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UV Induced Mutagenesis Elevates the Production of Laccase in *Enterobacter cloacae*

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The increasing commercial significance of microbial laccase in various fields compels the supply of enzymes in large quantities at an affordable price. Laccase has been produced by a wide variety of wild cultures of bacteria and fungi, the high yielding strains are always desired to meet the increasing demand of laccase. Although various approaches have been proposed for improving the yield of laccase, UV mutagenesis is known as the best method of improving the strains for better yield of microbial metabolites. However, the reports on the use of UV light in strain improvement for laccase production by the use of *Enterobacter* sp. are scarce and need to be explored fully. For this purpose present study was aimed to improve the laccase production in *Enterobacter cloacae* through UV mutagenesis. We report enhanced production of laccase in a high yielding strain of *E. cloacae* isolated from the soap industry waste. UV mutagenesis of a wild strain of *E. cloacae* resulted in 3.09 folds further improvement in the production. Optimum laccase synthesis was reported at 26 h, of incubation at 30ºC under neutral pH (7.0) conditions. This high yielding mutant strain will have great industrial significance for laccase production.

Keywords: *Enterobacter cloacae*, Laccase production, Optimization, UV mutation

Introduction

The recent decade has witnessed the biotechnological exploitation of microbial enzymes for a wide range of applications in various industries since they pose numerous merits over conventional chemical processes.[1–5] Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) is one such useful enzyme, it is a multicopper blue oxidase enzyme that functions over a wider range of pH and temperatures, and resistant to various inhibitors.[6] Such useful properties make it a multipotent enzyme for use in various sectors.[7] Enzyme laccase has great commercial significance in various fields like biomedical, bioremediation of dye, delignification in paper industries, in biosensor preparation, melanin degraders in cosmetics and the clarification of juices.

However, the low production yield has hampered its large scale commercial application.[8] To make the laccase production more cost-effective, improvements in laccase yields are vital. This warrants the urgent need to look or develop high yielding strains, which can be achieved through mutagenesis.[9] Ease of genetic manipulations allows utilizing bacterial laccases for industrial applications.[10] Hence the present work was focused on mutagenesis and the resulting mutant strain resulted in high laccase yield. The physical mutagenesis through UV rays is a common method employed in strain improvement as it results in quantitative yield enhancement.[11,12] Although various methods have been employed to improve the industrial strains, application of UV radiation appears as the most efficient method of strain improvement in the production of enzymes, siderophores, and other bioactive compounds[14,15], however, its use for laccase production in *Enterobacter* sp. needs to be fully explored. Therefore, present work was directed to improve the production of laccase through UV mutation in *E. cloacae*.

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Materials and Methods

Chemicals and soil samples

R and 2,2'-and-bis (ABTS) was procured from Sigma Aldrich (USA). Soil samples were collected from a waste disposal site in the industrial zone of the Kadi district (23.17.671N72.21.93E) of Gujarat, India.

Screening of isolate for laccase producing organisms

For this purpose, soil samples were collected from the waste disposal site in the industrial area of Kadi district of Gujarat (India). The samples were serially diluted and 10^{-5} dilution of each sample was separately grown on ABTS agar containing (gL^{-1}): peptone, 3.0; glucose, 10.0; KH_2PO_4, 0.6; $ZnSO_4$, 0.001; K_2HPO_4, 0.4; $FeSO_4$, 0.0005; $MnSO_4$, 0.05; $MgSO_4$, 0.5; agar 2% amended with 0.1% (w/v) ABTS[13,16] at 30ºC for 48 h. Plates were observed for the zone of hydrolysis of ABTS as an indication of the ability of the isolate to produce laccase. Isolates showing zone of ABTS hydrolysis were further enriched on ABTS agar and the isolate showing a higher degree of ABTS hydrolysis was chosen as potent laccase producing strain.

