
**JOSTIR Publication 2025 | ISSN: 1118-3896**

Special Issue December 2025 | 223-231

Journal of Science, Technology and Innovation Research
Volume 1 Special Issue | December 2025

# Efficient Decolorization of Textile Dye by Immobilized Thermotolerant Laccase from *Bacillus subtilis* CFB-09

Orungbemi, T. F., Akinola S., Adebayo B., and Olajuyigbe, F. M.*

Enzyme Biotechnology and Environmental Health Unit, Department of Biochemistry, School of Life Sciences, Federal University of Technology, Akure, Nigeria

**Correspondence:** folajuyi@futa.edu.ng

## ABSTRACT

The widespread discharge of textile dyes into aquatic environments has led to a growing public health concern, as a result of their toxicity in water bodies. Hence, there is an urgent need for sustainable dye removal strategies. Enzyme-based bioremediation has emerged as a promising eco-friendly approach, with laccase, a multicopper oxidase, gaining attention due to its ability to oxidize a wide range of phenolic compounds, including lignin-mimicking dyes. Therefore, this study explores the degradation of methyl blue dye using laccase from *Bacillus subtilis* CFB-09. The physicochemical properties of free and immobilized laccase were characterized, and their decolorization efficiency was evaluated across varied dye concentration (25-200 mg/L). Results showed that free and immobilized laccase exhibited optimal activity at 30 °C, pH 5.0 and 40 °C, pH 6.0, respectively. Free laccase demonstrated high thermal stability by retaining over 80% residual activity up to 70 °C for 180 min of incubation, while immobilized laccase exhibited a gradual decline in activity yet retained over 50% residual activity at 30-70 °C for 90 min. Decolorization studies of methyl blue showed that both free and immobilized laccase achieved maximum decolorization at lower dye concentrations. Remarkably, at 125 mg/L, immobilized laccase exhibited slightly higher decolorization (81.28%) than free laccase (78%) after 120 h, indicating enhanced stability and prolonged activity, while at higher concentration (200 mg/L), both free and immobilized laccase showed reduced efficiency (~6.87%). These findings suggest that both free and immobilized laccase hold promise as cost-effective and eco-friendly solutions for mitigating the environmental and health hazards associated with textile dye pollution.

**Keywords:** *Bacillus subtilis* CFB-09, Dye-decolorization, Laccase, Methyl blue, Thermal stability

## Introduction

Dyes are synthetic aromatic compounds that absorb light in the visible wavelength range of 400 to 700 nm (Ardila-Leal *et al.*, 2021). Among these, lignin-mimicking dyes (LMDs) are commonly used in the textile industry. These dyes possess complex aromatic structures derived from phenylpropanoid monolignols (Olajuyigbe *et al.*, 2021). The improper disposal of dye-containing effluents from textile industries has become a pressing environmental concern, causing significant ecological damage (Al-Tohamy *et al.*, 2022). Additionally, these effluents impair the aesthetic quality of water bodies and also obstruct light penetration, disrupt photosynthesis and reduce dissolved oxygen levels, ultimately threatening aquatic life (Islam *et al.*, 2023). Furthermore, many synthetic dyes exhibit carcinogenic and mutagenic properties (Ramamurthy *et al.*, 2024).

doi.org/10.51459/jostir.2025.1.Special-Issue.076

Conventional physical and chemical wastewater treatment methods are usually costly, inefficient, and unsustainable for effective dye decolorization. Consequently, there is an increasing need for affordable, environmentally friendly and efficient biological alternatives for dye degradation. Among the promising biological tools, laccase, a copper-containing oxidoreductase classified as EC 1.10.3.2, demonstrates potential for oxidizing a diverse range of phenolic and non-phenolic compounds (Olajuyigbe *et al.*, 2021). Numerous studies have been conducted on fungal laccases however, fewer studies have focused on bacteria laccases, despite their potential advantages such as faster growth rates, higher enzyme yields, and better adaptability to industrial conditions (Falade *et al.*, 2017). Moreover, limitations such as low operational stability, insufficient enzyme recovery, and high purification costs has hindered the industrial-scale application of free laccase (Zhang *et al.*, 2023). Enzyme immobilization, which is a technique involving the confinement of enzymes onto solid supports offers a promising solution to these challenges however, there is still a need to explore and optimize laccase immobilization for efficient dye degradation.

