Defence Life Science Journal, Vol. 7, No. 4, October 2022, pp. 276-281, DOI : 10.14429/dlsj.7.18040

Biodegradation of Indigo Carmine Dye by Laccase from *Bacillus licheniformis* NS2324

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ABSTRACT

Indigo carmine dye is used in many industries like textile, paper, cosmetics, etc. It is specially used in textile industries for dyeing denim. The untreated water discharged from these industries, especially the textile industry, poses a significant environmental threat. The conventional physicochemical methods used in treating industrial effluent are very expensive and cause secondary pollution. In the present study, an extracellular laccase-producing *Bacillus licheniformis* NS2324 was isolated, and a crude enzyme was used to degrade indigo carmine dye. The conditions needed for decolorisation were standardised. Optimisation studies for dye decolorisation by NS2324 laccase revealed that the enzyme was able to decolorize dye efficiently at 50 °C temperature, pH 8, with an enzyme dose of 10 IU/ml after 6 hours. Up to 96.79±1.06 per cent decolorisation was achieved under optimised conditions without the use of any mediator of laccase. Complete degradation of indigo dye by laccase in the absence of mediator makes the present study very useful for treating textile effluent from the denim industry.

Keywords: Laccase; Indigo carmine; Decolorisation; Bacillus licheniformis NS2324

1. INTRODUCTION

The employment of enzymes to substitute non-biological processes has grown in popularity. Laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) have been the focus of intense study in recent decades. Laccases are multicopper oxidases that are found in higher plants, bacteria, fungus, and insects.[1] They catalyse one-electron oxidation of phenols by reducing oxygen to water at the same time.[2] Laccases have a wide range of substrate preferences as both phenolic and non-phenolic substances are oxidised by them.[3]

Laccases are important biocatalysts with a variety of proven and new biotechnological uses, including biobleaching, xenobiotic bioremediation, textile dye degradation, biosensors, and food processing.[4]

Laccase is also used for the bioremediation of textile industry waste water.[5] The textile dyeing and finishing business produce a lot of highly colored effluent, which pollutes the environment.[6] Many dyes, as well as their breakdown products, are fatal for the environment and living beings.[7] The presence of even extremely tiny amounts of dyes in water (less than 1ppm) is readily apparent.[8] The absorption, as well as the reflection of sunlight in the water, is the root cause of the largest environmental problem with dyes as it is fatal for the aquatic flora and fauna of the receiving water body.[7] Because of their excellent thermal and photostability, dyes can last for a long time in the environment.[9] Currently, the effluent water released by the textile industry is treated using a combination of physicochemical and biological methods like flocculation, coagulation, sedimentation, electro-dialysis, etc.[10] However, many of these traditional and even modern treatment systems are limited in their ability to manage brightly colored textile industrial effluent.[11] Another disadvantage of these treatments is that they use lots of energy due to their inefficiency in decomposing the dyestuffs.[12]

Laccases from *Trametes versicolor, T. hirsuta, P. ostreatus, Pycnoporus cinnabarinus, Pyricularia oryzae,* etc., have been proposed for the degradation of a broad range of colours.[13] The use of fungal laccase for color degradation has yet to be commercialised, despite its promise because fungal enzymes perform well in acidic environments and are therefore appropriate for use in acid dye baths only which are used to color protein fibres.[14] In addition to their limited thermostability, fungal laccases are inhibited by high chloride ion concentrations as well.[15] Bacterial laccases, in contrast to fungal laccases, are extremely active and significantly more stable at higher temperatures and alkaline pH.[16] As a result, bacterial laccases may be used in a variety of industrial applications that need enzymes that are stable under harsh temperatures. In the present study, a bacterial laccase from *Bacillus licheniformis* NS2324 (further referred to as NS2324) was isolated and used to decolorize indigo carmine dye and further, the conditions for dye degradation *i.e.*, temperature, pH, time, and enzyme dose were optimised.

Received : 05 April 2022, Revised : 08 September 2022
Accepted : 03 October 2022, Online published : 05 December 2022

2. METHODOLOGY

2.1 Reagents

Guaiacol and indigo carmine were acquired from Sigma (USA) and Hi-media respectively. Additional reagents were purchased from Hi-media.

