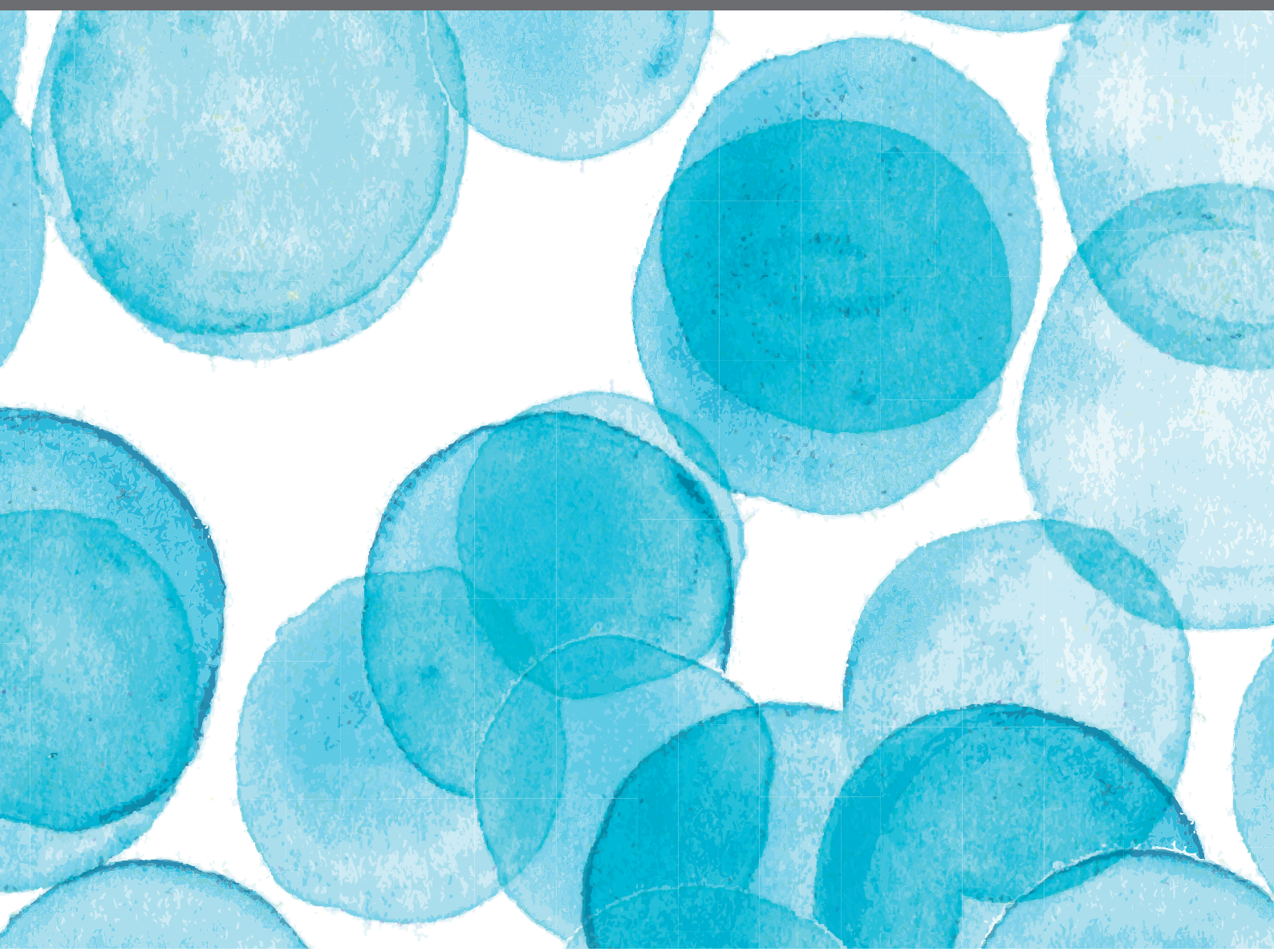
ECO-SUSTAINABLE BIOREMEDIATION OF TEXTILE DYE WASTEWATERS: INNOVATIVE MICROBIAL TREATMENT TECHNOLOGIES AND MECHANISTIC INSIGHTS OF TEXTILE DYE BIODEGRADATION

EDITED BY: Chirayu Desai, Raj Boopathy, Kunal R. Jain, Datta Madamwar and Eric D. van Hullebusch

PUBLISHED IN: Frontiers in Microbiology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88971-380-6
DOI 10.3389/978-2-88971-380-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

ECO-SUSTAINABLE BIOREMEDIATION OF TEXTILE DYE WASTEWATERS: INNOVATIVE MICROBIAL TREATMENT TECHNOLOGIES AND MECHANISTIC INSIGHTS OF TEXTILE DYE BIODEGRADATION

Topic Editors:
Chirayu Desai, Charotar University of Science and Technology, India
Raj Boopathy, Nicholls State University, United States
Kunal R. Jain, Sardar Patel University, India
Datta Madamwar, Charotar University of Science and Technology, India
Eric D. van Hullebusch, Université de Paris, France

Citation: Desai, C., Boopathy, R., Jain, K. R., Madamwar, D., van Hullebusch, E. D., eds. (2021). Eco-Sustainable Bioremediation of Textile Dye Wastewaters: Innovative Microbial Treatment Technologies and Mechanistic Insights of Textile Dye Biodegradation. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-380-6

Table of Contents

frontiers in Microbiology

EDITORIAL
published: 22 July 2021
doi: 10.3389/fmicb.2021.707083

Editorial: Eco-Sustainable Bioremediation of Textile Dye Wastewaters: Innovative Microbial Treatment Technologies and Mechanistic Insights of Textile Dye Biodegradation

Chirayu Desai[1]*, *Kunal R. Jain*[2], *Raj Boopathy*[3], *Eric D. van Hullebusch*[4] and *Datta Madamwar*[1]*

[1] Department of Biological Sciences, P. D. Patel Institute of Applied Sciences, Charotar University of Science and Technology, Changa, India, [2] Post Graduate Department of Biosciences, Sardar Patel University, Anand, India, [3] Department of Biological Sciences, Nicholls State University, Thibodaux, LA, United States, [4] Université de Paris, Institut de Physique Du Globe de Paris, CNRS, UMR 7154, Paris, France

Keywords: textile dye, decolorization, biodegradation, bioreactors, laccase, microbial fuel cells

OPEN ACCESS

Edited by:
Pratyoosh Shukla,
Banaras Hindu University, India

Reviewed by:
Julia Ines Fariña,
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

***Correspondence:**
Chirayu Desai
chirayurdesai@gmail.com
Datta Madamwar
datta_madamwar@yahoo.com

Specialty section:
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

Received: *08 May 2021*
Accepted: *30 June 2021*
Published: *22 July 2021*

Citation:
Desai C, Jain KR, Boopathy R, van Hullebusch ED and Madamwar D (2021) Editorial: Eco-Sustainable Bioremediation of Textile Dye Wastewaters: Innovative Microbial Treatment Technologies and Mechanistic Insights of Textile Dye Biodegradation. Front. Microbiol. 12:707083. doi: 10.3389/fmicb.2021.707083

Editorial on the Research Topic

Eco-Sustainable Bioremediation of Textile Dye Wastewaters: Innovative Microbial Treatment Technologies and Mechanistic Insights of Textile Dye Biodegradation

Textile dyeing processes utilize enormous volumes of potable water and auxiliary chemicals along with toxic dye compounds. Textile dye wastewaters are hazardous mixtures of unused textile dyes, partially degraded dye intermediates (aromatic amines) and several other xenobiotic compounds which resist biodegradation (Rathour et al., 2019). If these wastewaters are released untreated into the aquatic ecosystems, textile dyes can enter into the food webs, bioaccumulate, disrupt photosynthesis and exhibit a potential to induce ecotoxic, mutagenic and carcinogenic effects (Lellis et al., 2019). The xenobiotic and recalcitrant nature of constituent pollutants in the textile dye wastewaters makes their treatment extremely challenging. Therefore, it is imperative to devise eco-sustainable technologies for the remediation of textile dye wastewaters. Recently, various biological and physico-chemical technologies have been applied for the treatment of textile dye wastewater with varying efficacies (Deng et al., 2020). Recent research has demonstrated that microbiotechnolgy approaches, such as biodegradation of textile dyes using yeast, fungal, algal and bacterial processes are effective in the eco-friendly treatment of textile dye wastewaters (Deng et al., 2020). Similarly, enzymatic biodegradation of textile dyes by bacterial, fungal, and algal enzymes such as oxidoreductases, azo reductases, laccases, lignin peroxidases is also considered as a feasible alternative approach (Mishra and Maiti, 2019). Recent approaches utilizing bacterial communities (Rathour et al., 2019), bacterial-biofilm reactors (Rathour et al., 2021) and hybrid bioelectrochemical processes such as constructed wetland microbial fuel cells (CW-MFC) system or bacteria augmented CW-MFCs (Patel et al., 2021) have also shown promising results in the biological treatment of textile dye wastewaters. The aim of this Research Topic (RT) was to publish the recent advancements in microbial biotechnology approaches for effective bioremediation of textile dye wastewaters. This RT has published six papers including four original research papers and two mini-reviews.

The mini-review by Ceretta et al. emphasizes on an interdisciplinary approach for biological treatment of textile dye wastewater. In particular, this article provides a critical view on the state of the art of biological treatment, the degree of advancement and the prospects for their application underlining the importance of combining treatments processes while using toxicity tests on treated effluent in order to verify the toxicological quality of the treated effluents. In the mini-review by Morsy et al., various approaches used in dye decolorization processes by immobilized laccase enzymes have been summarized. The review briefly described the existing technologies based on physical, chemical and biological approaches. In the biological approach, the immobilization of laccase enzyme on solid matrix was discussed in detail with a case study on mechanism of enzyme activity of *Thermus thermophilus* HB27 (PDB code: 5JRR). Authors suggest using co-immobilization of laccase enzymes and redox mediators for the better efficiency and operational stability of the enzymes for textile dye decolorization.

In the study by Mani et al. a new approach was developed utilizing an enzymatic biocathode-MFC with *Shewanella oneidensis* MR-1 as an anodic biocatalyst for the decolorization of Acid orange 7 (AO7) dye. In this study, the *Trametes versicolor* laccase was immobilized by using three different approaches such as crosslinking with electropolymerized polyaniline (PANI), entrapment in copper alginate beads (Cu-Alg), and encapsulation in Nafion micelles, in the absence of redox mediators. Comparative analysis in this study found that biocathodes with laccase cross-linked with PANI were most suitable for efficient dye decolorization, enzyme activity retention, power production and reusability in the enzymatic-biocathode MFC systems. The paper by Dai et al. used recombinant (rlac1338) and mutant laccase (lac2-9) for decolorization of various dye compounds. The error-prone PCR approach was used to induce mutation in rlac1338 and four mutant enzymes were obtained, among which lac2-9 showed the highest activity. The observed results suggested that the expression of mutant enzyme increased by 22 $\pm$ 2% with an increase in the specific enzymatic activity. This study suggested that the error-prone PCR can be utilized in order to improve the catalytic efficiency of laccase or other dye decolorizing enzymes.

In a research paper, John et al., demonstrated the decolorization and degradation potential of halophilic bacterial strain of *Salinivibrio kushneri* HTSP for three dyes: Safranin, Congo red and CBB G-250. The bacterium under experimental conditions decolorized nearly 80% of CBB G-250 and Congo red at a wide range of dye concentrations within 48 h of incubation. They observed complete decolorization of Safranin at lower concentration (<150 mg/L), however the decolorization decreased at higher dye concentrations. Twelve different genes involved in dye degradation were annotated in the genome sequence of *Salinivibrio kushneri* HTSP. Authors conclude that, *Salinivibrio kushneri* HTSP strain has a potential to be used in textile dye wastewater treatment, however biotoxicity studies should be performed before its large-scale application. The research of Zaveri et al. utilized sodium benzoate as a model system to simulate the biodegradation of textile wastewater pollutants by *Pseudomonas citronellolis*. The study investigates how an experimental design approach enabled to understand the interplay of additional carbon and nitrogen sources as well as micronutrients on sodium benzoate degradation by *P. citronellolis*. This work clearly underlines the importance of correcting the nutrient balance in order to ensure an efficient biodegradation of aromatic pollutants present in textile effluents.