This study aims to evaluate the dye-degradation potential of free and immobilized laccase from *Bacillus subtilis* CFB-09 under various process conditions. By investigating the decolorization of methyl blue using both enzyme forms, this research is expected to provide insights into the stability and efficiency of laccase in dye degradation, highlight the benefits of enzyme immobilization for enhanced operational performance and contribute to the development of sustainable biotechnological process for textile wastewater treatment.

## Materials and Methods

### Chemicals and Reagents

All chemicals and reagents used in this study were of analytical grade.

### Microorganism, medium and inoculum preparation

*Bacillus subtilis* CFB-09 was gotten from the culture collection at the Enzyme and Microbial Technology Laboratory, Department of Biochemistry, Federal University of Technology, Akure. Seed culture of *Bacillus subtilis* CFB-09 was prepared by inoculating 10 mL of sterile nutrient broth with a loopful of the slant culture (pH 7.4), which contained peptone (5.0 g/L), NaCl (5.0 g/L), beef extract (1.5 g/L), and yeast extract (1.5 g/L) (Olajuyigbe *et al.*, 2021). For the fermentation process, 10% inoculum was added to a mineral salt medium (pH 8.0) which was made up of palm kernel shell (10.0 g/L), $NH_4NO_3$ (2.0 g/L), $KH_2PO_4$ (0.8 g/L), $K_2HPO_4$ (0.2 g/L), $MgSO_4$ (0.2 g/L), $CuSO_4.5H_2O$ (0.25 g/L) and $MnSO_4.H_2O$ (0.25 g/L).

### Production and immobilization of Laccase

Laccase production was done according to established optimal growth conditions. Following the completion of the cultivation phase, the fermentation broth was subjected to centrifugation at 10,000 rpm for 20 minutes at 4 °C. Clear supernatant was recovered and immobilized by entrapment method, using sodium alginate as the carrier material (Niladevi and Prema, 2008). The resulting mixture was extruded dropwise through a 0.4-mm diameter syringe into 0.2 M calcium chloride solution, which facilitated the formation of beads. These beads were left to cure for 1 h after which they were filtered and continuously washed until there was no detectable laccase activity.

### Determination of Laccase Activity

Activity of laccase was assayed using a modified method reported by Olajuyigbe et al (2019). Activity of laccase activity was determined by the oxidation of 1 mM ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) in a 50 mM sodium acetate buffer at 25 $^oC$. Reaction mixture for free laccase (FL) was made up of 250 µL of FL crude enzyme solution combined with 750 µL of the substrate solution (1 mM ABTS in 50 mM buffer at pH 5.0). For the immobilized laccase assay, 12 beads were used

which provided an equivalent volume and activity to 250 µL of the free enzyme, and were combined with 750 µL of the same substrate solution. Change in absorbance was monitored spectrophotometrically at 420 nm at 1 min intervals over a total period of 5 min. The extinction coefficient of ABTS at 420 nm and 25 °C was Ɛ420nm = 36,000 $M^{-1}$ $cm^{-1}$. A unit of laccase activity was defined as the quantity of enzyme required to catalyze the oxidation of 1 µmol of ABTS per minute under standard assay conditions. All assays were conducted in triplicates, and the results were reported as mean ± standard deviation.

$$\text{Activity} = \frac{\text{Absorbance/minute} \times \text{Total volume of the mixture}}{\text{Total time} \times \text{Extinction coefficient } (\varepsilon) \times \text{Volume of enzyme}} \quad (1)$$

### Characterization of Free and Immobilized Laccase from *Bacillus subtilis* CFB-09

Effect of pH and temperature were investigated on the activity and stability of free and immobilized laccase from *Bacillus subtilis* CFB-09 according to Olajuyigbe *et al.,* (2019).

Effect of pH on laccase activity was determined by assaying the enzyme with a 1% ABTS substrate prepared in a series of buffers, followed by incubation at 30 °C for 20 minutes. Buffers include glycine-HCl (pH 3.0), sodium acetate (pH 4.0 – 6.0) and Tris-HCL (pH 7.0 – 8.0). For the immobilized laccase, the reaction mixture contained 12 beads of immobilized enzyme combined with 750 µL of the substrate solution. The activity was determined using standard assay procedure as earlier reported.

Effect of temperature on laccase activity was investigated by incubating the reaction mixture at temperatures ranging from 30 °C – 80 °C for a period of 15 min prior to determining enzyme activity.

Thermal stability of free and immobilized laccase was investigated by incubating both enzyme forms in 50 mM sodium acetate buffer solution at pH 5.0 and pH 6.0, respectively, in the absence of substrate at 30 °C - 80 °C for 180 min. Aliquots of the reaction mixture were collected every 30 min and assayed for enzyme activity as previously outlined.

