



P-ISSN: 2349-8528  
E-ISSN: 2321-4902  
IJCS 2020; 8(1): 04-08  
© 2020 IJCS  
Received: 04-11-2019  
Accepted: 08-12-2019

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## Hyper-production of laccase a green catalyst by RS2 (*Pseudomonas stutzeri* CW202) through Mutagenesis

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DOI: <https://doi.org/10.22271/chemi.2020.v8.i1a.8296>

**Abstract**

Extracellular Laccase (EC 1.10.3.2) which is well known as a 'Ecofriendly' enzyme / Green catalyst has grown significantly in recent years and has wide range of industrial application from textile industries, pulp and paper industries to food industry and bioremediation processes. In this study the wild strain of *Pseudomonas stutzeri* CW202 was improved for enhanced Laccase production by using physical mutagen (ultraviolet rays). Mutant (M3) with hyper Laccase production was obtained after treating wild strain with ultraviolet rays on the basis of Laccase activity. Mutant M3 exhibited maximum extracellular Laccase activity after 24 hrs of incubation period at 35 °C which was assessed by calculating enzyme activity at different pH and Temperature. The optimum pH for higher Laccase activity was found at pH 8 using syringaldehyde substrate which was 1098 U/L i.e. six fold increase as compared with the wild which has 213U/L. Whereas using ABTS 499U/L activity was achieved at pH 4. The optimum temperature found at 45 °C. The effectiveness of hyper Laccase producing mutant M3 indicates its possible applicability in various biotechnological and industrial processes.

**Keywords:** hyper-production, green catalyst, RS2, *Pseudomonas stutzeri*, mutagenesis

**Introduction**

Increasing population thrown many challenges to feed and to provide daily necessities and increase in the demand of organic product is day by day increasing and due to this many industries focused on the more environmentally acceptable alternative to chemical treatment by replacing with some green catalyst. Laccases are one of the group of oxidative enzymes whose exploitation as biocatalysts in organic synthesis has been neglected in the past, probably because they were not commercially available. In recent years, many studies aimed to improve the strains using physical and chemical mutagenesis for the increase in production of industrial enzymes.

Microbial enzymes continue to draw greater attention as alternative to chemical processes which would enable the industries to meet the increasingly stringent environment requirements to reduce the pollution load. The highly-stable bacterial Laccases can function within a wider pH range and at high temperatures and these are less dependent on metal ions and less susceptible to inhibitory agents (Sharma P, Goel R, Caplash N (2007). Furthermore, bacterial systems are easier to handle than fungal ones (Brissos V, *et al.* (2009) [5]. Laccase activity has also been demonstrated in a number of bacteria including *Bacillus subtilis*, *Bacillus vallismortis*, *Bacillus pumilus*, *Bordetella compestris*, *Caulobacter crescentus*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Pseudomonas putida*, *Pseudomonas syringae*, *Pseudomonas aeruginosa*, *Yersinia pestis* and *Geobacillus thermocatenulatus* (Alexandre G, Zhulin IB (2000) and a Verma A, Shirkot P (2014) [6, 7].

The UV-induced mutagenesis is a frequently used method for mutagenesis in breeding. The pyrimidine bases have strong UV absorption. When UV ray is absorbed, the neighboring double thymines in the DNA chain form a thymine dimer that mainly causes the mutations. When DNA reproduction begins, the site of the single thymine dimer could run over and then

the gap is left, thereby inserting the false bases, causing the thymine-guanine (AT) → cytosine-adenine (GC) base pairs mutation (Xu A, *et al.* (2010) [8]. UV-induced mutagenesis produces point mutations, small deficiencies, chromosome breaks and chromosome rearrangements at different relative frequencies in many organisms (Ali MJ, *et al.* (2011) [9]. Earlier studies of enhancing laccase production by improving the strain of *Pseudomonas putida* LUA15.1, was attempted for higher laccase production and activity. (Verma A, Dhiman K, Shirkot P (2016) [1].

In this study the of RS2 bacterial isolate which was isolated for bioremediation of industrial azo dyes which showed 98% similarity with *Pseudomonas stutzeri* CW202 strain was attempted to mutate for checking the laccase activity against wild.