This RT highlights the bacterial biodegradation of textile dyes and integrated biological treatment of textile dye wastewater. In particular, the advancements made in enzymatic bioremediation such as biochemical characteristics of recombinant and mutant laccase enzymes, methods of immobilizing laccase enzymes and their applications in the decolorization of synthetic textile dyes as well as in bio-cathode enzymatic microbial fuel cells. This RT also addresses the current limitations, research gaps and potential solutions for future research in achieving sustainable bioremediation of textile dye wastewaters.

AUTHOR CONTRIBUTIONS

All the authors of this editorial article have contributed in conceptualization, writing, reviewing, revising, and have approved it for publication.

FUNDING

The subject area of this Research Topic compilation is based on the theme of research projects supported by Science and Engineering Research Board (Grant No. ECR/2015/000549) and Department of Biotechnology (Grant No. BT/PR18965/BCE/8/1401/2016), New Delhi, India.

ACKNOWLEDGMENTS

The Editors generously thank all the authors who have contributed to this Research Topic. Editors are also grateful to the expert reviewers for their critical assessment and precious time to improve the quality of research presented in the manuscripts submitted to this Research Topic.

REFERENCES

Deng, D., Lamssali, M., Aryal, N., Ofori-Boadu, A., Jha, M. K., and Samuel, R. E. (2020), Textiles wastewater treatment technology: a review. *Water Environ. Res.* 92, 1805–1810. doi: 10.1002/wer.1437

Lellis, B., Fávaro-Polonio, Z. C., Pamphile, J. A., and Polonio, J. C. (2019). Effects of textile dyes on health and the environment and bioremediation potential of living organisms. *Biotechnol. Res. Innov.* 3, 275–290. doi: 10.1016/j.biori.2019.09.001

Mishra, S., and Maiti, A. (2019). Applicability of enzymes produced from different biotic species for biodegradation of textile dyes. *Clean Techn. Environ. Policy* 21, 763–781. doi: 10.1007/s10098-019-01681-5

Patel, D., Bapodra, S. L., Madamwar, D., and Desai, C. (2021). Electroactive bacterial community augmentation enhances the performance of a pilot

scale constructed wetland microbial fuel cell for treatment of textile dye wastewater. *Bioresour. Technol.* 322:125088. doi: 10.1016/j.biortech.2021.125088

Rathour, R., Jain, K., Madamwar, D., and Desai, C. (2019). Microaerophilic biodegradation of raw textile effluent by synergistic activity of bacterial community DR4. *J. Environ. Manage.* 250:109549. doi: 10.1016/j.jenvman.2019.109549

Rathour, R., Jain, K., Madamwar, D., and Desai, C. (2021). Performance and biofilm-associated bacterial community dynamics of an upflow fixed-film microaerophilic-aerobic bioreactor system treating raw textile effluent. *J Clean. Prod.* 295:126380. doi: 10.1016/j.jclepro.2021.126380

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

MINI REVIEW
published: 30 September 2020
doi: 10.3389/fmicb.2020.572309

Current Development in Decolorization of Synthetic Dyes by Immobilized Laccases

*Sherine Ahmed Gamal Zakaria Morsy[1], Asilah Ahmad Tajudin[1,2], Mohd. Shukuri Mohamad Ali[1,3] and Fairolniza Mohd. Shariff[1,2]**

[1] Enzyme and Microbial Technology Research Centre, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia, [2] Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia, [3] Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia

The world today is in a quest for new means of environmental remediation as the methods currently used are not sufficient to halt the damage. Mostly, a global direction is headed toward a shift from traditional chemical-based methods to a more ecofriendly alternative. In this context, biocatalysis is seen as a cost-effective, energy saving, and clean alternative. It is meant to catalyze degradation of recalcitrant chemicals in an easy, rapid, green, and sustainable manner. One already established application of biocatalysis is the removal of dyes from natural water bodies using enzymes, notably oxidoreductases like laccases, due to their wide range of substrate specificity. In order to boost their catalytic activity, various methods of enhancements have been pursued including immobilization of the enzyme on different support materials. Aside from increased catalysis, immobilized laccases have the advantages of higher stability, better durability against harsh environment conditions, longer half-lives, resistance against protease enzymes, and the ability to be recovered for reuse. This review briefly outlines the current methods used for detoxification and decolorization of dye effluents stressing on the importance of laccases as a revolutionary biocatalytic solution to this environmental problem. This work highlights the significance of laccase immobilization and also points out some of the challenges and opportunities of this technology.

Keywords: immobilization, laccase, oxidoreductase, textile dye wastewater, decolorization, detoxification, biodegradation

OPEN ACCESS

Edited by:
Datta Madamwar,
Sardar Patel University, India

Reviewed by:
Pradeep Verma,
Central University of Rajasthan, India
Jingwei Hou,
The University of Queensland, Australia

***Correspondence:**
Fairolniza Mohd. Shariff
fairolniza@upm.edu.my

Specialty section:
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

Received: *13 June 2020*
Accepted: *01 September 2020*
Published: *30 September 2020*

Citation:
Morsy SAGZ, Ahmad Tajudin A, Ali MSM and Shariff FM (2020) Current Development in Decolorization of Synthetic Dyes by Immobilized Laccases. Front. Microbiol. 11:572309. doi: 10.3389/fmicb.2020.572309

INTRODUCTION

Throughout the last century, several industries have inflicted a high demand for synthetic dyes. An estimate of 700,000 tons of different synthetic dyes are produced annually (Holkar et al., 2016; Bilal et al., 2017; Katheresan et al., 2018). Those dyes have stable chemical structures that make them resistant to degradation by heat, light, or water (Lu et al., 2012a,b; Singh and Gupta, 2020). Some even form water-soluble complexes that are toxic to human, animal, and marine life (Bilal et al., 2019). Moreover, hazardous chemicals used in the dye industry, like corrosive acids, hydrogen peroxide, and caustic soda, are abundantly found in dye wastewater (Katheresan et al., 2018). Major industries as leather and textile are guilty of discarding

untreated used dyes in waste water. Other high dye-demanding fields, including electroplating, paper, pulp, tannery, plastic, pharmaceutical, and cosmetic industries (Husain, 2010), are also responsible of causing irreparable damage to the ecosystem through the way they release their wastes containing dyes into clean water bodies (Holkar et al., 2016; Katheresan et al., 2018; Shakerian et al., 2020).

The conventional chemical processes currently used are effective in dye degradation; nevertheless, they lead to the production of toxic intermediate products (Bilal et al., 2017, 2019; Rasheed et al., 2019). Consequently, utilizing technologies that are effective, cheap, and environmentally friendly are being highly favored by governments and dye manufacturers (Ashrafi et al., 2013; Sharma et al., 2018; Deska and Kończak, 2019; Shakerian et al., 2020). Accordingly, biocatalysis is considered a clean process of dye degradation. Various biocatalysts like oxidoreductases are used in degrading hazardous compounds, including phenolic pollutants, and natural or synthetic dye wastes (Bilal et al., 2018, 2019). Laccase is one widely used oxidoreductase catalyst of interest due to its catalysis potential of different dyes (Bilal et al., 2019). As a matter of fact, new sources for fungal and bacterial laccases have been increasingly demanded to degrade dye effluents, or most commonly known as dye waste (Dauda and Erkurt, 2020; Jeon and Park, 2020; Joshi et al., 2020; Singh and Gupta, 2020).

In the past decade, scientists have been working on the development of novel immobilization methods and support materials for laccases to improve their performance and reusability. This improvement is due to the noteworthy enhancement in pH and thermal stability profile range, as well as the capability of working under a wider range of environmental conditions (Dai et al., 2016; Ba and Vinoth Kumar, 2017; Skoronski et al., 2017; Ali et al., 2020).

The aim of this review is to highlight the recent and distinctive application of immobilized laccases in the degradation of dyes in waste water, and how enzyme immobilization enhanced its biocatalysis activity. Finally, limitations and future opportunities of using immobilized laccase are discussed.

RECENT DYE REMOVAL METHODS

Dyes are complex unsaturated organic molecules that are able to absorb light and give color, through reflecting the fraction of light not absorbed by the dye. They are categorized based on their chromophore structure, particle charge after dissolution, color index number, and industrial application as summarized in **Supplementary Table 1** (Hunger, 2004; Yagub et al., 2014; Zhou et al., 2019; Benkhaya et al., 2020). Dyes and metal ions are the most prevalent detrimental materials found in dye wastewater that are very harmful to water and soil (Gosavi and Sharma, 2014; Holkar et al., 2016). Attention has been drawn lately toward remediation of dye wastewater for reuse, due to scarcity of clean natural water sources. An effective dye removal method ideally does its task rapidly, cost effectively, and without producing secondary contaminants (Rodríguez-Couto et al., 2009; Katheresan et al., 2018). The current established dye removal methods are classified into three main categories: physical, oxidation, and biological methods.

Physical

The first physical dye removal method is the coagulation (flocculation) method. This method is mainly used for good removal of disperse dyes (Liang et al., 2014; Yeap et al., 2014), but it has the disadvantage of increased generation of sludge volume (Crini and Lichtfouse, 2019). Another physical method is adsorption, which has higher efficiency in discoloring more types of dyes than the coagulation method (Jadhav and Srivastava, 2013). It is also considered a cheap method of water remediation if low cost adsorbents, like polymeric resins and bentonite clay, are used, but this is not a cost-effective method as the adsorbent is usually used once, generating sludge, with no chance of regeneration for future use (Gupta et al., 2011). Third is the filtration method, where techniques like reverse osmosis and ultrafiltration are used to restore the effluent dye for commercial reuse, but the constant problems of these techniques are the high cost of filtration membranes and their maintenance (Holkar et al., 2016; Katheresan et al., 2018).

Oxidation

This is an easily applied method for dye degradation; hence, it is the most commonly used, starting with advanced oxidation processes, where they can oxidize a wide range of chemicals, including organic and inorganic compounds found in wastewater, but it has the limitation of forming a precipitating sludge (Babuponnusami and Muthukumar, 2014). Then there are the chemical oxidation processes that are very powerful and can break down even the chemical structures of dyes with double bonds and complex aromatic rings using ozone (O_3) molecules (Asghar et al., 2015). Nevertheless, using these molecules releases toxic secondary products as well as being expensive to purchase (Holkar et al., 2016). Finally, there are the synergistic hybrid advanced oxidation processes. It is a combination of the two previous methods, which is advised to be used when synergistic oxidative decolorization effect is desired (Holkar et al., 2016). However, it still has the disadvantages of the previous two methods.

Biological

This method is the most preferred nowadays for degrading, detoxifying, and remediation of recalcitrant dyes from factory effluents. Its mechanism is based on the adaptability of the selected microorganisms and the strength of the biological enzymes either secreted directly from microorganisms or free enzymes (Solís et al., 2012). Preference of biological methods over physical and oxidation practices is attributed to their environmental benefits as there is no production of hazardous byproducts as well as less sludge formation. Moreover, it is a cost-effective method, which makes it a better candidate to be used in industrial scale (Hayat et al., 2015). Up to this point, many microorganisms and their enzymes have been studied and tested for their potential ability in degrading dyes found in wastewater (D'Souza-Ticlo et al., 2009; Wikee et al., 2019). Apart from the enzyme source, those enzymes degrade dyes

through biocatalytic oxidation of their chromophores; hence, the increased interest in studying proprieties of different biocatalysts lately (Chapman et al., 2018). A number of research reports have documented the high efficiency of using enzymes in dye removal (Chiong et al., 2016; Yang X. et al., 2016; Katheresan et al., 2018; Kashefi et al., 2019b).

As promising as using enzymes commercially may seem, they still have their limitations like low stability and lack of recovery decreasing their potential for reuse. Also, rapid loss of catalytic activity may occur in case of altering their favorable operating conditions. Hence, they usually fail to perform under harsh industrial conditions. Therefore, new methods are being developed to enhance the durability of enzymes through immobilization. This is particularly crucial when intracellular enzymes are desired to be used in a cell free system. Nowadays, the usage of enzymes immobilized on solid carriers is gaining popularity more than the free enzyme, as immobilization stabilizes the protein structure giving it longer shelf-life, resistance against proteases, thermal and pH stability, and repeatability of use, thus, reducing operational cost, which makes a good candidate for commercial and industrial use (Dodor et al., 2004; Fernández-Fernández et al., 2013; Nair et al., 2013; Li et al., 2014; Sun et al., 2015; Bilal et al., 2019; Deska and Kończak, 2019).