### Decolorization of Methyl Blue by Free and Immobilized Laccase

The ability of laccase from *Bacillus subtilis* CFB-09 to decolorize methyl blue (MB) was determined following the method previously reported by Olajuyigbe *et al.,* (2021). Methyl blue dye solution was prepared at varying concentrations (25 mg/L, 50 mg/L, 75 mg/L, 100 mg/L, 125 mg/L, 150 mg/L, 175 mg/L and 200 mg/L) for quantitative decolorization and enzyme activity assay. A 20 mL reaction mixture containing 19 mL of dye solution (dye dissolved in 50 mM sodium acetate buffer (pH 5.0)) and 1 mL of free laccase was used for this experiment. The reaction mixture was incubated at 30 °C and 200 rpm for 120 h. Aliquots of the mixture (2 mL) were collected every 24 h intervals and centrifuged at 10,000 rpm for 15 min at 4 °C. Supernatants collected at intervals during decolorization process were used for quantitative estimation of dyes spectrophotometrically at 600 nm (Bandounas *et al.*, 2011). Percentage of dye decolourization was calculated according to the method of Aftab *et al.*, (2011) as follows:

$$\text{Decolorization \%} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100 \quad (2)$$

### Statistical Analysis

Results were carried out in triplicates and statistical analysis (Mean ± Standard Deviation) was evaluated and plotted using Microsoft Excel 2019.

## Results and Discussion

### Immobilization of Crude Laccase

Immobilization of laccase using calcium chloride ($CaCl_2$) as a cross-linking agent yielded uniformly spherical shaped calcium alginate beads (Figure 1). The beads had an average diameter ranging from 0.7 to 0.8 mm, indicating consistent bead formation, which is critical for achieving uniform mass transfer

and predictable catalytic activity in biocatalytic processes. The morphology of the beads suggests effective gelation during the ionic cross-linking process, likely due to optimal interaction between alginate molecules and $Ca^{2+}$ ions (Cerciello *et al.*, 2017). The beads exhibited a light grey coloration. This visualization provides confirmation of a successful enzyme immobilization.

**Figure 1.** Laccase Immobilized in Sodium Alginate

### Effect of pH and Temperature on Activity of Free and Immobilized Laccase

The Optimum pH for immobilized and free laccase were 6.0 and 5.0, respectively (Figure 2A). Laccase in both forms retained over 70% activity across the pH range tested however, as pH progressed towards alkalinity, enzyme activity slightly decreased. This reduction in activity at higher pH values may be attributed to the inhibition of laccase by hydroxide ions, which can bind to the copper atoms at the active site of enzymes and disrupt electron transfer (Reinhammar, 2018). Immobilized laccase exhibited slightly greater sensitivity to pH changes in comparison to free laccase possibly as a result of the buffering capacity of the alginate matrix, which can create microenvironmental pH conditions that differ from the bulk solution (Ranimol *et al.,* 2021). The observed difference in optimum pH reflects the phenomenon where the physicochemical environment of an immobilized enzyme differs from that of free enzyme. Such differences are usually influenced by the nature of the immobilization matrix and its interaction with the enzyme. Laccase activity is known to be source-dependent and highly influenced by immobilization, as reported by Ranimol *et al.,* (2021), which supports the findings of this study. Although laccases generally exhibit activity across a wide pH range, their catalytic performance typically diminishes outside their optimal range, as shown in Figure 2A.

Optimum temperature of free and immobilized laccase were 30°C and 40°C, respectively (Figure 2B). This alteration in optimum temperature for the immobilized enzyme may be attributed to structural modifications induced during immobilization, which often result in increased enzyme rigidity and altered thermal stability (Bié *et al.,* 2022). Remarkably, both enzyme forms retained more than 40% of their activity at 80°C, indicating significant thermotolerance. However, free laccase retained slightly higher activity than the immobilized form at elevated temperatures, suggesting that while immobilization enhanced optimum temperature, it may have also introduced some diffusion or conformational limitations at high thermal conditions. The ability of immobilized laccase to withstand elevated temperatures could be linked to the stabilizing interactions between laccase and the alginate matrix, which may enhance its structural resilience under thermal stress. This is in line with findings by Chauhan *et al.,* (2017) and Peter and Kumar, 2014), who reported optimum temperatures for bacterial and fungal laccases within the range of 30–50°C. The thermotolerant behavior observed in Figure 2B highlights the potential application of both enzyme forms, particularly in industrial processes that operate under moderately high temperatures.