Production of laccase

Laccase production was performed at shake flask level by submerged fermentation in minimal media (MM). For this purpose $6x10^{-4}$ cells mL^{-1} of log culture of isolate were grown in MM containing (mgL^{-1}) glucose, 3000; KH_2PO_4, 1000, $(NH_4)_2SO_4$, 260; $MgSO_4.7H_2O$, 500; 2,2-dimethylsuccinic acid, 2200; $CaCl_2.2H_2O$, 74000; $CuSO_4.7H_2O$, 0.5; $ZnSO_4.7H_2O$, 6; $FeSO_4.7H_2O$, 5; $MnSO_4.4H_2O$, 5; $CoCl_2.6H_2O$, 1; vitamin solution 500 µl, pH set to 4.5 at 30°C for 48 h.[17] The cell-free supernatant obtained after centrifugation at 10,000 rpm for 15 min at 4°C, was subjected to laccase assayed.

Laccase assay

Laccase activity was estimated in terms of the oxidation of substrate ABTS. For this, $6x10^{-4}$ cells mL^{-1} of the log culture of the isolate was grown for 24 h in nutrient broth at 30°C at 100 rpm. After incubation, the broth was centrifuged at 10,000 g, 4°C for 10 min, and cell-free supernatant was used as a source of crude enzyme, and 800 µL of this supernatant mixed with 200 µL of 2 mM ABTS solution was added into the 96 well plates. The plates were covered with parafilm, gently shaken to mix the contents, incubated at 30°C for 10 min. Change in absorbance due to the oxidation of ABTS in the reaction mixture was measured spectrophotometrically at 420 nm[17,18] One unit of laccase activity was defined as the amount of enzyme that oxidizes 1 µM of ABTS per min under assay conditions.

Molecular identification of the isolate

The genomic DNA of the isolate was separated according to the method of Sambrook and Russel.[19] The 16S rRNA gene of the isolates was amplified by using a ready mix™ reaction mixture with a universal forward primer (8F) and reverse primer (U1492R). The polymerase chain reaction (PCR) amplification was performed with an Applied Biosystems Verti thermal cycler (USA) and these sequences were analyzed by BLAST on NCBI (www.ncbi.nlm.nih.gov). The PCR was set up with 20–50 ng of DNA 16. The 16S rRNA gene was amplified according to the method of Sonawane *et al*.[20] Sequencing of the 16S rRNA gene amplicon was carried out on ABI 3730Xl automated sequencer using a ready reaction kit (Perkin Elmer, CA). Identification of amplified sequences was carried out by using BLAST on NCBI (www.ncbi.nlm.nih.gov) database and a phylogenetic tree was build up with the neighbor-joining method using MEGA5 software.[21] The isolate was identified based on the phylogenetic relationship.

Ultraviolet (UV) mutagenesis of *E. cloacae*

To induce physical mutagenesis, the log culture of the isolate (6×10^{-4} cells mL^{-1}) suspended in Luria broth was exposed to short UV (280 nm). A 15-Watt germicidal lamp with UV output at 2537 A with a dosage of ca 6,000 ergs/mm^2 was used for irradiation. Plates were incubated n dark. To determine the optimal distance of exposure, the plates were exposed to UV light from different distances i.e. 10 cm 15, 20, 25, and 30 cm at different time intervals in the range of 0.5 to 6 minutes. Samples of the irradiated suspension were plated and the survival rate was determined by using following formulae.[17]

$$\text{The survival rate} = \frac{\text{Number of colonies on the irradiated plate}}{\text{Number of colonies on non-irradiated plates}}$$

Selection of potent laccase producing colonies

Following the exposure to UV light, the culture was subjected to the screening of mutants on method on tannic acid agar (TAA) plate that was containing 0.5% tannic acid, 3% malt extract, 0.5% mycological peptone and 2 % agar.[22] Wild strain was

simultaneously grown on the TAA at 30°C for 24 h. Colonies that produced a maximum black zone were taken as laccase positive mutants and were further confirmed by enzyme assay. Colonies showing maximum black zone and maximum laccase activity were selected as potent strain.