IMMOBILIZED LACCASE IN REMOVAL OF DYES

One enzyme of interest that has been around for some time, is laccase. Scientists have regained interest in it due to its promising catalytic and physiochemical properties, making it the perfect candidate for bioremediation processes (Rao et al., 2014; Legerska et al., 2016). Laccase is a monomeric, dimeric, or a tetrameric glycoprotein, which oxidizes a broad spectrum of phenolic and non-phenolic substrates (Giardina et al., 2010). By now, many laccases have been discovered, and as a family, they show different structures and functions depending on their source as they can be widely found in eukaryotes as well as prokaryotes (Deska and Kończak, 2019); including higher plants, bacteria, insects, fungi, and recently mammals (Giardina et al., 2010; Janusz et al., 2020). As for the mechanism of action of laccases, they catalyze three types of reactions using oxygen atom and releasing water molecule: (i) direct oxidation of phenolic substrates, (ii) indirect oxidation of non-phenolic substrates with high redox potential in the presence of a natural or synthetic low molecular weight mediator (Breen and Singleton, 1999; Agrawal et al., 2018), and (iii) coupling reactions with reactive intermediate radicals formed during direct oxidation (Polak and Jarosz-Wilkolazka, 2012). The active site of laccases, where biocatalysis occurs, has three copper centers/types/domains; (1) blue copper center (type I), (2) normal copper center (type II), and (3) coupled binuclear copper centers (type 3) (Messerschmidt and Huber, 1990; Solomon et al., 1992; Dwivedi et al., 2011).

A great number of research articles have reported the increased biocatalytic performance of laccase for a longer stretch of time when immobilized. As a general definition, to immobilize an enzyme is when the soluble form of the enzyme is attached to a solid support or, most recently, through formation of aggregates. In this context, different ways of immobilization include adsorption on beads or a matrix, covalent binding to a solid support, entrapment or encapsulation in polymers, and crosslinking as illustrated in **Supplementary Figure 1** (Shrivastava et al., 2012; Datta et al., 2013; Sirisha et al., 2016). Whichever method is chosen, one point ought to be taken into consideration: no immobilization method should influence the enzymatic conformation so that the activity shall not be affected (Shaheen et al., 2017). Other factors to bear in mind while considering the different immobilization methods shall be more elaborated in **Supplementary Data 1**. A simple proposed mechanism of dye degradation by immobilized laccase is illustrated in **Figure 1** with the aid of the crystal structure of laccase from *Thermus thermophilus HB27* (PDB code: 5JRR). In the figure, the dye structure, substrate, is being oxidized by only oxygen in the presence of laccase as a catalyst. When the dye is oxidized, it loses an electron that moves from one Cu^{2+} atom to another inside the catalytic site, until it eventually reduces oxygen to release water. More information about laccase-catalyzed descoloration of dyes and the exact oxidized products have been reported in literature (Zille et al., 2005; Yang et al., 2015; Kagalkar et al., 2015).

Immobilized laccases proved to be worthy challengers for the effective decolorization, degradation, and removal of dyes (Bilal et al., 2019). For example, laccase from the *Cerrena* sp. strain HYB07 was immobilized by preparing cross-linked enzyme aggregates (CLEAs) of the enzyme to degrade Remazol Brilliant Blue Reactive dye. Almost 90% of the dye was eliminated from the solution in just 40 min without the help of a redox mediator (Yang J. et al., 2016). In another study, Arica et al. (2017) demonstrated the immobilization of laccase on fibrous polymer-grafted polypropylene chloride film, where three dyes, namely, Procion Green H4G, Brilliant Blue G, and Crystal Violet, were tested for removal by both the free and immobilized enzyme. The immobilized laccase gave better dye degradation results than the free one (Arica et al., 2017).

In addition to the improved biocatalytic properties, immobilized laccase shows notable storage stability and repeated use capability, while maintaining satisfactory efficiency (Deska and Kończak, 2019). In a study, by Ma et al. (2018), genipin-activated chitosan beads were introduced as a highly stable laccase biocatalyst from *Trametes pubescens*. The resulting immobilized laccase reached to a decolorization of 77.49% of Acid Black 172 dye. Moreover, the carrier-supported laccase displayed excellent reuse potential with > 55% of remaining activity reserved after 11 cycles of constant use. It also showed high storage stability retaining over 57.14% of its original activity after 30 days of storage at 4°C (Ma et al., 2018).

Also, increased pH stability is a notable characteristic of the immobilized laccase as stated by Wen et al. (2019), when the enzyme was physically adsorbed on kaolinite for the removal of malachite green dye. The immobilized biocatalyst exhibited outstanding stability over a broad pH range from 3 to 6, with the lowest relative activity being 60% at pH 5.5 and the highest

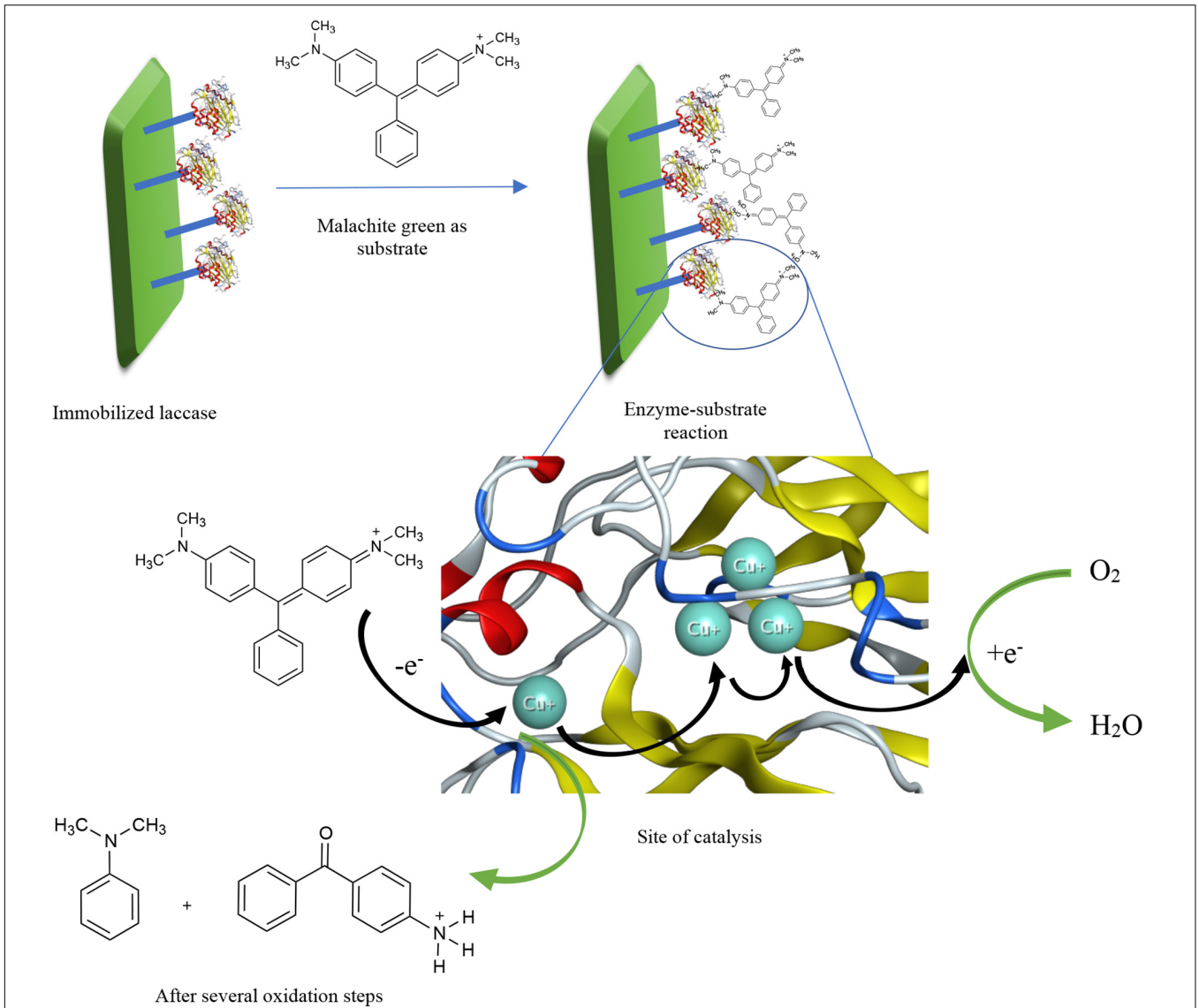


FIGURE 1 | A proposed mechanism of dye degradation by immobilized laccase with the aid of the crystal structure of laccase from *Thermus thermophilus HB27* (PDB code: 5JRR).

is 100% at pH 4.5. The authors justified this enhanced pH tolerance to a relative stable proton production on the surface of support and assured that it could retain the activity of laccase (Wen et al., 2019).

Furthermore, immobilized catalysts often show remarkable improved thermal steadiness than free enzymes. A recent report presented a novel support carrier; laccase covalently bound to crosslinked graphene oxide–zeolite nanocomposites. The obtained catalytic system was exploited for the degradation of Direct Red 23 dye. Notably, the immobilized laccase was very stable at 80°C and preserved 84% of its initial catalytic activity, unlike its free counterpart, which could only retain 18%. The author attributed this thermal protection to the covalent bonding between laccase and supporting carrier (Mahmoodi and Saffar-Dastgerdi, 2020).

Many literature reports confirm that using immobilized laccases is a favorable technology in the treatment of dye effluents. Several recent reports regarding this matter are listed in **Table 1**.

LIMITATIONS AND FUTURE OPPORTUNITIES

As narrated throughout this minireview, the current established dye treatment methodologies still have, to some extent, several limitations. The fact that no certain decolorization method is adopted universally for all types of dye effluents needs more study, as currently, using a single dye removal method, either physical, oxidation, or biological, is not enough.

TABLE 1 | Some recent reports about the degradation of dyes by immobilized laccases from different microbial sources.

Laccase source	Immobilization method	Immobilization matrix	Target molecule (dye)	Degradation (%)	References
White rot fungus *T. trogii*	Covalent binding	Thiolated chitosan–Fe_3O_4 hybrid composite	Reactive Blue 171, Acid Blue 74	Using 6 mg of immobilized NPs, 79% after 10 cycles and 56% after 8 cycles respectively	Ulu et al., 2020
Trametes versicolor	Adsorption Covalent binding	PMMA/PANI electrospun fibers	Remazol Brilliant Blue R	87% using adsorbed laccase and 58% using covalently bonded laccase	Jankowska et al., 2020
Trametes versicolor	Adsorption	Carbon nanotube nanocomposites	Congo Red	96% within 3 h	Zhang et al., 2020
N/A	Adsorption	3D PVA-co-PE HPNM for immobilizing laccase-$Cu_2(PO_4)_3{\cdot}3H_2O$ HNF .	Reactive Blue 2, Acid Blue 25, Acid Yellow 76, Indigo Carmine	83.59%, 86.35%, 90.2% in 10 h, 99.5% in 3 h respectively	Luo et al., 2020
N/A	Adsorption	CNT/GO&Lac@UF	Methylene Blue	80%	Zhu et al., 2020
Trametes versicolor	Adsorption	Poly(2-hydroxyethyl methacrylate-glycidyl methacrylate) [p(HEMA-GMA)] cryogels	Brilliant Blue R, Brilliant Green, Orange G, Procion Red MX-5B, Congo Red, Sunset Yellow	63.16%, 77.27%, 52.27%, 34.71%, 46.67%, 52.08% respectively	Bayraktaroğlu et al., 2020
Trametes versicolor	Entrapment	PILM	Remazol Brillant Blue R	75%	HajKacem et al., 2020
Boletus edulis	Adsorption	Modified rice husks	Reactive Blue-19	91%	Tuncay and Yagar, 2020
N/A	Adsorption	TiO_2 sol–gel-coated PAN/O-MMT composite nanofibers	Crystal Violet	95% in 6 h	Wang et al., 2020
Genetically modified *Aspergillus*	Covalent attachment	Graphene oxide nano-sheets	Direct Red 23, Acid Blue 92	88.7%, 48.7% respectively	Kashefi et al., 2019a
Trametes versicolor	Cross-linking	Laccase-$Cu_3(PO_4)_2{\cdot}3H_2O$ hybrid NFs	Bromophenol Blue, CBBR-250, Xylene cyanol	41.2%, 73.2%, 73.0% respectively (without use of mediator)	Patel et al., 2018
Myceliophthora thermophila	Covalent attachment	Epoxy-functionalized silica	Malachite Green, Acid Red 52, Acid Orange 156, Coomassie Brilliant Blue, Methyl Violet	100%, 99%, 98%, 97%, 78% respectively; all in the presence of DMHBA as redox mediator	Salami et al., 2018
Trametes versicolor	Adsorption	Methacrylyol group and amino] group functionalized Fe_3O_4@SiO_2	Methyl Red	Removal efficiency was more than 80% for the first 3 days	Lin et al., 2017
Trametes pubescens	Entrapment	Chitosan beads	Blue R, Indigo Blue, Remazol Brilliant, Reactive Brilliant, Blue X-B, Methylene Blue, Acid Black 172, Neutral red, Congo Red, Naphthol Green B	68.84%, 56.28%, 54.24%, 52.26%, 48.23%, 45.12%, 44.58%, 37.18%, 25.39%, 20.81% respectively	Zheng et al., 2016
Trametes versicolor	Encapsulation	Sponge-like chitosan grafted polyacrylamide hydrogel	Malachite Green	90% in the first cycle	Sun et al., 2015

DMHBA, 3,5-dimethoxy-4-hydroxybenzaldehyde; PMMA/PANI, poly(methyl methacrylate)/polyaniline; HNF, hybrid nanoflower; HPNM, hierarchically porous nanofibrous membrane; CNT/GO&Lac@UF, carbon nanotubes/graphene oxide and laccase on ultrafiltration membrane; PILM, polymeric ionic liquid membrane; PAN/O-MMT, polyacrylonitrile/organically modified montmorillonite; ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); NFs, nanoflowers.

