, its production was optimized, 27-fold increase in laccase activity was achieved with OVAT method, and CuSO_4 and xylose were most effective as they enhanced the enzyme yield by 1.5-fold and 2.45-fold, respectively. Cu^{2+} is a known inducer of laccase and has been reported to increase the enzyme yield by many fold (Sondhi et al. 2015). Ravikumar et al. (2012) have shown 17-fold increase in laccase yield from *Hypsizygus ulmarius* on the addition of xylose.

To further enhance the yield, optimization was done using statistical designs including PB and RSM. Out of 19 different parameters tested in PB, 10 were having a positive effect on laccase production, yeast extract followed by $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, tryptone and vanillic acid had maximum effect. The effect of nitrogen source on laccase production has been reported to

vary from organism to organism (Collins and Dobson 1997). In case of basidiomycetes, it has been reported that limited concentration of nitrogen in culture medium favoured the production of lignolytic enzymes including laccase (Buswell 1992; Calvo et al. 1998). Whereas in some reports, yeast extract has been reported as not only a good nitrogen source but also as an inducer of laccase. More than twofold increase in laccase yield has been reported in case of *Pleurotus ostreatus* (ATCC 52857) when yeast extract concentration was increased from 0.5 to 1.0% (Zhu et al. 2016). Along with yeast extract, tryptone has also been reported to enhance the laccase production (Patel and Akshaya 2016). Mostly Cu^{2+} is known to regulate the gene expression of laccase but, other metal ions are also known to be potent inducers of laccase (Gonzalez et al. 2013). $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ has been reported to induce laccase from *Trametes multicolor* and *Agaricus blazei* (Hess et al. 2002; Valle et al. 2014). Vanillic acid is a known inducer of laccase in case of *T. versicolor*, about twofold increase enzyme yield was observed when vanillic acid was added to the culture medium (Wang et al. 2016). Vanillic acid is known to generate an oxidative stress which upgrades the expression of laccase gene (Collins and Dobson 1997; Dong et al. 2005; Piscitelli et al. 2011).

These four variables were selected to further optimize the enzyme production by RSM. A 746-fold increase in enzyme production is observed in comparison with unoptimized values. This enzyme yield is higher than laccases obtained from most of the reported organisms (Chenthamarakshan et al. 2017). Moreover, as no laccase inducers such as veratryl alcohol, and ferulic acid has been used it makes enzyme production from *Bacillus subtilis* DS economical.

In the only report where polymerization of natural precursors occurred for the development of colors using laccase by Jeon et al. (2010), they developed colors using a combination of different precursors at acidic pH in 22 h of the reaction period. Hair dyeing process has to be carried out at alkaline pH as the hair swells up and helps in the penetration of dye. If the hair is dyed at acidic and neutral pH, dye color comes out easily during washing. As Lac DS was active and stable in alkaline range, it was explored for color development from natural precursors. A wide range of colors black, brown, golden, etc. was developed from different natural precursors. The color development took place within 30 min of the reaction which is much less than Jeon et al. (2010) where it was 22 h. The precursors giving black color are especially useful as they can be used as an alternative to synthetic dye PPD for hair blackening. Further studies can be done to polymerize various combinations of precursors to develop more range of colors. Therefore, Lac DS can be used to develop a user-friendly hair dyeing process.

Conclusion

The present investigation had led to the isolation of an extracellular bacterial laccase (Lac DS). The enzyme could polymerize a number of phenolic and non-phenolic natural precursors to synthesize a range of colors at alkaline pH in a very short time. The production of enzyme has been optimized to give higher yield at significantly low cost. Lac DS is a potential candidate for its application in developing a natural, user-friendly hair dyeing process.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest

Human and animal rights and informed consent Research involving Human Participants and/or Animals/Informed consent: not applicable for the study.

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