## Material and Methods

### Buffers, solutions and reagents

Preparation of buffers and solutions for estimation of enzyme activity

**50mm sodium citrate buffer (pH 5 and pH 6):** 50 ml of 0.1M sodium citrate buffer was diluted with 50 ml of distilled water to make 100 ml 50mM sodium citrate buffer. Solutions for the preparation of buffer was prepared as follows:

**0.1M citric acid:** Prepared by dissolving 2.101 g of citric acid in 100 ml distilled water.

**0.1M sodium citrate:** Prepared by dissolving 2.941 g of sodium citrate in 100 ml distilled water.

**0.1M sodium citrate buffer:** 46.5 ml of 0.1M citric acid was added to 3.5 ml of 0.1M sodium citrate and pH was adjusted. Volume was made up to 100 ml with distilled water.

**1M Potassium phosphate buffer (pH 7)** The solutions were prepared as follows 1M Dipotassium hydrogen phosphate Dissolved 17.4 g of  $K_2HPO_4$  in 100 ml of distilled water.

**1M Potassium hydrogen phosphate:** Dissolved 13.6 g of  $KH_2PO_4$  in 100 ml of distilled water.

To prepare 1M Potassium phosphate buffer (pH 7) 48 ml of  $K_2HPO_4$  solution was added to 25 ml of  $K_2HPO_4$  and final volume was made up 100 ml by adding distilled water. The pH was adjusted to 7 before making up the final volume.

**50mm Tris HCl buffer (pH 8):** 12.5 ml of 1M Tris HCL buffer was diluted buffer to 87.5 ml of distilled water. 1M Tris HCl  
Dissolved 12.1 g of Tris base in 100 ml of distilled water and adjusted the pH to 8 with conc. HCl before final volume make up.

**20% Glucose solution (20g/100ml):** Dissolved 20g of glucose/dextrose to 100 ml of distilled water, and filter sterilized.

**0.22 M Guaiacol (Pyrocatechol monoethyl ether ( $C_7H_8O_2$ ) solution:** 0.22 M solution of guaiacol was prepared in 0.1 M phosphate buffer (pH 6.0) and put in dark colour bottle and stored in refrigerator.

**10 mM ABTS:** 0.055 g of ABTS was dissolved in 10 ml of autoclaved distilled water and kept covered.

**0.2mM Syringaldehyde:** 0.036 g Syringaldehyde was dissolved in 100ml of 95% ethanol.

### Microbial strain

In this study *Pseudomonas stutzeri* CW202, which was isolated from industrial sludge samples collected from Yavatmal district, Maharashtra state (India) and maintained on slants of Luria Bertani agar medium at 4 °C by repeated sub culturing.

### Culture conditions and enzyme extraction

*Pseudomonas stutzeri* CW202 was inoculated into the LB broth and mixed thoroughly by keeping the flasks on a rotary shaker at 110 rpm for 24-48 hours at 28 °C. The culture supernatant was obtained by centrifugation of overnight culture of *Pseudomonas putida* LUA15.1 at 10,000rpm, for 10 minutes at 4 °C and used for the enzyme assay.

### Mutation of wild strain of *Pseudomonas stutzeri* CW202 ultraviolet (UV) mutagenesis:

Mutation of strain of *Pseudomonas stutzeri* CW202 was done by UV mutagenesis. For induction mutation of 24hr old culture was exposed to short Ultraviolet light (280 nm) from a distance of 15 cm for various time intervals (20, 40, 60, 80, 100 and 120 minutes) as describe by Verma A, Dhiman K, Shirkot P (2016) [1].

### Screening of mutants

Liquid state cultivation of the mutants was done in the LB medium supplemented with the 0.5 mM guaiacol as a substrate. Cultures were incubated for 24-72 hours at an optimum incubation temperature of 35 °C. Oxidation of guaiacol substrate in the liquid state cultivation of mutants indicated the production of Laccase based on which the culture showing maximum oxidation of guaiacol was selected for further Laccase enzyme activity assay.

### Laccase activity assay

Laccase activity was measured by monitoring the oxidation of ABTS (Faramarzi MA, Forootanfar H (2011) [13]. Laccase activity was studied at 420nm with ABTS as a substrate spectrophotometrically using spectrophotometer. The reaction mixture contained 100µl aliquots of crude extracellular enzyme preparation and 0.2mM ABTS in 0.1M sodium acetate buffer (pH 5.0). The final volume was set to 2.0ml. The reaction was held at 37 °C for 15 minutes followed by addition of 0.5ml of 80% trichloroacetic acid to stop the reaction. One unit of enzyme was defined as the amount of enzyme required to oxidize 1.0µmol of ABTS per min. The molar extinction coefficient of ABTS was found to be 36,000 M<sup>-1</sup> cm<sup>-1</sup>. On the basis of maximum Laccase activity, hyper Laccase producing mutant of *Pseudomonas stutzeri* CW202, was selected for further studies.