Using a combination of different methods may be attributed to the complex and different chromophore structures of dying compounds. It is undeniable that the current enzyme immobilization methods are very useful and somehow effective in the issue at hand: decolorization and detoxification of dye wastewater. However, as auspicious as immobilized enzymes seem to be, the matter of loss of activity or stability should be addressed. Limitations of current immobilization methods are due to enzyme leakage from carrier, undesired reactions between carrier and enzyme or even the inability of the enzyme to react with its substrate. Nowadays, scientists are still developing new enzyme immobilization methods as it is a promising field that gives both better results and cost effectiveness for industrial use. However, from what is evident from recent research reports, they are merely focused on finding new materials, but the principles and methodologies of enzyme immobilization are still unchanged.

Despite the success of most of the reported laccase immobilization methods in dye removal, there is still one more aspect to be investigated when assessing their efficiency. The chemical structure of the target dye affects the results dramatically. Research reports often published assess the efficiency of their technologies by testing them on dyes with simple, low molecular weight structures that eventually show high decolorization results. Also, those promising technologies are still not implemented on an industrial scale. As the industrial sectors always seek the most economical technologies, they prefer using large quantities of mixed crude cultures or pure cultures over the immobilized one to limit the costs, overlooking the long-term advantages of reusage of immobilized enzymes. Most are

drawn toward fungal more than bacterial laccase, due to its higher redox potential that show a higher oxidative ability. However, bacterial laccases are reported to withstand the harsh industrial environments with higher temperatures and pH values than those tolerated by fungal laccases. It is advised to use a redox mediator to act as an electron shuttle for better decolorization results of dye structures with higher redox potentials.

For all the mentioned drawbacks, immobilized laccases represent a potential effective, eco-friendly and commercial alternative to the physical, chemical, and oxidative dye decolorization methods. The scientific and industrial communities have real chances and future opportunities to moderate the current situation. Improvements can be made in fields of developing novel immobilization strategies in order to avoid enzyme leakage and come up with a more stable, durable, sustainable, and economical immobilization systems. One solution to the problem is the immobilization of both enzyme and redox mediator on the same carrier. This method ensures that low and medium redox potential laccases have better efficiency and operational stability. Also, this co-immobilized system can be reused several times, which will eventually reduce the manufacturing cost, that is ultimately the main concern of the industrial sector. This ambitious suggestion shall introduce an environmentally conscious and cost-effective dye detoxification method to the market.

AUTHOR CONTRIBUTIONS

SAGZM: conceptualization, original draft preparation, and editing. AAT: manuscript reviewing. MSMA: supervision. FMS: project administration and supervision. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

SAGZM thanks Y. Bhg. Prof. Dato' Dr. Abu Bakar Bin Salleh and Mohammed Abdelbaset Mahmoud Farag for their support, guidance and constructive review of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.572309/full#supplementary-material

Supplementary Figure 1 | Basic methods of enzyme immobilization.

Supplementary Table 1 | Classification of dyes according to industrial application, particle charge after dissolution and chromophore structure.

Supplementary Data 1 | Types of immobilization methods.

REFERENCES

Agrawal, K., Chaturvedi, V., and Verma, P. (2018). Fungal laccase discovered but yet undiscovered. *Bioresour. Bioprocess.* 5, 1–12. doi: 10.1186/s40643-018-0190-z

Ali, E. A. M., Abd Ellatif, S., and Abdel Razik, E. S. (2020). Production, purification, characterization and immobilization of laccase from Phoma betae and its application in synthetic dyes decolorization. *Egyptian J. Bot.* 60, 301–312. doi: 10.21608/ejbo.2019.19226.1381

Arica, M. Y., Salih, B., Celikbicak, O., and Bayramoglu, G. (2017). Immobilization of laccase on the fibrous polymer-grafted film and study of textile dye degradation by MALDI–ToF-MS. *Chem. Eng. Res. Des.* 128, 107–119. doi: 10.1016/j.cherd.2017.09.023

Asghar, A., Raman, A. A. A., and Daud, W. M. A. W. (2015). Advanced oxidation processes for in-situ production of hydrogen peroxide/hydroxyl radical for textile wastewater treatment: a review. *J. Clean. Prod.* 87, 826–838. doi: 10.1016/j.jclepro.2014.09.010

Ashrafi, S. D., Rezaei, S., Forootanfar, H., Mahvi, A. H., and Faramarzi, M. A. (2013). The enzymatic decolorization and detoxification of synthetic dyes by the laccase from a soil-isolated ascomycete. *Paraconiothyrium variabile*. *Int. Biodeter. Biodegr.* 85, 173–181. doi: 10.1016/j.ibiod.2013.07.006

Ba, S., and Vinoth Kumar, V. (2017). Recent developments in the use of tyrosinase and laccase in environmental applications. *Crit. Rev. Biotechnol.* 37, 819–832. doi: 10.1080/07388551.2016.1261081

Babuponnusami, A., and Muthukumar, K. (2014). A review on Fenton and improvements to the Fenton process for wastewater treatment. *J. Environ. Chem. Eng.* 2, 557–572. doi: 10.1016/j.jece.2013.10.011

Bayraktaroğlu, M., Husein, Ý, AktaşUygun, D., and Uygun, M. (2020). Lectin-modified cryogels for laccase immobilization: a decolorization study. *Water Air Soil Pollut.* 231, 1–13. doi: 10.1007/s11270-020-4395-3

Benkhaya, S., M' rabet, S., and El Harfi, A. (2020). A review on classifications, recent synthesis and applications of textile dyes. *Inorg. Chem. Commun.* 115, 1–35. doi: 10.1016/j.inoche.2020.107891

Bilal, M., Asgher, M., Parra-Saldivar, R., Hu, H., Wang, W., Zhang, X., et al. (2017). Immobilized ligninolytic enzymes: an innovative and environmental responsive technology to tackle dye-based industrial pollutants – A review. *Sci. Total Environ.* 576, 646–659. doi: 10.1016/j.scitotenv.2016.10.137

Bilal, M., Rasheed, T., Iqbal, H. M. N., and Yan, Y. (2018). Peroxidases-assisted removal of environmentally-related hazardous pollutants with reference to the reaction mechanisms of industrial dyes. *Sci. Total Environ.* 644, 1–13. doi: 10.1016/j.scitotenv.2018.06.274

Bilal, M., Rasheed, T., Nabeel, F., Iqbal, H. M. N., and Zhao, Y. (2019). Hazardous contaminants in the environment and their laccase-assisted degradation – A review. *J. Environ. Manag.* 234, 253–264. doi: 10.1016/j.jenvman.2019.01.001

Breen, A., and Singleton, F. L. (1999). Fungi in lignocellulose breakdown and biopulping. *Curr. Opin. Biotechnol.* 10, 252–258. doi: 10.1016/S0958-1669(99)80044-5

Chapman, J., Ismail, A., and Dinu, C. (2018). Industrial applications of enzymes: recent advances. Techniques, and Outlooks. *Catalysts* 8:238. doi: 10.3390/catal8060238

Chiong, T., Lau, S. Y., Lek, Z. H., Koh, B. Y., and Danquah, M. K. (2016). Enzymatic treatment of methyl orange dye in synthetic wastewater by plant-based peroxidase enzymes. *J. Environ. Chem. Eng.* 4, 2500–2509. doi: 10.1016/j.jece.2016.04.030

Crini, G., and Lichtfouse, E. (2019). Advantages and disadvantages of techniques used for wastewater treatment. *Environ. Chem. Lett.* 17, 145–155. doi: 10.1007/s10311-018-0785-9

Dai, J., Wang, H., Chi, H., Wang, Y., and Zhao, J. (2016). Immobilization of laccase from Pleurotus ostreatus on magnetic separable SiO2 support and excellent activity towards azo dye decolorization. *J. Environ. Chem. Eng.* 4, 2585–2591. doi: 10.1016/j.jece.2016.04.037

Datta, S., Christena, L. R., and Rajaram, Y. R. S. (2013). Enzyme immobilization: an overview on techniques and support materials. *3 Biotech* 3, 1–9. doi: 10.1007/s13205-012-0071-7

Dauda, M. Y., and Erkurt, E. A. (2020). Investigation of reactive Blue 19 biodegradation and byproducts toxicity assessment using crude laccase extract from *Trametes versicolor*. *J. Hazard. Mater.* 393, 1–9. doi: 10.1016/j.jhazmat.2019.121555

Deska, M., and Kończak, B. (2019). Immobilized fungal laccase as "green catalyst" for the decolourization process – State of the art. *Process Biochem.* 84, 112–123. doi: 10.1016/j.procbio.2019.05.024

Dodor, D. E., Hwang, H. M., and Ekunwe, S. I. N. (2004). Oxidation of anthracene and benzo[a]pyrene by immobilized laccase from *Trametes versicolor*. *Enzyme Microb. Technol.* 35, 210–217. doi: 10.1016/j.enzmictec.2004.04.007

D'Souza-Ticlo, D., Sharma, D., and Raghukumar, C. (2009). A thermostable metal-tolerant laccase with bioremediation potential from a marine-derived fungus. *Mar. Biotechnol.* 11, 725–737. doi: 10.1007/s10126-009-9187-0

Dwivedi, U. N., Singh, P., Pandey, V. P., and Kumar, A. (2011). Structure-function relationship among bacterial, fungal and plant laccases. *J. Mol. Catal. B Enzym.* 68, 117–128. doi: 10.1016/j.molcatb.2010.11.002

Fernández-Fernández, M., Sanromán, M. Á, and Moldes, D. (2013). Recent developments and applications of immobilized laccase. *Biotechnol. Adv.* 31, 1808–1825. doi: 10.1016/j.biotechadv.2012.02.013

Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., and Sannia, G. (2010). Laccases: a never-ending story. *Cel. Mol. Life Sci.* 67, 369–385. doi: 10.1007/s00018-009-0169-1

Gosavi, V. D., and Sharma, S. (2014). A General review on various treatment methods for textile wastewater. *J. Environ. Sci. Comput. Sci. Eng. Technol.* 3, 29–39.