2.2 Isolation of Extracellular Laccase-Producing Bacteria

Using M162 basal medium supplemented with 2 mM guaiacol as substrate, laccase-producing bacteria were isolated.[17] Optimal dilutions of soil samples (collected from areas wherein dyes containing industrial effluent had been discharged) were plated on the same medium and incubated at 37 °C for 48 hours. The reddish brown-tinged colony was selected. Out of the selected colonies, the colonies producing extracellular laccase with the highest activity were selected for further studies.

2.3 Identification of the Selected Laccase-Producing Bacteria

The identification of the selected bacterial isolate was done using 16S rRNA sequencing. The 16S rRNA gene was PCR amplified using standard forward primer: 5'-AGAGTTTGATCCTGGCTCAG - 3' and reverse primer: 5'- ACGGCTACCTTGTTACGACTT - 3'[18] The conditions used for PCR are described in Table 1. The amplified gene was then sent for sequencing to Aristogene Biosciences, Banglore. The sequencing results were analysed on BLAST (Basic Local Alignment Search Tool). Phylogenetic tree analysis was also done to identify the Bacteria.

Table 1. The PCR conditions used for 16S rRNA gene amplification

Initial Denaturation	30 Cycles		
	Denaturation	Annealing	Extension
94 ˚C 2 min	94 ˚C 30 sec	58 ˚C 30 sec	72 ˚C 1 min 30 sec

2.4 Laccase Production

B. licheniformis NS2324 was grown in M162 medium having yeast extract (0.2%), tryptone (0.2%), and 100mM $CuSO_4$ to produce laccase. A 24 hour old culture of *B. licheniformis* NS2324 was used to inoculate the medium at 0.1 per cent concentration. Throughout the experiment, vessels were maintained at 37 ºC with continuous swirling at 150 rpm for 48 hours. Following the incubation time, centrifugation at 7826 × g for 15 minutes was done. Culture supernatant was used as extracellular laccase.

2.5 Laccase Assay

Laccase assay was done for 5 min at 50 °C with 2 mM guaiacol in 0.1 M Tris-HCl buffer (pH 8.0). Measurements of oxidation of guaiacol were done at 465 nm (12000 M^{-1} cm^{-1}). Using standard test procedures and enzyme concentrations, one unit of enzyme activity was defined as one micromole of substrate oxidised by one ml of enzyme in one minute.[19]

2.6 Degradation of Indigo Carmine

Degradation of indigo carmine was done in the absence of mediator. Before and following treatment, the dye's absorbance at 600 nm was compared. Percent degradation was calculated as discussed by Kaur and Sondhi.[19]

2.7 Standardisation of Indigo Carmine Degradation

Variable components were varied one at a time to visualise their effect on indigo carmine degradation. The preceding reaction's optimal conditions were implemented in the subsequent reactions.

2.7.1 Impact of Temperature

To study the influence of temperature on the degradation of the indigo carmine, a reaction mixture including enzymes and the dye was incubated at different temperatures ranging from 30-60 ºC. pH was maintained to 7.5. An enzyme dosage of 10 $IUml^{-1}$ NS2324 laccase was introduced to 50 ppm indigo carmine in 10 ml reaction volume. Proper substrate and enzyme controls were employed for each experiment. Equation 1 was used to determine the change in absorbance after treatment.

2.7.2 Impact of Time

Incubation time was varied from 1-8h to study the effect of incubation time on indigo carmine degradation. pH was maintained to 7.5. The enzyme dosage of 10 $IUml^{-1}$ NS2324 laccase was mixed with 50ppm indigo carmine dye in 10 ml reaction volume. To ensure proper substrate and enzyme controls, the same conditions were employed for each experiment. Equation 1 was used to determine the change in absorbance after treatment.

2.7.3 Impact of Enzyme Dose

The enzyme dose was varied from 0 to 40 $IUml^{-1}$ in 10 ml reaction volume containing 50 ppm indigo carmine dye. pH was maintained to 7.5. Substrates and enzyme controls were established for each reaction. Equation 1 was used to determine the change in absorbance after 6 hours of treatment.

2.7.4 Impact of pH

To study the influence of pH on the degradation of the dye, the dye solution was prepared in buffers ranging from pH 5.5 to 8.5. An optimum enzyme dosage of NS2324 laccase was added to 50 ppm indigo carmine dye in 10 ml reaction volume. The investigation was carried out at the optimal time and temperature for optimal results. To ensure proper substrate and enzyme

controls, the same conditions were employed for each experiment. Equation 1 was used to determine the change in absorbance after treatment.