### Optimization of conditions for maximum Laccase activity

The mutant of *Pseudomonas stutzeri* CW202 selected on the basis of maximum Laccase activity has further investigated to study the effect of different factors such as incubation temperature and pH on Laccase activity.

To determine the effect incubation temperature on Laccase activity, the enzyme substrate mixtures were incubated at 5 °C intervals (37-55 °C) for 15 minutes in a digital incubator. After 15 minutes of incubation, absorbance was measured using UV-Vis spectrophotometer.

The influence of pH on Laccase activity was studied by recording the absorbance of enzyme catalyzed reaction at

optimum temperature, using substrate dissolved in buffers of different pH (Acetate buffer replaced by sodium citrate pH 4, and pH 5, phosphate buffer pH 7 and tris-glycine buffer pH 8) and incubated at 25°C for 15min and absorbance were recorded using UV-Vis spectrophotometer. (Desai S. *et al.*, 2011)<sup>[12]</sup>.

## Results and Discussion

In this study an attempt was made to develop *Pseudomonas stutzeri* CW202 by mutagenesis and selection of efficient strain for production of Laccase enzyme. The mutation was induced to the wild strain by UV irradiation.

### Screening and selection of mutants by mutagenesis UV mutagenesis

The survival count at each interval (20, 40, 60 and 120 minutes) was determined. 99.75% of the cells were killed within 120 minutes exposure of UV rays. Four putative UV mutants of *Pseudomonas stutzeri* CW202 were inoculated in liquid state using Luria Bertani medium containing 0.5mM Guaicol to determine highest Laccase producing strain among the mutant by observing oxidation of guaicol substrate by Laccase produce by mutants. Of the 4 putative mutants which showed highest oxidation of guaicol substrate by producing brown color in the culture as compared to wild strain was selected for further study and that was M3 mutant obtained after 60 minute of UV treatment.

### Optimization of parameters for laccase enzyme activity at different temperature

Enzyme assay was set using ABTS and it was found that at 45 C mutant strain of *Pseudomonas stutzeri* CW202 showed 499U/L activity was found as compared to wild strain which was 264U/L. Mutant showed increase in activity at 50 C which was 315U/L as compared with wild which showed only 37U/L activity. At Temperature 37 C mutant showed 398U/L activity and wild strain showed 298U/L activity. It was observed that the activity of wild strain goes on decreasing as the temperature was increase.

Where as in other studies of Laccase production it was noted that, maximum enzyme production was achieved at 30 °C temperature in case of *Pseudomonas putida* MTCC 7525 [Kuddus M, *et al.* (2013)] and at 37 °C in case of  $\gamma$ -*proteobacterium* JB (Bains J, Capalash N, Sharma P (2003)<sup>[11]</sup>. Maximum growth OD of 1.51 and maximum extracellular

activity of 34.30 U/l was observed at pH: 8.0. case of *Pseudomonas putida* MTCC 7525 (Kuddus M, *et al.* (2013)) and at 37 °C in case of  $\gamma$ -*proteobacterium* JB (Bains J, Capalash N, Sharma P (2003)<sup>[11]</sup>.

### Optimization of parameters for Laccase enzyme activity at different pH using different substrates

#### Effect of pH on Laccase activity using ABTS substrate.

At pH 4 Maximum Laccase activity was showed by mutant M3 strain which was 499U/L using ABTS substrate against the wild strain of *Pseudomonas stutzeri* which was 263.52U/L. The activity found at pH 7 was also good which was 463U/L but the wild strain does not showed change in activity at pH 7. lowest activity was recorded at pH 5. Mutant strain of *Pseudomonas stutzeri* CW202 i.e M3 showed increase in enzyme activity as compare to wild strain at pH varied pH (4, 7 and 8). Increased in Laccase activity for mutant produced could be because of the reason that the gene responsible for production of Laccase might have increased in DNA due to mutation (this is due to increase in gene copy number or expression of genes or both) which resulted in increase of enzyme activity. Similarly, Laccase production has also been improved through UV radiations-based mutagenesis, and UV mutant i.e. GZ11K2 was reported as a preferable Laccase producer (Du W, *et al.* (2014)<sup>[10]</sup>.