Gupta, V. K., Gupta, B., Rastogi, A., Agarwal, S., and Nayak, A. (2011). A comparative investigation on adsorption performances of mesoporous activated carbon prepared from waste rubber tire and activated carbon for a hazardous azo dye-Acid Blue 113. *J. Hazard. Mater.* 186, 891–901. doi: 10.1016/j.jhazmat.2010.11.091

HajKacem, S., Galai, S., Hernández Fernandez, F. J., Pérez de los Ríos, A., Smaali, I., and Quesada Medina, J. (2020). Bioreactor membranes for laccase immobilization optimized by ionic liquids and cross-linking agents. *Appl. Biochem. Biotechnol.* 190, 1–17. doi: 10.1007/s12010-019-03085-z

Hayat, H., Mahmood, Q., Pervez, A., Bhatti, Z. A., and Baig, S. A. (2015). Comparative decolorization of dyes in textile wastewater using biological and chemical treatment. *Separat. Purificat. Technol.* 154, 149–153. doi: 10.1016/j.seppur.2015.09.025

Holkar, C. R., Jadhav, A. J., Pinjari, D. V., Mahamuni, N. M., and Pandit, A. B. (2016). A critical review on textile wastewater treatments: possible approaches. *J. Environ. Manag.* 182, 351–366. doi: 10.1016/j.jenvman.2016.07.090

Hunger, K. (2004). "Chapter 1: dyes, general survey," in *Industrial Dyes: Chemistry, Properties, Applications*, ed. K. Hunger (Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA), 1–12. doi: 10.1002/3527602011

Husain, Q. (2010). Peroxidase mediated decolorization and remediation of wastewater containing industrial dyes: a review. *Rev. Environ. Sci. Biotechnol.* 9, 117–140. doi: 10.1007/s11157-009-9184-9

Jadhav, A. J., and Srivastava, V. C. (2013). Adsorbed solution theory based modeling of binary adsorption of nitrobenzene, aniline and phenol onto granulated activated carbon. *Chem. Eng. J.* 229, 450–459. doi: 10.1016/j.cej.2013.06.021

Jankowska, K., Zdarta, J., Grzywaczyk, A., Kijeńska-Gawrońska, E., Biadasz, A., and Jesionowski, T. (2020). Electrospun poly(methyl methacrylate)/polyaniline fibres as a support for laccase immobilisation and use in dye decolourisation. *Environ. Res.* 184:Article109332. doi: 10.1016/j.envres.2020.109332

Janusz, G., Pawlik, A., Świderska-Burek, U., Polak, J., Sulej, J., Jarosz-Wilkołazka, A., et al. (2020). Laccase properties, physiological functions, and evolution. *Int. J. Mol. Sci.* 21, 1–25. doi: 10.3390/ijms21030966

Jeon, S. J., and Park, J. H. (2020). Refolding, characterization, and dye decolorization ability of a highly thermostable laccase from *Geobacillus* sp. JS12. *Protein Expr. Purif.* 173:105646. doi: 10.1016/j.pep.2020.105646

Jesionowski, T., Zdarta, J., and Krajewska, B. (2014). Enzyme immobilization by adsorption: a review. *Adsorption* 20, 801–821. doi: 10.1007/s10450-014-9623-y

Joshi, A. U., Hinsu, A. T., Kotadiya, R. J., Rank, J. K., Andharia, K. N., and Kothari, R. K. (2020). Decolorization and biodegradation of textile di-azo dye Acid Blue 113 by *Pseudomonas stutzeri* AK6. *3 Biotech* 10, 1–16. doi: 10.1007/s13205-020-02205-5

Kagalkar, A. N., Khandare, R. V., and Govindwar, S. P. (2015). Textile dye degradation potential of plant laccase significantly enhances upon augmentation with redox mediators. *RSC Adv.* 5, 80505–80517. doi: 10.1039/C5RA12454A

Kashefi, S., Borghei, S. M., and Mahmoodi, N. M. (2019a). Covalently immobilized laccase onto graphene oxide nanosheets: preparation, characterization, and biodegradation of azo dyes in colored wastewater. *J. Mol. Liqu.* 276, 153–162. doi: 10.1016/j.molliq.2018.11.156

Kashefi, S., Borghei, S. M., and Mahmoodi, N. M. (2019b). Superparamagnetic enzyme-graphene oxide magnetic nanocomposite as an environmentally friendly biocatalyst: synthesis and biodegradation of dye using response surface methodology. *Microchem. J.* 145, 547–558. doi: 10.1016/j.microc.2018.11.023

Katheresan, V., Kansedo, J., and Lau, S. Y. (2018). Efficiency of various recent wastewater dye removal methods: a review. *J. Environ. Chem. Eng.* 6, 4676–4697. doi: 10.1016/j.jece.2018.06.060

Legerska, B., Chmelova, D., and Ondrejovic, M. (2016). Degradation of synthetic dyes by laccases – A mini-review. *Nova Biotechnol. Chim.* 15, 90–106. doi: 10.1515/nbec-2016-0010

Li, Z., Shang, W., Liu, W., Li, H., and Tian, Y. M. (2014). Application of immobilized laccase in the removal of oil from wastewater. *Desalinat. Water Treatm.* 52, 2594–2600. doi: 10.1080/19443994.2013.797142

Liang, C. Z., Sun, S. P., Li, F. Y., Ong, Y. K., and Chung, T. S. (2014). Treatment of highly concentrated wastewater containing multiple synthetic dyes by a combined process of coagulation/flocculation and nanofiltration. *J. Memb. Sci.* 469, 306–315. doi: 10.1016/j.memsci.2014.06.057

Lin, J., Lai, Q., Liu, Y., Chen, S., Le, X., and Zhou, X. (2017). Laccase – methacrylyol functionalized magnetic particles: highly immobilized, reusable, and efficacious for methyl red decolourization. *Int. J. Biol. Macromol.* 102, 144–152. doi: 10.1016/j.ijbiomac.2017.03.169

Lu, L., Zhao, M., Li, G. F., Li, J., Wang, T. N., Li, D.-B., et al. (2012a). Decolorization of synthetic dyes by immobilized spore from *Bacillus amyloliquefaciens*. *Catal. Commun.* 26, 58–62. doi: 10.1016/j.catcom.2012.04.024

Lu, L., Zhao, M., Wang, T. N., Zhao, L. Y., Du, M. H., and Li, T. L. (2012b). Characterization and dye decolorization ability of an alkaline resistant and organic solvents tolerant laccase from Bacillus licheniformis LS04. *Bioresour. Technol.* 115, 35–40. doi: 10.1016/j.biortech.2011.07.111

Luo, M., Li, M., Jiang, S., Shao, H., Razal, J., Wang, D., et al. (2020). Supported growth of inorganic-organic nanoflowers on 3D hierarchically porous nanofibrous membrane for enhanced enzymatic water treatment. *J. Hazard. Mater.* 381:Article120947. doi: 10.1016/j.jhazmat.2019.120947

Ma, H. F., Meng, G., Cui, B. K., Si, J., and Dai, Y. C. (2018). Chitosan crosslinked with genipin as supporting matrix for biodegradation of synthetic dyes: laccase immobilization and characterization. *Chem. Eng. Res. Des.* 132, 664–676. doi: 10.1016/j.cherd.2018.02.008

Mahmoodi, N. M., and Saffar-Dastgerdi, M. H. (2020). Clean laccase immobilized nanobiocatalysts (graphene oxide - zeolite nanocomposites): from production to detailed biocatalytic degradation of organic pollutant. *Appl. Catal. B Environ.* 268:Article118443. doi: 10.1016/j.apcatb.2019.118443

Messerschmidt, A., and Huber, R. (1990). The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin modelling and structural relationships. *Eur. J. Biochem.* 187, 341–352. doi: 10.1111/j.1432-1033.1990.tb15311.x

Nair, R. R., Demarche, P., and Agathos, S. N. (2013). Formulation and characterization of an immobilized laccase biocatalyst and its application to eliminate organic micropollutants in wastewater. *New Biotechnol.* 30, 814–823. doi: 10.1016/j.nbt.2012.12.004

Patel, S. K. S., Otari, S. V., Li, J., Kim, D. R., Kim, S. C., Cho, B. K., et al. (2018). Synthesis of cross-linked protein-metal hybrid nanoflowers and its application in repeated batch decolorization of synthetic dyes. *J. Hazard. Mater.* 347, 442–450. doi: 10.1016/j.jhazmat.2018.01.003

Polak, J., and Jarosz-Wilkolazka, A. (2012). Fungal laccases as green catalysts for dye synthesis. *Process Biochem.* 47, 1295–1307. doi: 10.1016/j.procbio.2012.05.006

Rao, M. A., Scelza, R., Acevedo, F., Diez, M. C., and Gianfreda, L. (2014). Enzymes as useful tools for environmental purposes. *Chemosphere* 107, 145–162. doi: 10.1016/j.chemosphere.2013.12.059

Rasheed, T., Bilal, M., Nabeel, F., Adeel, M., and Iqbal, H. M. N. (2019). Environmentally-related contaminants of high concern: potential sources and analytical modalities for detection, quantification, and treatment. *Environ. Int.* 122, 52–66. doi: 10.1016/j.envint.2018.11.038

Rodríguez-Couto, S., Osma, J. F., and Toca-Herrera, J. L. (2009). Removal of synthetic dyes by an eco-friendly strategy. *Eng. Life Sci.* 9, 116–123. doi: 10.1002/elsc.200800088

Salami, F., Habibi, Z., Yousefi, M., and Mohammadi, M. (2018). Covalent immobilization of laccase by one pot three component reaction and its

application in the decolorization of textile dyes. *Int. J. Biol. Macromol.* 120, 144–151. doi: 10.1016/j.ijbiomac.2018.08.077

Shaheen, R., Asgher, M., Hussain, F., and Bhatti, H. N. (2017). Immobilized lignin peroxidase from Ganoderma lucidum IBL-05 with improved dye decolorization and cytotoxicity reduction properties. *Int. J. Biol. Macromol.* 103, 57–64. doi: 10.1016/j.ijbiomac.2017.04.040

Shakerian, F., Zhao, J., and Li, S. P. (2020). Recent development in the application of immobilized oxidative enzymes for bioremediation of hazardous micropollutants – A review. *Chemosphere* 239, 1–12. doi: 10.1016/j.chemosphere.2019.124716

Sharma, B., Dangi, A. K., and Shukla, P. (2018). Contemporary enzyme based technologies for bioremediation: a review. *J. Environ. Manag.* 210, 10–22. doi: 10.1016/j.jenvman.2017.12.075

Sheldon, R. A., and van Pelt, S. (2013). Enzyme immobilisation in biocatalysis: why, what and how. *Chem. Soc. Rev.* 42, 6223–6235. doi: 10.1039/c3cs60075k

Shrivastava, S., Lata, S., and Shukla, P. (2012). An insight on recent advances on immobilization methods for industrial enzymes and its relevance to xylanases. *Dyn. Biochem. Process Biotechnol. Mol. Biol.* 6, 57–61.

Singh, D., and Gupta, N. (2020). *Microbial Laccase: A Robust Enzyme and Its Industrial Applications*. Berlin: Springer, doi: 10.2478/s11756-019-00414-9

Sirisha, V. L., Jain, A., and Jain, A. (2016). Enzyme immobilization: an overview on methods, support material, and applications of immobilized enzymes. *Adv. Food Nutr. Res.* 79, 179–211. doi: 10.1016/bs.afnr.2016.07.004

Skoronski, E., Souza, D. H., Ely, C., Broilo, F., Fernandes, M., Fúrigo, A., et al. (2017). Immobilization of laccase from *Aspergillus oryzae* on graphene nanosheets. *Int. J. Biol. Macromol.* 99, 121–127. doi: 10.1016/j.ijbiomac.2017.02.076

Solís, M., Solís, A., Pérez, H. I., Manjarrez, N., and Flores, M. (2012). Microbial decolouration of azo dyes: a review. *Process Biochem.* 47, 1723–1748. doi: 10.1016/j.procbio.2012.08.014

Solomon, E. I., Baldwin, M. J., and Lowery, M. D. (1992). Electronic structures of active sites in copper proteins: contributions to reactivity. *Chem. Rev.* 92, 521–542. doi: 10.1021/cr00012a003

Sun, H., Yang, H., Huang, W., and Zhang, S. (2015). Immobilization of laccase in a sponge-like hydrogel for enhanced durability in enzymatic degradation of dye pollutants. *J. Colloid Interface Sci.* 450, 353–360. doi: 10.1016/j.jcis.2015.03.037

Tuncay, D., and Yagar, H. (2020). Decolorization of Reactive Blue-19 textile dye by Boletus edulis laccase immobilized onto rice husks. *Int. J. Environ. Sci. Technol.* 17, 3177–3188. doi: 10.1007/s13762-020-02641-z