Optimization of media parameters for maximum production of laccase in mutant

As *E. cloacae* produced maximum laccase activity, it was subjected to study the effect of various parameters viz. incubation temperature, pH, and incubation time on laccase production as well as the growth of the bacterial isolate. The positive bacteria obtained were grown on the nutrient agar medium with 0.1% tannic acid.

Growth and laccase production as a function of time

To ascertain the precise time for maximum growth and laccase production, the log culture of the isolate was grown in basal medium for 72 h at 30ºC at 120 rpm.[16,22] Samples collected after every 6 h were subjected to estimation of bacterial growth and laccase activity as per the method of Jeon and Lim.[18]

Influence of pH on growth and laccase production

The effect of pH on growth and laccase production was carried out in basal medium[16,22], basal media separately prepared with pH values in the range of 4–10 pH with the help of acetate buffer (pH 4), phosphate buffer (pH 6), Tris HCl buffer (pH 8) and glycine NaOH buffer (pH 10). The log phase culture of the isolate was grown separately grown in each basal agar plate and medium for 72 h at 30°C at 120 rpm. Samples withdrawn after every 6 h from each flask were analyzed for growth estimation and laccase assay.[18]

Effect of temperature on growth and laccase production

For this purpose log culture of the isolate was separately grown on four different basal agar plates and simultaneously into each basal medium for 72 h at 30°C at 120 rpm. Plates and flasks were incubated at different temperature 10°C, 20°C, 30°C, 40°C, 50°C and 60°C. Plates were observed for the zone of hydrolysis of ABTS. Samples withdrawn after every 6 h from each flask were subjected for estimation of bacterial growth and laccase activity.[18]

Statistical analysis

Each experiment was performed in three replicates and the average of triplicates value was statistically analyzed by using the Student's t-test. $P \leq 0.05$ values were considered taken as statistically significant.[23]

Results

Screening for laccase production and selection of potent laccase producing isolate

A total of 12 bacterial isolates were obtained from 3 soil samples of the soap industry. Among these; 4 isolates showed zone of hydrolysis of ABTS on a basal agar plate (Fig. 1a). However, isolate EC showed a larger zone of hydrolysis of ABTS and therefore was selected as the best laccase producer. Isolate *E. cloacae* showing larger zones of ABTS hydrolysis produced the best laccase activity of 22.6 UmL^{-1}.

Molecular identification of the isolate

The 16s rRNA gene sequences analysis and its comparison with the NCBI database revealed 99.00% similarity with *Enterobacter cloacae* (Fig. 1b). Thus the isolate was identified as *Enterobacter cloacae* and the gene sequences of the isolates were submitted to NCBI gene bank under the name *Enterobacter cloacae* with Genbank accession number KJ794112.

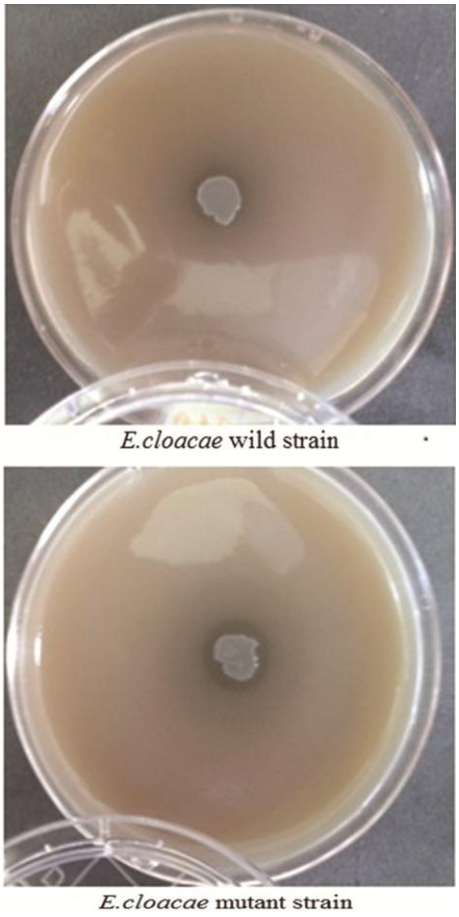

Fig. 1a — Laccase production in wild and mutant strains of *E. cloacae* after 48 h growth on basal media containing ABTS as the only source of carbon