3. RESULTS

3.1 Isolation of Extracellular Laccase-producing Bacteria

A total of 20 bacterial isolates were isolated on M162 agar plates supplemented with guaiacol. Out of them, 8 showed reddish brown colonies, and only 5 isolates produced extracellular laccase. Of the extracellular bacteria, the one exhibiting the highest extracellular activity of 16.979 U/ml/min was selected for further investigation (Fig. 1).

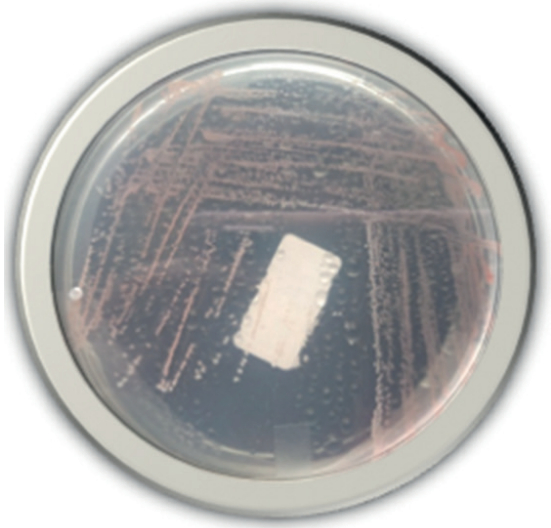

Figure 1. *Bacillus licheniformis* NS2324 laccase-producing bacteria showing reddish brown colonies on M162 media.

3.2 Identification of the Selected Laccase-Producing Bacteria

The PCR amplification of the 16S rRNA gene produced a single band of 1.5 kb. Sequencing results produced a sequence of 994 nucleotides (Fig. 2). On BLAST analysis, the sequence showed 99.78 per cent similarity with *Bacillus licheniformis,* and the phylogenetic tree analysis also depicted that it was most closely related to the *Bacillus licheniformis* (Fig. 3). The identified strain was later submitted in MTCC 13026. The retrieved sequence was then submitted to Genbank (*MT186173*).

3.3 Optimisation of Treatment Condition for Indigo Carmine Degradation

3.3.1 Temperature

Indigo carmine was degraded to maximum extent (88.68 ± 1.08%) at 50°C (Fig. 4). Further rise in temperature resulted in decreased decolorisation.

3.3.2 Time

The degradation of indigo carmine dye was observed to increase with an increase in time till 6 hours (90.48±0.79%),

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TCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGT
GGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTT
GATTGAACCGCATGGTTCAATCATAAAAGGTGGCTTTCAGCTACCACTTGCAGATGGACC
CGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGAC
CTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGA
AGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCG
GTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAA
TACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCT
TAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTT
GAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAG
GAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTG
GGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTG
TTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGT
ACGGTCGCAAGACTGAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGAAGCAACGCGAAGAA
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Figure 2. Sequencing result of 16S rRNA gene of isolated laccase-producing bacteria.

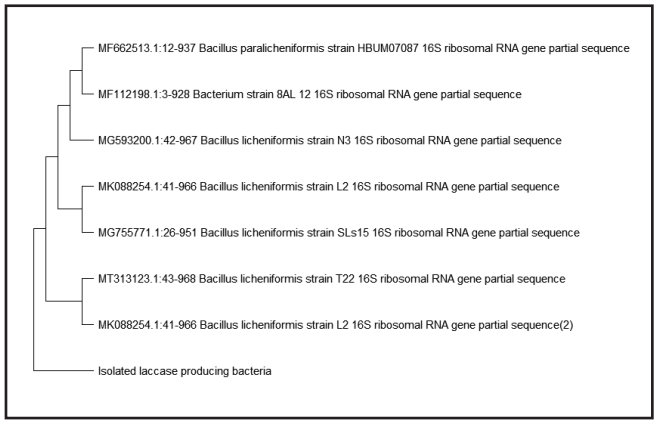

Figure 3. Phylogenetic tree analysis of 16S rRNA sequence by Maximum likelihood tree method.