Ulu, A., Birhanli, E., Boran, F., Köytepe, S., Yesilada, O., and Ateş, B. (2020). Laccase-conjugated thiolated chitosan-Fe3O4 hybrid composite for biocatalytic degradation of organic dyes. *Int. J. Biol. Macromol.* 150, 871–884. doi: 10.1016/j.ijbiomac.2020.02.006

Wang, Q., Wang, T., Lv, Z., Cui, M., Zhao, Z., Cao, X., et al. (2020). TiO2 sol-gel coated PAN/O-MMT Multi-functional composite nanofibrous membrane used as the support for laccase immobilization: synergistic effect between the membrane support and enzyme for dye degradation. *Polymers* 12, 1–13. doi: 10.3390/polym12010139

Wen, X., Du, C., Wan, J., Zeng, G., Huang, D., Yin, L., et al. (2019). Immobilizing laccase on kaolinite and its application in treatment of malachite green effluent with the coexistence of Cd (⊓). *Chemosphere* 217, 843–850. doi: 10.1016/j.chemosphere.2018.11.073

Wikee, H., Hatton, J., Turbé-Doan, A., Mathieu, Y., Daou, M., Lomascolo, A., et al. (2019). Characterization and dye decolorization potential of two laccases from the marine-derived fungus *Pestalotiopsis* sp. *Int. J. Mol. Sci.* 20, 1–20. doi: 10.3390/ijms20081864

Yagub, M. T., Sen, T. K., Ang, H. M., and Afroze, S. (2014). Dye and its removal from aqueous solution by adsorption: a review | Elsevier Enhanced Reader. *Adv. Colloid Interface Sci.* 209, 172–184. doi: 10.1016/j.cis.2014.04.002

Yang, J., Xu, X., Yang, X., Ye, X., and Lin, J. (2016). Cross-linked enzyme aggregates of Cerrena laccase: preparation, enhanced NaCl tolerance and decolorization of remazol brilliant blue reactive. *J. Taiwan Inst. Chem. Eng.* 65, 1–7. doi: 10.1016/j.jtice.2016.04.025

Yang, J., Yang, X., Lin, Y., Ng, T. B., Lin, J., and Ye, X. (2015). Laccase-catalyzed decolorization of malachite green: performance optimization and degradation mechanism. *PLoS One* 10:e0127714. doi: 10.1371/journal.pone.0127714

Yang, X., Zheng, J., Lu, Y., and Jia, R. (2016). Degradation and detoxification of the triphenylmethane dye malachite green catalyzed by crude manganese peroxidase from Irpex lacteus F17. *Environ. Sci. Pollut. Res.* 23, 9585–9597. doi: 10.1007/s11356-016-6164-9

Yeap, K. L., Teng, T. T., Poh, B. T., Morad, N., and Lee, K. E. (2014). Preparation and characterization of coagulation/flocculation behavior of a novel inorganic-organic hybrid polymer for reactive and disperse dyes removal. *Chem. Eng. J.* 243, 305–314. doi: 10.1016/j.cej.2014.01.004

Zhang, W., Yang, Q., Luo, Q., Shi, L., and Meng, S. (2020). Laccase-Carbon nanotube nanocomposites for enhancing dyes removal. *J. Clean. Product.* 242:118425. doi: 10.1016/j.jclepro.2019.118425

Zheng, F., Cui, B. K., Wu, X. J., Meng, G., Liu, H. X., and Si, J. (2016). Immobilization of laccase onto chitosan beads to enhance its capability to degrade synthetic dyes. *Int. Biodeter. Biodegr.* 110, 69–78. doi: 10.1016/j.ibiod.2016.03.004

Zhou, Y., Lu, J., Zhou, Y., and Liu, Y. (2019). Recent advances for dyes removal using novel adsorbents: a review. *Environ. Pollut.* 252, 352–365. doi: 10.1016/j.envpol.2019.05.072

Zhu, Z., Chen, Z., Luo, X., Liang, W., Li, S., He, J., et al. (2020). Biomimetic dynamic membrane (BDM): fabrication method and roles of carriers and laccase. *Chemosphere* 240, 1–7. doi: 10.1016/j.chemosphere.2019.124882

Zille, A., Górnacka, B., Rehorek, A., and Cavaco-Paulo, A. (2005). Degradation of azo dyes by *Trametes villosa* laccase over long periods of oxidative conditions. *Appl. Environ. Microbiol.* 71, 6711–6718. doi: 10.1128/AEM.71.11.6711-6718.2005

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 21 December 2020
doi: 10.3389/fmicb.2020.594011

Bio-Decolorization of Synthetic Dyes by a Halophilic Bacterium *Salinivibrio* sp.

Jojy John[1,2], Ramadoss Dineshram[1], Kaveripakam Raman Hemalatha[3], Magesh Peter Dhassiah[4], Dharani Gopal[4] and Amit Kumar[2]**

[1]Biological Oceanography Division, CSIR-National Institute of Oceanography, Dona Paula, India, [2]International Research Centre for Climate Change Studies, Sathyabama Institute of Science and Technology, Chennai, India, [3]Department of Microbiology, Annamalai University, Chidambaram, India, [4]Department of Marine Biotechnology, National Institute of Ocean Technology, Chennai, India

Synthetic dyes, extensively used in various industries, act as pollutants in the aquatic environment, and pose a significant threat to living beings. In the present study, we assessed the potential of a halophilic bacterium *Salinivibrio kushneri* HTSP isolated from a saltpan for decolorization and bioremediation of synthetic dyes. The genomic assessment of this strain revealed the presence of genes encoding the enzymes involved in decolorization mechanisms including FMN-dependent NADH azoreductase Clade III, which cleave the azo bond of the dye, and the enzymes involved in deamination and isomerization of intermediate compounds. The dye decolorization assay was performed using this bacterial strain on three water-soluble dyes in different concentrations: Coomassie brilliant blue (CBB) G-250 (500–3,000 mg/L), Safranin, and Congo red (50–800 mg/L). Within 48 h, more than 80% of decolorization was observed in all tested concentrations of CBB G-250 and Congo red dyes. The rate of decolorization was the highest for Congo red followed by CBB G-250 and then Safranin. Using UV-Visible spectrometer and Fourier Transform Infrared (FTIR) analysis, peaks were observed in the colored and decolorized solutions. The results indicated a breakdown of dyes upon decolorization, as some peaks were shifted and lost for different vibrations of aromatic rings, aliphatic groups (–CH_2, –CH_3) and functional groups (–NH, –SO_3H, and –SO_3^-) in decolorized solutions. This study has shown the potential of *S. kushneri* HTSP to decolorize dyes in higher concentrations at a faster pace than previously reported bacterial strains. Thus, we propose that our isolated strain can be utilized as a potential dye decolorizer and biodegradative for wastewater treatment.

Keywords: phylogenetic analysis, bioremediation, azoreductase, dye decolorization, halophilic bacteria

OPEN ACCESS

Edited by:
Datta Madamwar, Sardar Patel University, India

Reviewed by:
Mayur B. Kurade, Hanyang University, South Korea
Liang Tan, Liaoning Normal University, China

***Correspondence:**
Ramadoss Dineshram
dinesh@nio.org;
dinbiot@gmail.com
Amit Kumar
amit.kumar.szn@gmail.com

Specialty section:
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

Received: *12 August 2020*
Accepted: *30 November 2020*
Published: *21 December 2020*

Citation:
John J, Dineshram R, Hemalatha KR, Dhassiah MP, Gopal D and Kumar A (2020) Bio-Decolorization of Synthetic Dyes by a Halophilic Bacterium Salinivibrio sp.. Front. Microbiol. 11:594011. doi: 10.3389/fmicb.2020.594011

INTRODUCTION

Dyes are organic coloring agents which are extensively used for coloring the products in various industries including textile, paper, cosmetic, and food (Vaghela et al., 2005). These coloring agents are complex aromatic structures which remain stable by resisting the impacts of temperature and other environmental factors (Pandey et al., 2007). Overall, more than

thousands of dyes are generated commercially and 7×10^5 metric tons of dyes are produced annually (Amoozegar et al., 2011). Generally, based on their origin, the dyes are classified into natural and synthetic dyes. Natural dyes are obtained directly from plants, microbes, insects, and animals (Shahid et al., 2013). Although natural dyes are eco-friendly and biodegradable, they are unstable (Yamjala et al., 2016). On the other hand, synthetic dyes produced in controlled laboratory conditions show greater stability than natural dyes. Globally, the textile industry utilizes the highest proportion of dyes (Fang et al., 2004), which are often toxic to living beings and difficult to degrade (Fu and Viraraghavan, 2001). Upon coloring the fabrics, 10–15% of used dyes, fibers, and other components generally get discharged into the aquatic ecosystem (Chen et al., 2003). The dyes in the aquatic environment pose a major threat to the inhabitants (Sandhya et al., 2008). The presence of dyes inhibits photosynthetic activity and oxygen solubility at deeper layers of the water body by reducing the light penetration (Saratale et al., 2009). The dyes form a source of aromatic amines upon degradation, which, in turn, are considered as mutagenic, toxic, and carcinogenic, posing threats to living beings (Robinson et al., 2001; Ayed et al., 2011). Considering these detrimental effects of dye on the environment, safe disposal and successful decolorization of dyes becomes the utmost priority.

For dye decolorization and degradation, several methods such as coagulation/adsorption, electrolysis, ozonation, chemical oxidation, and ultrafiltration have generally been used (Zhang et al., 2004; Zhu et al., 2004). However, these methods have limited applicability due to their inefficiency, higher budget, and toxic intermediates (Verma et al., 2003; Zhang et al., 2004). The municipal sewage systems are not adequate to decolorize the water discharge efficiently due to the complex nature of pollutants and residuals from the by-products (Champagne and Ramsay, 2010). Hence, the biological degradation of dyes involving microbes is considered as one of the best methods (O'neill et al., 2000). This has been proven as a cost-effective and eco-friendly method that generates a significantly lesser amount of intermediate toxic compounds (Robinson et al., 2001; Chen et al., 2003). Biological methods include dye degradation by metabolic pathways, absorption, and accumulation by bacteria, fungi, yeast, and algae (Solís et al., 2012). Several studies have reported that bacterial strains possessing genes for azoreductase, laccase, and peroxidase, which act on the amine and aromatic structure of dye, make them suitable candidates for dye degradation (Chen et al., 2003; Babu et al., 2015). Several bacterial strains including Gram-negative and Gram-positive bacteria had been reported to show the dye decolorization ability, for example, *Bacillus subtilus* and *Aeromonas hydrophila* (Wuhrmann et al., 1980); *Proteus mirabilis* and *Pseudomonas* sp. (Saratale et al., 2011); *Shewanella* sp.; and other bacterial consortia (Moosvi et al., 2005).

Major challenges in bacterial dye degradation in the wastewaters include high salt concentration, various metals, and complex nature of wastes. Hence, most of the bacteria, though have shown a promise in the early experiments, fail to work efficiently at a larger scale (Mellado and Ventosa, 2003). In recent years, halophilic bacterial strains have shown promise in dye decolorization (Amoozegar et al., 2011). With the potential to degrade dye, they can grow in a wide range of salinity, temperature, pH, and elevated heavy metal concentrations (Margesin and Schinner, 2001; John et al., 2020).

In the present study, we have assessed the dye degradation potential of a halophilic bacterial strain *Salinivibrio kushneri* HTSP isolated from Marakkanam saltpan, Tamil Nadu, India. This saltpan is characterized by a large seasonal fluctuation of salinity, temperature, dissolved oxygen, and elevated levels of several heavy metals (John et al., 2019, 2020). Previous studies on this halophilic bacterium, *S. kushneri* HTSP (Proteobacteria, Gamma-proteobacteria, Vibrionales, and Vibrionaceae), revealed its ability to grow in a wide salinity range of 15–210 ppt and pH range of 5–10, and it has tolerance to heavy metals including Cu, Zn, Co, Hg, Cr, Pb, and As (John et al., 2019). The analysis of whole-genome sequencing showed the presence of genes conferring resistance/tolerance to heavy metals and UV radiation, as well as those responsible for hydrocarbon degradation, by producing various enzymes, thus indicating the bioremediation potential of this bacterium in various industries and ecosystems (John et al., 2019). Though this species shows potential in dye decolorization, no studies have been attempted to prove this claim. Hence, to address this knowledge gap, we studied bio-decolorization of three dyes belonging to different classes using the *S. kushneri* HTSP strain: (i) Coomassie brilliant blue (CBB) G-250, a triphenylmethane dye commonly used in the textile industry and molecular biology laboratories (Rayaroth et al., 2015; Abbas et al., 2016); (ii) Safranin O, a quinone imine dye commonly used as a biological stain in the laboratories (Drabik et al., 2010; Sabnis, 2010); and (iii) Congo red, an azo dye, a known carcinogen, widely used in the paper, textile and other industries (Babu et al., 2013).