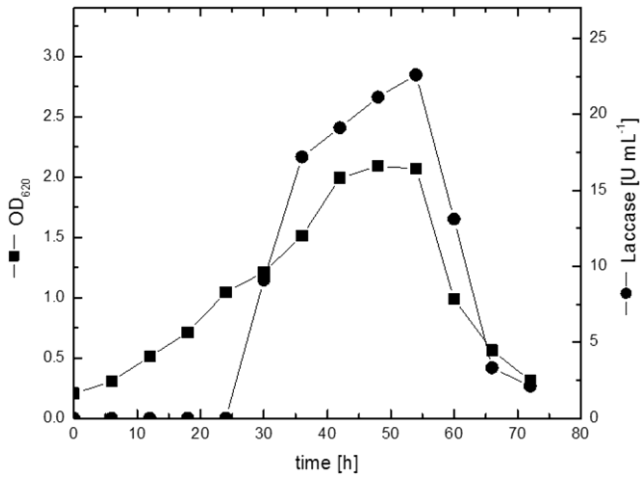

Fig. 1b — Influence of incubation period on growth of *E .cloacae* and its laccase production in basal medium

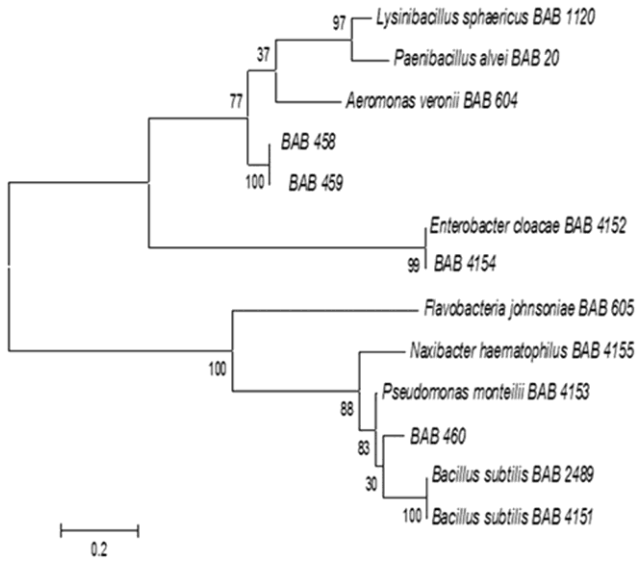

Fig. 1c — Phylogenetic analysis of isolate based on 16s rRNA gene sequence drawn using the neighbor joining method showing 99% similarity to *E. Cloacae*

Ultraviolet (UV) mutagenesis of *E. cloacae*

Treatment of wild strain of *E. cloacae* with UV rays of 280 nm with a dosage of 6,000 ergsmm^{-2} produced mutation. Among the various periods employed for UV treatment, treatment of 40 minutes resulted in the formation of more number of mutants. Among the various distances of exposure and time of exposure, a UV light exposure at the distance of 30 cm and a time of 10 min exposure resulted in a good number of mutant colonies. A 20% to 50% survival was considered as the optimal dose for inducing reversions and mutations.