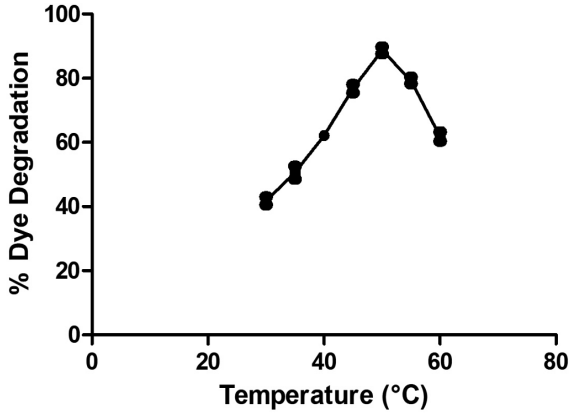

Figure 4. Impact of temperature on degradation of indigo carmine dye.

and then it remained constant. Thus, the optimum incubation time for indigo carmine decolorisation was observed to be 6 hours (Fig. 5).

3.3.3 Enzyme dose

There was an initial increase in degradation with the increase in enzyme until 10 IU/ml (93.57±1.53%), therefore, optimum enzyme dose was 10 IU/ml (Fig. 6).

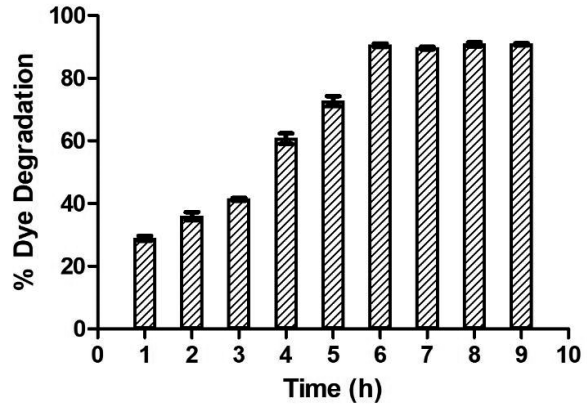

Figure 5. Impact of time on the degradation of indigo carmine dye.

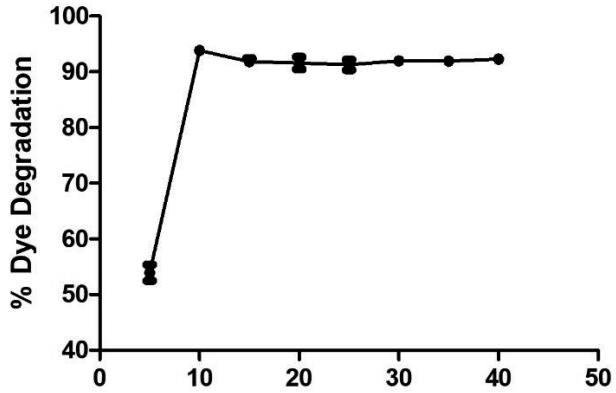

Figure 6. Effect of enzyme dose on indigo carmine degradation.

3.3.4 The Impact of pH

The rate of degradation of indigo carmine increased with increasing pH, with the highest degradation being 96.79 ± 1.06 per cent reported at pH 8 (Fig. 7). As pH was increased further, the rate of degradation of indigo carmine decreased. The decolorisation of indigo dye is shown in Fig. 8.

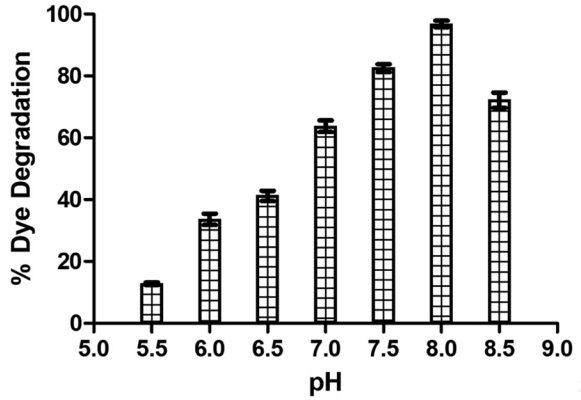

Figure 7. Effect of pH on indigo carmine degradation.

4. DISCUSSION

Laccases have been reported to oxidize indigo pigments by sequentially removing four electrons from the dye molecule. The initial phase in the degradation of Indigo and its derivatives is electrochemical oxidation leading

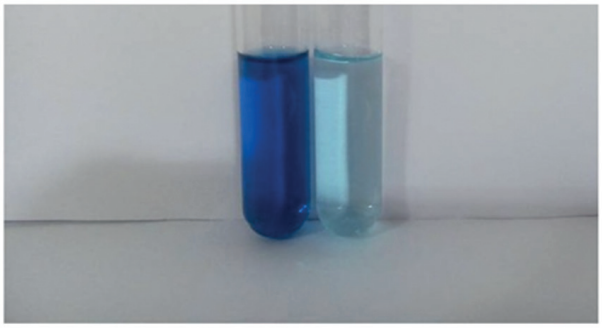

Figure 8. Biodegradation of indigo carmine dye by enzyme laccase from *Bacillus licheniformis* NS2324.