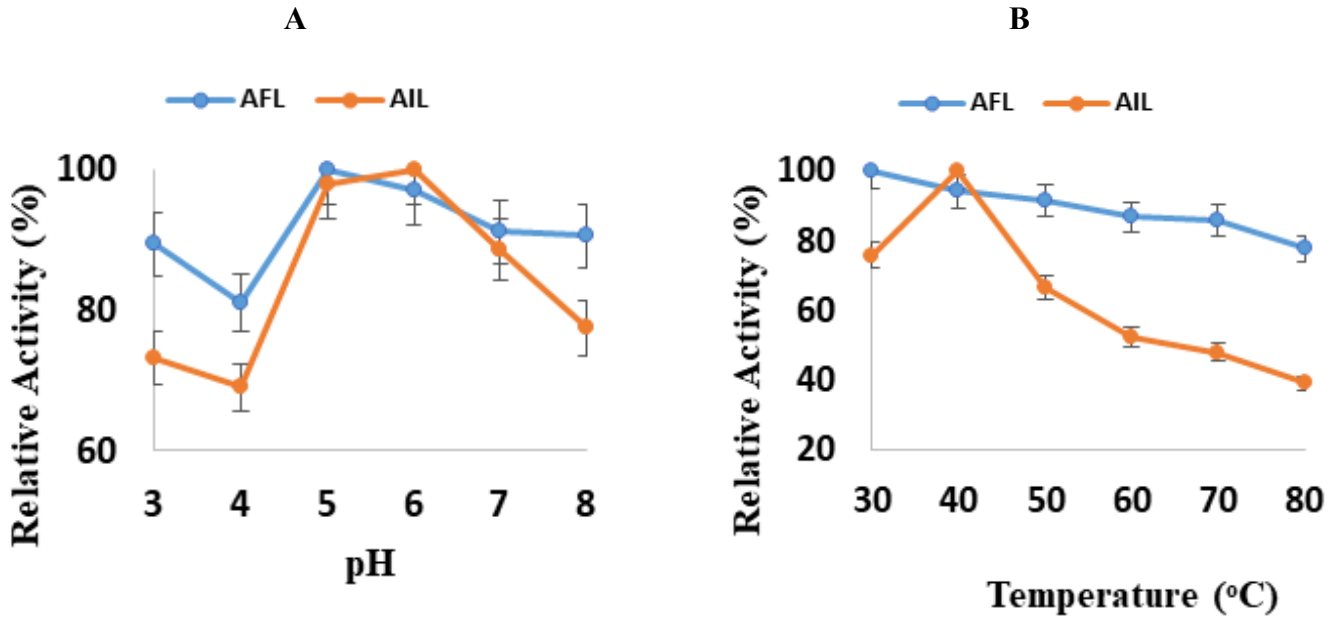


**Figure 2 A.** Effect of pH on activity of free and immobilized Laccase; **B.** Effect of temperature on activity of free and immobilized laccase; AFL - Activity of Free Laccase, AIL - Activity of Immobilized Laccase

### Effect of Temperature on Laccase Stability

Figure 3A shows the effect of temperature on the stability of free laccase from *Bacillus subtilis* CFB-09 at varying temperature. At 30°C, residual activity of free laccase decreased slightly from 99.64% to 84.74% throughout the 180 min incubation period. This indicates excellent baseline thermostability. Remarkably, free laccase maintained high stability at 40 – 70°C, retaining over 84% residual activity even at 180 min at each temperature. The best stability was observed around 60–70°C, where residual activity remained above 85%. At 80°C, though a more noticeable decrease in activity occurred (69.29%), free laccase still showed considerable stability. This result points to a degree of thermostability uncommon among bacterial laccases (Panwar *et al.*, 2023). In contrast to free laccase, immobilized laccase exhibited a significantly lower thermal stability across all temperatures tested (Figure 3B). At 30°C, residual activity dropped sharply from 93.96% to 32.97% over the 180 min incubation period. This indicates that immobilization matrix negatively affects enzyme conformational stability at lower temperatures. Although residual activity was generally higher at 40–50°C than at 30°C, it still followed a continuous downward trend over time, stabilizing at 49.45% to 52.33% after 180 min. At higher temperatures (60–80°C), thermal inactivation became more pronounced with residual activity dropping to 19.07% at 80 °C after 180 min. These results suggest that the immobilization support lacked thermal protection or that the enzyme-support interactions weakened at higher temperatures (Cottone *et al.,* 2019). These results suggest that free laccase from *Bacillus subtilis* CFB-09 is highly thermotolerant, while immobilization reduces its stability, likely due to conformational changes or mass transfer limitations.