#### Effect of pH on laccase activity using syringaldehyde substrate

Enzyme activity of mutant and wild strain was assessed at different pH using syringaldehyde as a substrate and it was found that, at pH 8 Maximum Laccase activity was showed by mutant M3 strain which was 1098 U/L using Syringaldehyde substrate against the wild strain of *Pseudomonas stutzeri* which was 213.2U/L. At pH 4, 5 and 7 mutant M3 showed 399U/L, 303U/L AND 171U/L Laccase activity respectively.

#### Effect of pH on laccase activity using guaicol substrate

Enzyme activity of mutant and wild strain was assessed at different pH using Guaicol as a substrate and it was found that, At pH 5 Mutant M 3 showed laccase activity which was 160 U/L using guaicol and the wild strain showed 91.476U/L activity which was very less than ABTS and syringaldehyde substrate. At pH 8 both mutant and wild strain showed less activity.

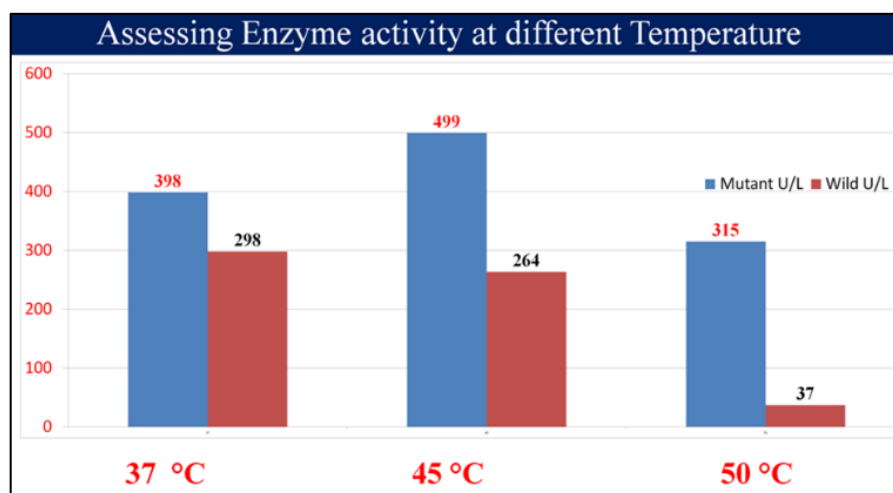
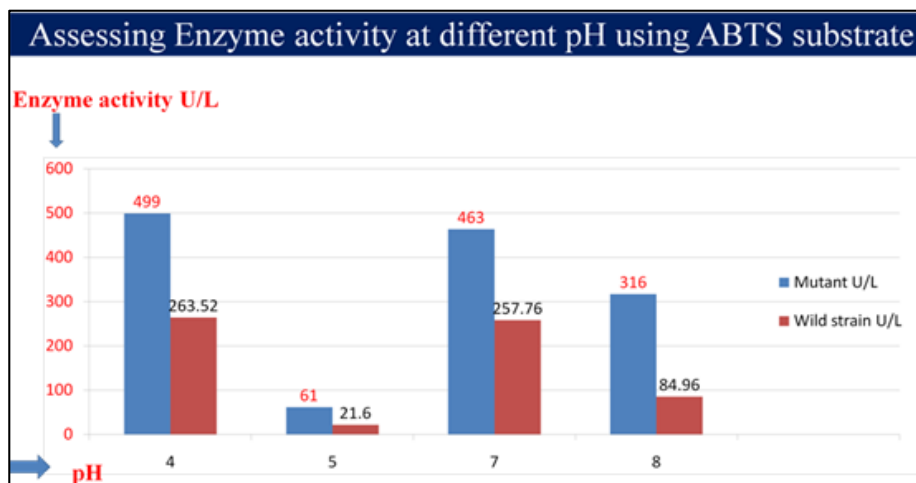
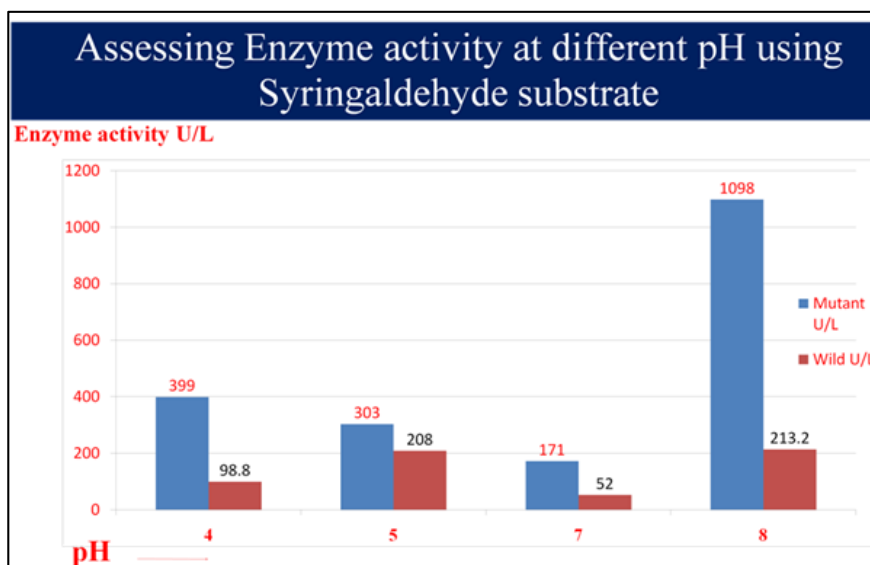