to the formation of dehydroindigo[20], after which it is attacked by a nucleophile (like water), which results in the integration of oxygen atoms leading to the degradation products.[21] Laccases are capable to degrade indigo by enabling the synthesis of isatine (i.eindol-2,3-dion) in the reaction. Afterward, isatine is converted to anthranilic acid (an intermediate formed hydrolytically as a result of the breakdown of isatine) via isatic acid decarboxylation.[20,22] This is a spontaneous decarboxylation reaction.

Laccases have been implicated in the breakdown of indigo carmine in several studies.[23–25] Using the fungal laccase from *Scytalidium thermophillum*, Younes and Sayadi (2013)[26] reported that the enzymatically breakdown of Indigo Carmine by the fungal laccase attained 98 per cent decolorisation after 24 hours. The bacterial laccase from γ-proteobacterium JB was reported to be capable of degrading indigo carmine into anthranilic acid as well as isatin-5-sulfonic acid in the vicinity of mediators such as vanillin, syringaldehyde, and p-hydroxybenzoic acid by Singh, *et al.*[25]

Campos, *et al.*[21], also reported degradation of indigo by fungal laccase from *T. hirsuta* and a bacterial laccase from *Sclerotium rolfsii*, leading to the formation of anthranilic acid with isatin after enzymatic degradation by both of them. However, fungal laccases are also reported to be unstable at alkaline pH and higher temperatures[27], their potential for degrading indigo carmine hasn't been fully realised despite this.

As compared to fungal laccases, bacterial ones can function under high temperature and pH environments found in industries.[28] Spore-bound laccases from *Bacillus* WD23 and *Bacillus amyloliquefaciens* are reported to degrade indigo carmine by 70 per cent and 85 per cent, respectively.[28,29] In another study, Singh, *et al.*[22] reported complete degradation of indigo carmine dye in the presence of acetosyring one as a mediator in 10 mins by laccase from *Bacillus sp.* SS4, however, only 25 per cent of degradation was reported without the use of any mediator after 4 hours.

In the present research, the maximum degradation was observed under alkaline pH of 8 at 50 °C after 6 hours using an enzyme dose of 10U/ml. The NS2324 laccase from *Bacillus licheniformis* was able to degrade indigo carmine dye up to 96.79±1.06 % without the use

of any mediator or inducer. The optimum pH at which an enzyme works can be attributed to the source of the enzyme.[27] Since NS2324 laccase has been isolated from bacteria, it catalyses the degradation of Indigo carmine dye best at pH 8. The enzyme activity of NS2324 laccase was observed to increase with the increasing temperature up to 50 °C after which it decreases because the further increase in temperature might have led to the unfolding of the protein.[30]

The degradation capability of NS2342 was observed to increase with the increase in enzyme dose, but a plateau was observed after 10 U/ml of enzyme dose. It can be attributed to the fact the catalysation reaction increases with the increase in enzyme concentration till the time all the substrate binds to the enzyme, after which the rate of reaction remains same.[31] The optimisation conditions of indigo carmine degradation by NS2324 laccase suggest that the enzyme can withstand high pH and temperatures in an industrial environment and can efficiently remove dye without the use of any mediator or inducer; hence it has great potential to help in the removal of synthetic dyes at industrial level.

5. CONCLUSION

The present research revealed that the bacterial laccase from *B. licheniformis* NS2324 could degrade indigo carmine dye very efficiently at an alkaline pH after six hours. The findings of this work suggest that the laccase enzyme under study can be used at the industrial level. Hence it can provide an efficient, environmentally friendly, and cost-effective approach for degrading the Indigo carmine that does not require the use of mediators. As mostly, dye degradation studies using laccase have been done using mediators, which are costly and known to cause secondary pollution. The aforementioned study may be used to conduct more investigations on the use of enzymes for dye degradation, allowing the impurity generated by industrial outflow to be handled successfully.

ACKNOWLEDGEMENT

The authors are obliged to the Department of Biotechnology, I.K. Gujral Punjab Technical University, Kapurthala-144603, Punjab, India, for their guidance and encouragement.

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