### Decolorization of Methyl Blue by Laccase from *B. subtilis* CFB-09

Decolorization of methyl blue (MB) at 25 mg/L, 50 mg/L, 75 mg/L and 100 mg/L by free and immobilized laccase is shown in Figure 4, while decolorization at 125 mg/L, 150 mg/L, 175 mg/L and 200 mg/L by free and immobilized laccase is shown in Figure 5. Remarkably, both forms of laccase were able to achieve optimum decolorization percentage of 100% at lower concentration (Figure 4). The decolorization of methyl blue (MB) by free and immobilized

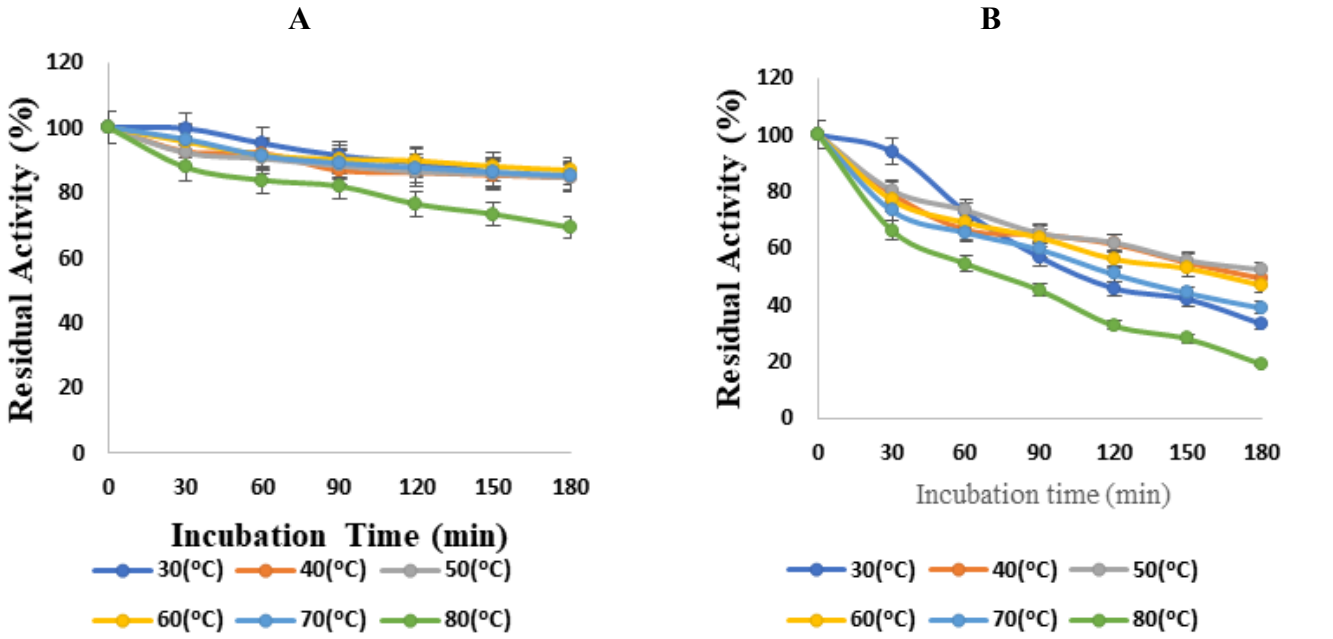


**Figure 3 A**. Effect of temperature on stability of free laccase from *B. subtilis* CFB-09; B. Effect of temperature on stability of immobilized laccase from *B. subtilis* CFB-09