Selection of potent laccase producing colonies

Colonies of *E. cloacae* treated with UV rays produced maximum black zone vis-à-vis wild strain. These colonies were separated as mutants of *E. cloacae*. Two putative UV mutants UV1 and UV2 of *E. cloacae* were plated onto nutrient agar having 0.1% tannic acid to determine the size of the black zone. Of these two putative mutants, mutant UV1 and UV2 resulted in the larger sized black zone as compared to the wild strain. The zone difference between the wild strain and mutant UV1 was 0.2 while the zone difference between the wild strain and mutant UV2 was and 0.4 cm.

Optimization of media parameters for maximum production of laccase in mutant

E. cloacae mutant UV2 showed maximum laccase activity and hence it was chosen for optimizing the best conditions for growth and laccase production. Each organism or strain has its requirements for physical and chemical factors for the optimum production of any metabolite. These factors have been reported as crucial ones for promotion, stimulation, enhancement, and optimization of the production of enzymes.[24-26]

The effect of incubation time on laccase production revealed, a lag phase of 8 h, exponential phase began after 12 h and stationary phase started after 36 h. Laccase synthesis began during 36 h and reached to the optimum level in 48 h and declined thereafter. Maximum (22.6 UmL^{-1}) laccase activity was observed at 48 h of incubation (Fig 1c).

Optimum laccase activity was obtained at neutral pH (7.0), while alkaline pH affected the production of laccase (Table 1).

Optimum laccase activity (22.6 UmL^{-1}) was obtained at 30°C. An increase in temperature beyond 30°C resulted in a decrease in the enzyme. The lowest enzyme activity (9.1 UmL^{-1}) was observed at 20°C (Table 1).

Discussion

Change in the color of minimal medium reflected the laccase mediated oxidative conversion of ABTS to the cationic chromophore.[25] Odeniyi *et al*.[27] have reported the production of thermostable laccase in *Cornybacerium efficiens* and *E. ludwigii*. Bhamare *et al*.[5] have reported the oxidation of ABTS due to the activity of laccase produced by *Aspergillus* sp. Many bacterial cultures namely *Cornybacterium* sp., *Enterobacter* sp.[26], and *Bacillus* sp.[15] are known to produce copious amounts of laccase. Desai[28] has

Table 1 — Influence of pH and temperature on growth and laccase production in *E. cloacae* UV2 mutant

pH	Growth (OD 620 nm)	Laccase (UmL^{-1})	Temperature (°C)	Growth (OD 620 nm)	Laccase (UmL^{-1})
4.0	0.201 (0.01)	0 (0.01)	10	0.201 (0.01)	9.10 (0.01)
5.0	0.305 (0.02)	0 (0.12)	20	1.045 (0.12)	15.20 (0.15)
5.5	0.510 (0.02)	0 (0.15)	30	2.091 (0.15)	22.60 (0.19)
6.0	0.710 (0.01)	9.10 (0.37)	35	2.001 (.037)	13.11 (0.41)
6.5	1.045 (0.51)	15.20 (0.46)	40	0. 950 (0.46)	9.21 (0.46)
7.0	2.091 (0. 51)	22.60 (0.55)	45	0. 790 (0.52)	3.32 (0.53)
7.5	1.982 (0.51)	13.11 (0.32)	50	0. 413 (0.30)	2.11 (0.29)
8.0	0.987 (0.21)	9.21 (0.29)	55	0.211 (0.28)	1.57 (0.21)
9.0	0.561 (0.27)	3.32 (0.15)	60	0.103 (0.15)	0.55 (0.11)
10.0	0.311 (0.221)	1.21 (0.11)			

reported laccase production (4.9 UmL^{-1}) in *Enterobacter* sp. producing obtained from contaminated sites.