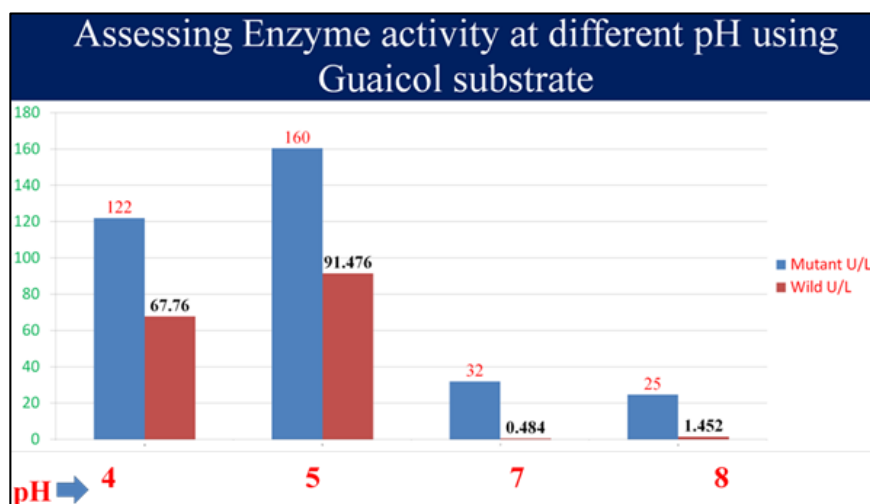
Fig 1: Assessing Enzyme activity at different temperature using ABTS



**Fig 2:** Assessing Enzyme activity at different pH using ABTS substrate



**Fig 3:** Assessing Enzyme activity at different pH using Syringaldehyde substrate



**Fig 4:** Assessing Enzyme activity at different pH using Guaicol substrate

### Conclusion

In this study the yield of Laccase enzyme from *pseudomonas stutzeri* CW202 strain was increased by mutagenesis. Optimum temperature for highest Laccase activity was found to be 45 °C Optimum pH for highest Laccase activity was found to be pH 4.0 for ABTS substrate and for Syringaldehyde substrate pH 8.0 was found best. Using

mutant highest activity of 1098 U/L activity was obtained as compared to wild strain which showed 213 U/L only i.e. 6 fold increase in Laccase production was achieved. Using mutant highest activity of 1098 U/L activity was obtained as compared to wild strain which showed 213 U/L only i.e. 6 fold increase in Laccase production was achieved. Industries can use this mutated strain of *pseudomonas stutzeri* for

getting higher amount of Laccase production which helps them for producing various ingredients. It is a cost-effective technique require minimum cost than any other biotechnological methods. Laccases are ecofriendly enzyme thus it does not provide harmful effects on the environment, Human Health, soil health

### Acknowledgements

We wish to thank Dr. N.D. Parlavar, Associate Dean, Vasantnao Naik College of Agricultural Biotechnology, Yavatmal, Maharashtra, India for financial support, valuable encouragement and kind assistance.

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