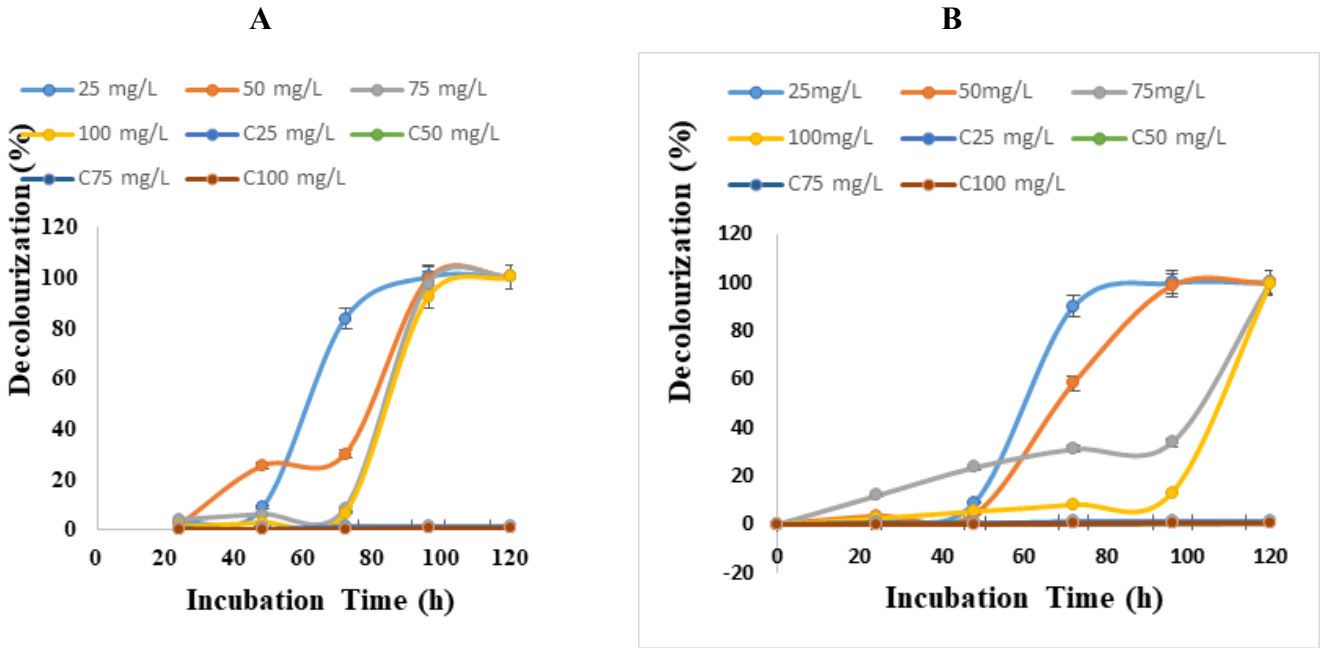


**Figure 4 A:** Decolorization of lower concentration of methyl blue by free laccase from *B. subtilis*; **B.** Decolorization of lower concentration of methyl blue by immobilized laccase from *B. subtilis.* C – Control

laccase from *Bacillus subtilis* was equally assessed at higher dye concentration (125, 150, 175, and 200 mg/L) over a 120 h incubation period. Both free and immobilized laccase demonstrated time-dependent decolorization, with the percentage of dye removal increasing progressively at each point in time. At 125 mg/L, free laccase exhibited notable decolorization, reaching 78% by 120 h.