The incubation period is a crucial factor that gives the idea of a beginning of synthesis, optimum secretion of any metabolite as well its degradation/denaturation. Thus any metabolite should be recovered at the time when it is produced in an optimum amount.[28] We report a higher amount of laccase (22.6 UmL^{-1}) in 48 h vis-à-vis laccase yield (15.96 UmL^{-1}) recently reported in *E. cloacae* strain KSB_4 (8.31 UmL^{-1})[29] in *E. ludwigii* (4.7 UmL^{-1})[26] and *Aspergillus* sp. HB_RZ4.[5] Bhamare *et al.*[5] and Odeniyi *et al.*[27] observed that the prolonged incubation results in significant loss of enzyme yield. Muthukumarasamy *et al.*[30] have reported 96 h as optimum incubation period for the maximum production of laccase in *Bacillus subtilis* MTCC 2414. Prolonged incubation of 9 days has been reported to lose the laccase activity in *A. flavus* and *Botrytis cinerea*.[22,31] A higher yield of laccase in a shorter duration of 48 h suggested the metabolic potential of isolate and its suitability for the economically feasible production of laccase at the industrial scale.

Further increase in laccase yield due to the mutation can be attributed to the higher expression of laccase synthesizing genes. UV mutagenesis has been found as the best method of strains improvement for an increase in the yield of metabolites in improving enzyme production in *A.niger*[16] and *Rhizopus oryzea*[12] and mycelia and sporophore production in *P. florida* and *P. sajor-caju*.[14]

Ghosh and Ghosh[24] reported higher laccase yields in *A. flavus* on the 20th day while Kumar *et al.*[32] reported higher laccase yields in *A. flavus* on the 12th day of incubation. Fungi are slow-growing organisms and generally require a longer incubation time i.e. 12–30 days for the secretion of metabolites.[23,17] Sivakumar *et al*.[30] reported a higher laccase yield after 12 days of incubation under static conditions. Also, it has been reported that the optimum yield of 8.31 UmL^{-1} was produced by *E. cloacae* strain KSB_4.[28] Besides an increase in incubation period the utilization of substrate is decreased and loss of viability of the organism is significantly lost.

Optimization of laccase production by mutant strain further boosted the enzyme yield by 3.09 folds. Du *et al.*[12] have reported improvement in laccase production following UV mutagenesis in *Shiraiab* sp. GZS1. It has been also reported that increased laccase productivity in UV mutant of the white-rot fungus, UV mutant of the fungus produced 11.57 UmL^{-1} laccase vis-à-vis 7.60 UmL^{-1} produced by a wild strain of fungus.[25] UV induced mutation has been found as an effective method for increasing the productivity of cellulose in *Aspergillus terreus* D34.[32]

The temperature of 30°C and neutral pH (7.0) was best suited for the optimum production of laccase. Temperature influences all cell reactions, the rate of metabolism, nutritional requirement, and the concentration of cell mass. The level of H^+ ions affects the biological activities including the activities of enzymes.[31] The ability of the isolates to grow and produce laccase at alkaline pH makes this enzyme more suitable for industrial applications.[33] Odeniyi *et al.*[27] reported the best laccase activity in *P. pulmonarius* at 30°C. They found a good response by isolate to cultural conditions for enhancing the production of laccase. Similar results were obtained by Mishra *et al.*[34] and Bhamare *et al*.[5] they observed maximum laccase production at 30°C in *Streptomyces lavendulae* and *Aspergillus* sp. respectively. The occurrence of laccase producing *E. cloacae* from industrial waste and its higher laccase activity in a very shorter period of 48 h

indicated the biotechnological potential of this isolate for its exploitation at a commercial level and its possible role in biodegradation of organic matter.

Conclusions

E. cloacae isolated from industrial waste produced a higher amount of laccase in a shorter period of 48 h; a 3.09 fold increase in laccase yield was evident following the UV induced mutation. This study demonstrates that UV induced mutant bacteria are capable of producing higher amounts of laccase. Saprophytic organisms are known to degrade decaying organic matter present in the waste and other decomposing matters through the secretion of various enzymes, laccase being one of the principal biodegrading enzymes. The ability of *E. cloacae* to produce laccase enzyme indicated its potential for applications in various fields like biomedical, bioremediation, delignification, biosensor, and cosmetics.

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