Industrialization is increasing at a faster pace and causing the rise of environmental pollution by producing millions of different chemicals including hydrocarbons, herbicides, pesticides toxic metals, and different dyes as either direct products or by-products (Amoozegar et al., 2011). Synthetic dyes and toxic structures of the dye-containing effluents pose a serious threat to aquatic life forms by causing toxicity to them (Georgiou et al., 2004; Supaka et al., 2004). Biological dye decolorization has been advocated as one of the most suitable methods to treat waste materials before discharge (Stolz, 2001). In this regard, we assessed the suitability of halophilic bacteria *S. kushneri* HTSP isolated from Marakkanam saltpan for decolorization of synthetic dyes. We analyzed the rate of bacterial decolorization of dyes using UV-Vis spectrophotometer and Fourier Transform Infrared (FTIR) spectrometer. We also performed *in silico* analysis to identify genes involved in dye decolorization in the previously reported *S. kushneri* HTSP genome.

MATERIALS AND METHODS

Dyes and Chemicals

Three dyes were used for dye decolorization experiments: Safranin, Congo red, and CBB G-250. All the dyes used in

this study were purchased from Sigma-Aldrich (United States). Different concentrations were used for decolorization experiments and the desired concentrations were selected based on preliminary screening from 50 to 5,000 mg/L. For conducting the experiments, 500–3,000 and 50–800 mg/L was selected for CBB G-250, Congo red, and Safranin, respectively. All the dye solutions were prepared in filter-sterilized (0.2 µm) dye decolorization broth for avoiding nutrient loss. The working solutions were prepared using sterilized decolorization media for compensating the nutrient loss.

Microorganisms and Inoculum Preparation

Salinivibrio kushneri HTSP isolated from the Marakkanam salt pan (12.13°02° N; 79.58°12° E) was previously identified through a polyphasic taxonomic approach including morphological, biochemical, and molecular analysis (John et al., 2019). For the present study, the culture was revived from a stock kept in 80% glycerol at −80°C. These were transferred on to a fresh nutrient agar plate prepared in the source seawater (120 ppt). The culture conditions including optimum salinity, temperature, pH, and days of incubation were standardized. The bacterial strain was inoculated under aerobic conditions onto nutrient broth as well as agar with different ranges of salinity, from 15 to 60 ppt, and incubated at 27–37°C for 12 h to 2 days. The required salinity was obtained by mixing the source seawater (200 ppt) with sterilized Milli Q water.

One loopful of overnight-grown culture was inoculated into the decolorization broth (5-g glucose, 2.5-g yeast extract, and 2.5-g NaCl in a final volume of 500 ml) and incubated in a shaker incubator (aerobic) at 37°C for 6 h. After 6 h (log phase of bacterial growth), the cells were harvested by a quick centrifuge at 3,000 rpm for 5 min. The bacterial cells were resuspended in 0.8% NaCl, and the optical density at 600 nm was adjusted to ~0.6–0.8 (6×10^8 cells/ml). The cell suspensions were used for further analysis.

Decolorization Experiment

Two hundred microliters of the bacterial cell suspension were inoculated onto different concentrations of different dyes (for 20 ml) and the tubes were incubated at 37°C for 48 h against a negative control, which contained only the dye in respective concentrations without the inoculum. The solution was withdrawn at an interval of 4 h for CBB G-250 and 6 h for Congo red and Safranin. The absorbance of the decolorized media solution (blank) was measured at 580 nm (CBB G-250), 490 nm (Congo red), and 530 nm (Safranin), respectively, using UV-Vis spectrophotometer against a blank of decolorization media without dye. The solution was centrifuged at 12,000 rpm for 1 min before taking the absorbance for removing any suspended precipitates. All the experiments were performed in triplicates. The trial experiment was also conducted with a higher volume (250 ml) and the same result was obtained. For the qualitative and quantitative analysis, the experiment was performed in 20 ml volume.

Decolorization percentage was calculated using the formula:

$$\text{Decolorization}\,(\%) = \left[\frac{\text{Initial absorbance} - \text{final absorbance}}{\text{Initial absorbance}}\right] * 100$$

Biodegradation Assay

The dye degradation was further confirmed through spectral analysis of UV-Vis spectroscopy and FTIR spectroscopy.

UV-Visible Spectroscopy

For the UV-Vis spectral analysis of dye decolorization, the decolorized solution was scanned (200–1,000 nm) against a dye control and the peaks were cross-matched using a spectrophotometer (Shimadzu, UV-1800). The highest concentration of dye which was completely decolorized was chosen for this analysis against a dye control. The peaks obtained before and after decolorization were analyzed (Ali et al., 2009; Ayed et al., 2011).

Fourier Transform Infrared Spectroscopy

The functional groups of the degraded dyes were analyzed with FTIR spectroscopy. The biodegraded dye was collected after the experiment and the bacterial suspension was removed by centrifugation at 5,000 rpm for 5 min. The resulting supernatant solution was lyophilized and used for FTIR analysis. The highest concentration at which the dyes were completely decolorized was used as a test sample for FTIR analysis and the dyes in powder form were used as control. The mid-IR spectra of degraded dyes were obtained in the FTIR spectrophotometer by Shimadzu, IR Infinity 1. The degraded dye sample was prepared by mixing 1 weight % of dye with 99 weight % of KBr and the mixture was ground well to make a paste of uniform consistency. The sample matrix was loaded in a 7-mm Pellet Die of 7-mm Disc Holder Mount and pressed in 2 T Mini-Pellet Press (Specac Ltd.). The sample mixture was then analyzed using the instrument by mounting the sample on the sample holder using the ring holder and the transmittance scanned from the range of 4,000–400 cm^{-1} with a resolution of 1 cm^{-1} set for 20 scans per min (Babu et al., 2013). The obtained peaks were compared with the published reference dye peaks (Sigma-Aldrich, n.d.; Sun et al., 2013; Maity et al., 2015; Sahu and Patel, 2015; Asses et al., 2018).

In silico Analysis of *Salinivibrio kushneri* HTSP Dye-Decolorizing

The genome of *S. kushneri* HTSP was retrieved from the NCBI genome database (John et al., 2019, NCBI genome accession no. PXUD00000000) for analyzing dye-decolorizing genes. The genome was subjected to automatic annotation on the Rapid Annotations using Subsystems Technology webserver (Overbeek et al., 2014). The annotated genome was visually searched to identify the target genes, and a BLAST search was performed using the annotated genome against well-studied genes.

Azoreductase Clade III, a well-known decolorizing enzyme, was identified in the genome and compared with other bacterial species through phylogenetic analysis. The phylogenetic tree was constructed based on an amino acid sequence from the best BLASTp hits along with previously reported sequences

(Kumaran et al., 2020). Pairwise and multiple sequence alignment was performed using the CLUSTAL W program and phylogeny was constructed using the Neighbor-Joining (NJ) method in MEGA (v 7.0, Kumar et al., 2016). Multispecies nitroreductase family protein of *Bacilli* (NCBI GenBank accession: WP_002358386) was used as an outgroup in the phylogenetic tree.

Statistical Analysis

All the data from dye decolorization assays were tested for statistical significance by comparing the mean of different test conditions using One-way ANOVA with the Tukey Scheffe alpha multiple comparison test. The data were checked for the normality by visual inspection and homogeneity of variance by Levene's test. The data were considered significant if $p < 0.05$. The statistical analysis was performed using SPSS [V. 22, IBM SPSS Statistics for Windows, Armonk, NY (SPSS, 2013)].

RESULTS AND DISCUSSION

In silico Analysis for Dye Decolorization Genes

A total of 12 genes involved in the dye decolorization pathway were identified. These include genes encoding FMN-dependent NADH azoreductase Clade III, hydroxymuconate delta isomerase, maleylacetoacetate isomerase, cytidine deaminase, cytosine deaminase, ornithine cyclodeaminase, deoxycytidine triphosphate deaminase, tRNA-specific adenosine-34 deaminase, diamino hydroxyl phosphoribosyl amino pyrimidine deaminase, glucosamine-6-phosphate deaminase, adenosine deaminase, and porphobilinogen deaminase.

To ascertain the identity of the FMN-dependent NADPH azoreductase Clade III, the first enzyme in the decolorizing pathway (Dave et al., 2015), the amino acid sequence of the gene was compared with previously reported sequences through BLAST and NJ-based phylogeny. The phylogenetic reconstruction revealed that azoreductase of *S. kushneri* HTSP forms a separate clade supported by high bootstrap confidence (**Figure 1**).

The genomic assessment of this strain revealed the presence of genes involved in various mechanisms of dye decolorization, for example, the FMN-dependent NADH azoreductase is involved in cleaving the azo bonds of the dye and converting the dye into aromatic amines (Dave et al., 2015). The resultant aromatic amines and other functional groups would be further cleaved or reduced by genes involved in deamination and isomerization processes. Based on the genes found in the genome, we have predicted a dye decolorization pathway (**Figure 2**). However further analysis like NMR; GC/LC-MS is needed for the confirmation of formed intermediates. AZO reductase, a primary enzyme in the decolorization process, was compared with other known sequences from different bacterial strains. Though the sequences from the genus *Salinivibrio* including *S. kushneri* HTSP formed a separate clade in the phylogenetic tree, it was comparatively closer to clade III azoreductase than other classes (I, II, and IV) of azoreductases (Suzuki, 2019; Kumaran et al., 2020). Class III azoreductase is a flavin-dependent enzyme with a significant difference in substrate specificities (Bafana and Chakrabarti, 2008; Misal and Gawai, 2018). In general, azo, nitro compounds, and quinone are a better substrate for class III azoreductase (Liu et al., 2008; Suzuki, 2019) such as Congo red (Yang et al., 2011; Gao et al., 2015), dyes with methyl groups (Eslami et al., 2016).

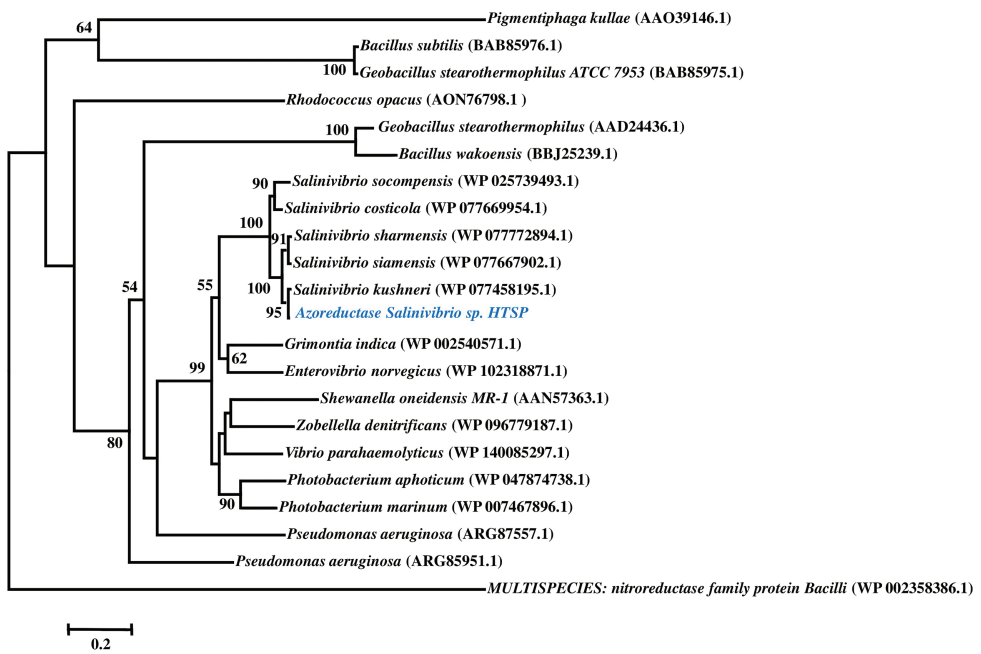


FIGURE 1 | Phylogenetic tree of azoreductase based on amino acid sequences. Amino acid sequences for nitroreductase is used as an outgroup.