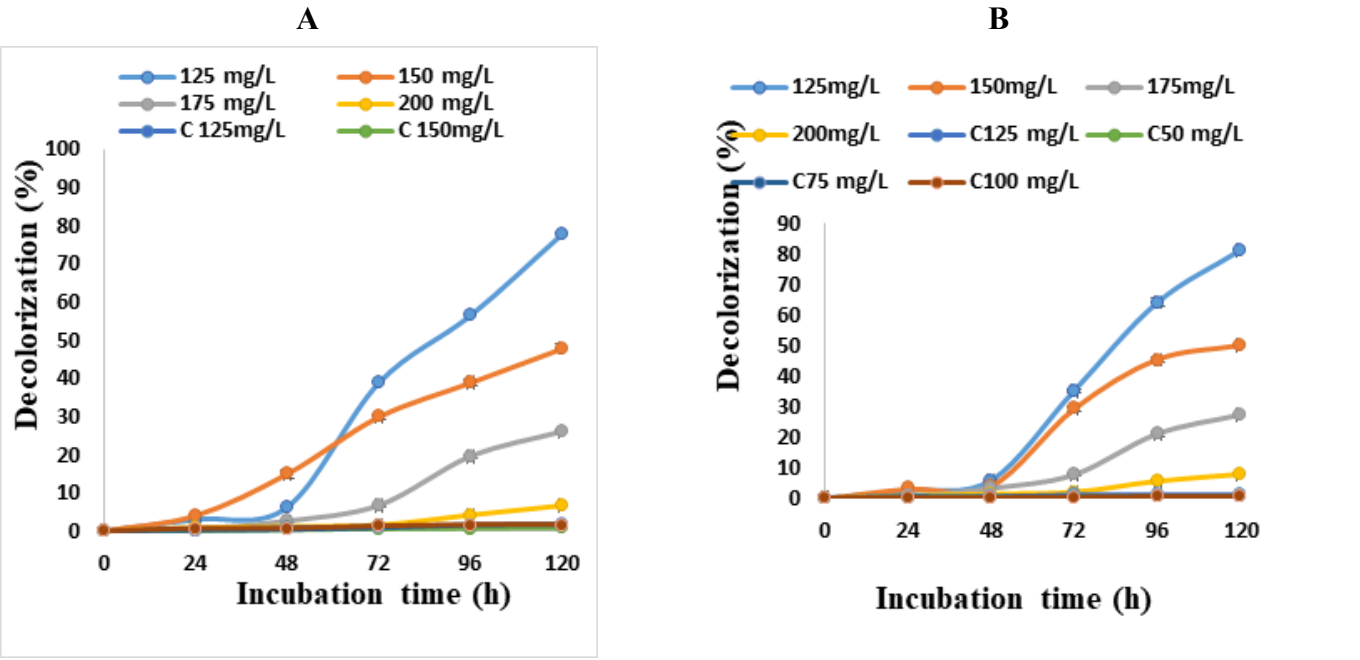


**Figure 5 A:** Decolorization of higher concentration of methyl blue by free laccase from *B.* subtilis; **B**. Decolorization of higher concentration of methyl blue by immobilized laccase from *B. subtilis.* C – Control

Similarly, immobilized laccase showed a slightly higher decolorization of 81.28% under the same condition. This suggests that immobilization enhanced catalytic performance of the enzyme by increasing its stability and protecting the enzyme from inactivation over time (Bié *et al.*, 2022). At 150 mg/L, decolorization by free and immobilized laccase dropped to 48% and 49.95%, respectively, after 120 hours. A further reduction in decolorization was observed at 175 mg/L, with free laccase achieving only 26% and immobilized laccase reaching 27.2% decolorization. At the highest concentration tested (200 mg/L), both enzyme forms exhibited minimal decolorization at 6.87%. This distinct decrease in decolorization efficiency at higher dye concentrations can be attributed to possible substrate inhibition, where excessive dye molecules may block active sites or alter enzyme conformation, thereby reducing catalytic activity (Rajendran *et al.*, 2025). Additionally, the intense coloration at higher concentrations may hinder enzyme-substrate interactions by affecting diffusion or causing steric hindrance (Dubey and Tripathi, 2021).

Despite similar decolorization patterns, immobilized laccase had higher decolorization efficiency than free laccase at lower concentrations, particularly at 125 mg/L. This supports previous reports that enzyme immobilization enhance functional stability and reusability (Atiroğlu *et al.*, 2024; Defaei *et al.*, 2018). Immobilized enzymes are known to resist environmental stresses better and maintain activity over extended periods, which could account for the slightly higher decolorization efficiency observed. However, at higher dye concentrations (175–200 mg/L), the advantage of immobilization diminished, likely due to the overwhelming inhibitory effects of methyl blue.

## Conclusion

This study revealed that the dye decolorization efficiency of laccase was improved by immobilization in sodium alginate beads. This result demonstrates sodium-alginate as a cost-effective and eco-friendly support material for laccase immobilization. Both free and immobilized laccase actively decolorized methyl blue at lower concentrations compared to

higher concentrations. Overall, immobilized laccase was more effective in decolorizing methyl blue than free laccase. Thermotolerance of immobilized laccase from *B. subtilis* demonstrates its potential for various biotechnological applications than free laccase especially in decolorization of dye-contaminated effluents. The striking pH-versatility and thermotolerance of both enzyme forms in this study further demonstrates the potential of the enzyme in decolorization of dye-contaminated water and effluents.

## References

Al-Tohamy, R., Ali, S. S., Li, F., Okasha, K. M., Mahmoud, Y. A. G., Elsamahy, T., and Sun, J. (2022). A critical review on the treatment of dye-containing wastewater: Ecotoxicological and health concerns of textile dyes and possible remediation approaches for environmental safety. *Ecotoxicology and Environmental Safety, 231*, 113160.

Ardila-Leal, L. D., Poutou-Piñales, R. A., Pedroza-Rodríguez, A. M., and Quevedo-Hidalgo, B. E. (2021). A brief history of colour, the environmental impact of synthetic dyes and removal by using laccases. *Molecules, 26(13)*, 3813.

Atiroğlu, V., Atiroğlu, A., Atiroğlu, A., Al-Hajri, A. S., and Özacar, M. (2024). Green immobilization: Enhancing enzyme stability and reusability on eco-friendly support. *Food Chemistry, 448*, 138978.

Bandounas, L., Wierckx, N. J., de Winde, J. H., and Ruijssenaars, H. J. (2011). Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential. *Bmc Biotechnology*, 11, 1-11.

Bié, J., Sepodes, B., Fernandes, P. C., and Ribeiro, M. H. (2022). Enzyme immobilization and co-immobilization: main framework, advances and some applications. Processes, *10(3)*, 494.

Cerciello, A., Del Gaudio, P., Granata, V., Sala, M., Aquino, R. P., and Russo, P. (2017). Synergistic effect of divalent cations in improving technological properties of cross-linked alginate beads. *International Journal of Biological Macromolecules, 101*, 100-106.

Chauhan, P. S., Goradia, B., and Saxena, A. (2017). Bacterial laccase: recent update on production, properties and industrial applications. *3 Biotech, 7(5)*, 323.

Cottone, G., Giuffrida, S., Bettati, S., Bruno, S., Campanini, B., Marchetti, M., and Ronda, L. (2019). More than a confinement:"Soft" and "hard" enzyme entrapment modulates biological catalyst function. *Catalysts, 9(12)*, 1024.

Defaei, M., Taheri-Kafrani, A., Miroliaei, M., and Yaghmaei, P. (2018). Improvement of stability and reusability of α-amylase immobilized on naringin functionalized magnetic nanoparticles: A robust nanobiocatalyst. *International Journal of Biological Macromolecules, 113*, 354-360.

Dubey, N. C., and Tripathi, B. P. (2021). Nature inspired multienzyme immobilization: Strategies and concepts. *ACS Applied Bio Materials, 4(2)*, 1077-1114.

Falade, A. O., Nwodo, U. U., Iweriebor, B. C., Green, E., Mabinya, L. V., and Okoh, A. I. (2017). Lignin peroxidase functionalities and prospective applications. *MicrobiologyOpen, 6(1)*, e00394.

Islam, T., Repon, M. R., Islam, T., Sarwar, Z., and Rahman, M. M. (2023). Impact of textile dyes on health and ecosystem: a review of structure, causes, and potential solutions. *Environmental Science and Pollution Research, 30(4)*, 9207-9242.

Niladevi, K. N., and Prema, P. (2008). Effect of inducers and process parameters on laccase production by Streptomyces psammoticus and its application in dye decolourization. *Bioresource Technology, 99(11)*, 4583-4589.

Olajuyigbe, F. M., Adetuyi, O. Y., and Fatokun, C. O. (2019). Characterization of free and immobilized laccase from Cyberlindnera fabianii and application in degradation of bisphenol A. *International Journal of Biological Macromolecules, 125,* 856-864.

Olajuyigbe, F. M., Afere, F. P., Adetuyi, O. Y., and Fatokun, C. O. (2021). Decolorization of lignin-mimicking dyes by Stenotrophomonas sp. CFB-09: Enzyme activity, transformation dynamics and process optimization. *Biocatalysis and Biotransformation, 40(5)*, 351-364.

Panwar, V., Lzaod, S., and Dutta, T. (2023). Thermostable bacterial laccase: catalytic properties and its application in biotransformation of emerging pollutants. *ACS omega, 8(38)*, 34710-34719.

Peter, J. K., and Kumar, P. V. (2014). Production optimization and partial purification of laccases from bacterial consortium. *International Journal of Engineering Resources 3*, 458-465.

Rajendran, S., Kalairaj, A., and Senthilvelan, T. J. B. C. (2025). A comprehensive review on enzymatic decolorization of various azo dyes using laccase for the abatement of industrial pollution. *Biomass Conversion and Biorefinery, 15(9)*, 13079-13101.

Ramamurthy, K., Priya, P. S., Murugan, R., and Arockiaraj, J. (2024). Hues of risk: investigating genotoxicity and environmental impacts of azo textile dyes. *Environmental Science and Pollution Research, 31(23)*, 33190-33211.

Ranimol, G., Paul, C., and Sunkar, S. (2021). Optimization and efficacy studies of Laccase immobilized on Zein-Polyvinyl pyrrolidone nano fibrous membrane in decolorization of Acid Red 1. *Water Science and Technology, 84(10-11)*, 2703-2717.

Reinhammar, B. (2018). Laccase. In Copper proteins and copper enzymes (pp. 1-36). CRC press.

Zhang, W., Zhang, Z., Ji, L., Lu, Z., Liu, R., Nian, B., and Hu, Y. (2023). Laccase immobilized on nanocomposites for wastewater pollutants degradation: current status and future prospects. *Bioprocess and Biosystems Engineering, 46(11),* 1513-1531.