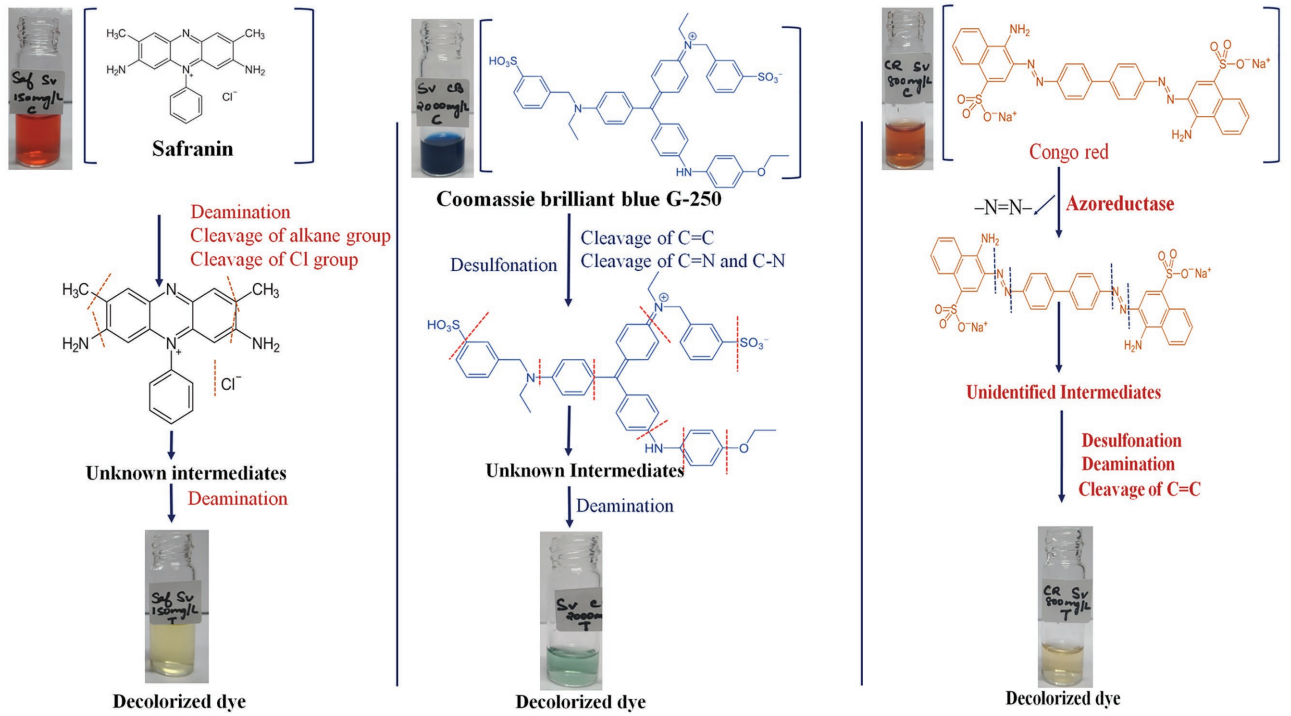


FIGURE 2 | Predicted mechanism of dye decolorization by *Salinivibrio kushneri* HTSP based on Fourier Transform Infrared (FTIR) peaks and functions of the genes identified. Functional group and bond cleavages are identified through the FTIR peaks.

Hence, it can be assumed that *S. kushneri* HTSP uses azoreductase to decolorize Congo red, Safranin (a dye with methyl group), and CBB G-250, indicating its potential for dye decolorization. The ability of different bacterial strains to decolorize different classes of dyes through the action of azoreductase has been reported earlier (Eichlerová et al., 2006; Amoozegar et al., 2011). For example, *Halomonas elongate*, a halophilic bacterium, is reported to decolorize mono and di azo dyes including methyl red, remazol black, and sulphonyl blue TLE (Eslami et al., 2016; Cao et al., 2017). Further, to ascertain our claim based on genomic analysis that the isolated strain can achieve dye decolorization, we performed decolorization assays on three dyes Congo red, Safranin, and CBB G-250 and analyzed using analytical methods of UV-Vis spectrophotometer and FTIR spectrometer, which is considered a gold standard for decolorization studies (Chen et al., 2008).

Dye Decolorization by *Salinivibrio kushneri* HTSP

The bacterial strain showed a luxuriant growth on a 60 ppt solid plate after overnight incubation at room temperature (~29°C) with a pH of 7.4, and in the broth, it took 6 h to reach an optical density of 0.8–1 at 600 nm. Hence, decolorization assays were performed in 60 ppt salinity conditions. Single-factor analysis on ANOVA after complete decolorization of each dye at same time point showed the rate of decolorization between the different concentrations of dyes was statistically significant ($p < 0.05$). *Salinivibrio kushneri* HTSP significantly decolorized CBB G-250 dye for all concentrations within 48 h (**Figure 3A**). The percentage of decolorization was slow in the initial period of incubation and by 48 h almost 90% of the dye at all tested concentrations were completely decolorized. Within 48 h, 96 ± 0.00, 88 ± 0.00, 85 ± 0.00, 82 ± 0.02, and 73 ± 0.00% dye was decolorized in 500, 800, 1,000, 2,000, and 3,000 mg/L dye, respectively (**Figure 3A**).

For Congo red dye decolorization, a significant color reduction was observed within 36 h of incubation. At 24 h, 92 ± 0.00% dye was decolorized in 50 ppm and for 150, 300, 600, and 800 ppm the decolorization percentage was found to be 88 ± 0.00, 78 ± 0.01, 80 ± 0.02, and 69 ± 0.00%, respectively. After 36 h, more than 90% of dye was decolorized in all concentrations (**Figure 3B**).

Salinivibrio kushneri HTSP decolorized Safranin completely in the lower concentration of 50 and 150 mg/L within 48 h. However, with increasing dye concentration, the rate of decolorization decreased. A low level of decolorization, such as 79 ± 0.02% for 300 mg/L, 25 ± 0.00% for 600 mg/L, and 22 ± 0.00 for 800 mg/L was obtained after 48 h (**Figure 3C**).

Characterization of Samples After Degradation

Shifts in the peaks were observed under UV-Vis spectroscopy between the test decolorized samples and the control. For CBB G-250, only one peak was observed in the control (at 585 nm) and three peaks were observed in the test decolorized sample. For Congo red, three different peaks were observed in the

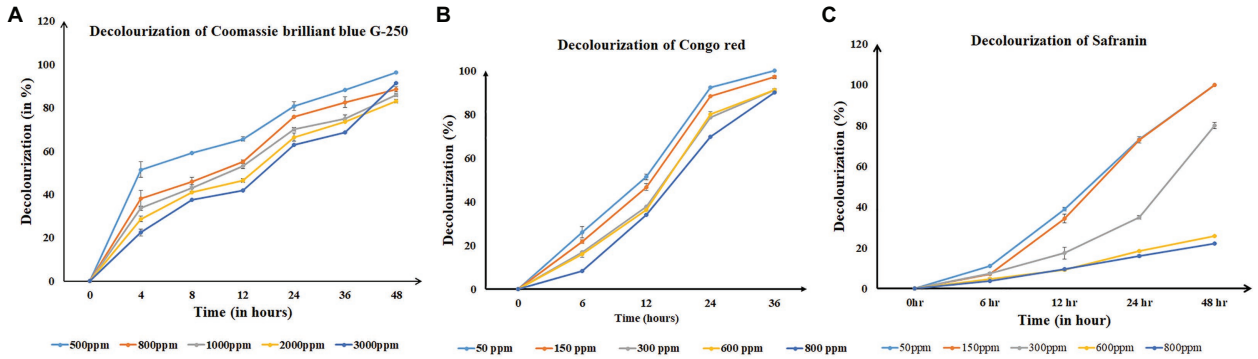


FIGURE 3 | Time-series graph denoting the decolorizing percentage of all three dyes at regular time intervals for *S. kushneri* HTSP, where, **(A)** is Coomassie brilliant blue G-250 (CBB), **(B)** is Congo red, and **(C)** is Safranin.

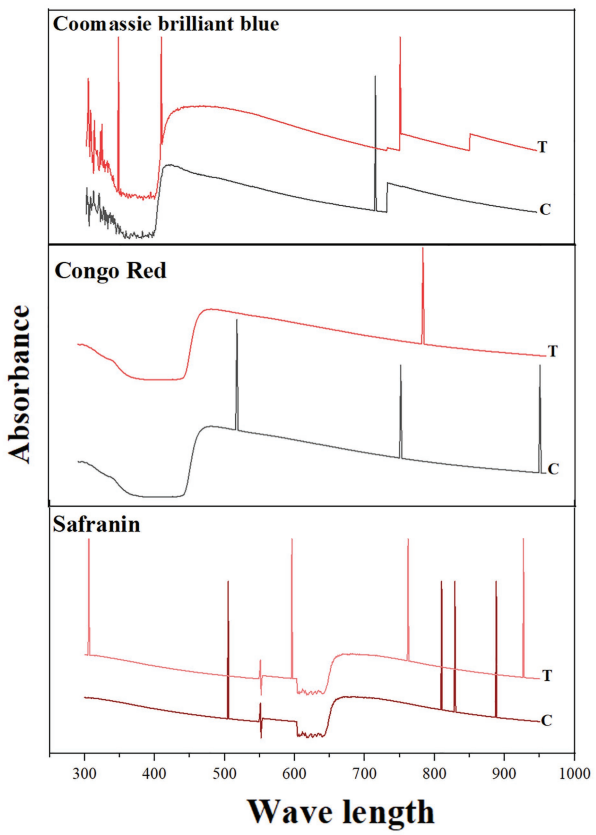


FIGURE 4 | Spectrophotometric analysis of dye decolorization by *S. kushneri* HTSP. Here, Control indicates dye before decolorization and test indicates dye after decolorization. CBB for CBB G-250.

control, while only one peak after decolorization. For Safranin, four peaks were observed in both the control and the decolorized sample. However, the absorption wavelengths were different before and after decolorization: Peaks were observed at 888, 829, 810, and 505 nm before decolorization and 927, 732, 596, and 306 nm after decolorization (**Figure 4**).

Further, various peaks were obtained in FTIR analysis of all three dyes corresponding to different vibrations of aromatic rings, aliphatic groups (–CH_2, –CH_3), and functional groups (–NH, –SO_3H, and –SO_3^-). There were considerable variations observed in the functional groups before and after dye decolorization. For Congo red, 12 absorption peaks were observed before decolorization; however, no relevant peak was observed after decolorization (**Figure 5A**). For CBB G-250, 33 peaks were observed in the colored solution, while the number of peaks reduced to 30 after decolorization. The peaks spanned in the range of 422–3,200 nm wavelength with a majority between 1,000 and 2,000 nm. However, peaks were not the same between colorized and decolorized solution, except for one at 454 nm (**Figure 5B**). For Safranin, 25 peaks in color solution and 24 peaks in decolorized solutions were observed. However, colored solution peaks were in the range of 3,400–4,000 nm, and decolorized solution peaks in the range of 600–3,600 nm (**Figure 5C**). We used smoothened data for plotting the graph.

The functional group analysis using FTIR and peak analysis using UV-Vis spectroscopy before and after 36 h of decolorization of CBB G-250 (2,000 mg/L), Safranin (150 mg/L), and Congo red (800 mg/L) supported our predicted mechanism shown in **Figure 2**. In general, the peaks which are responsible for several different bonds and functional groups cleavage were identified and matched. The major peaks corresponded with cleavage of N=N bonds, cleavage of aromatic rings, OH releasing, conversion of SO^{3-} to –OH groups, cleavage of C=C and C=H bonding, and cleavage of alkane group. On the contrary, after decolorization, the majority of the peaks were identified as hydroxy groups (–OH; Maity et al., 2015).

There were no significant peaks were observed after decolorization for Congo red. For Congo red dye (**Figure 5A**), observed peaks were identified as N–H stretching (3,468 cm^{-1}), C–O stretching (1,226–1,179 cm^{-1}), S=O stretching (1,059 cm^{-1}), and several unknown peaks found in the control sample were absent in the test conditions (Babu et al., 2013; Sun et al., 2013; Asses et al., 2018). For CBB G-250, the peaks in the range of 1,397–1,585 nm that corresponded with aromatic rings observed in the colored solution were mostly absent in the

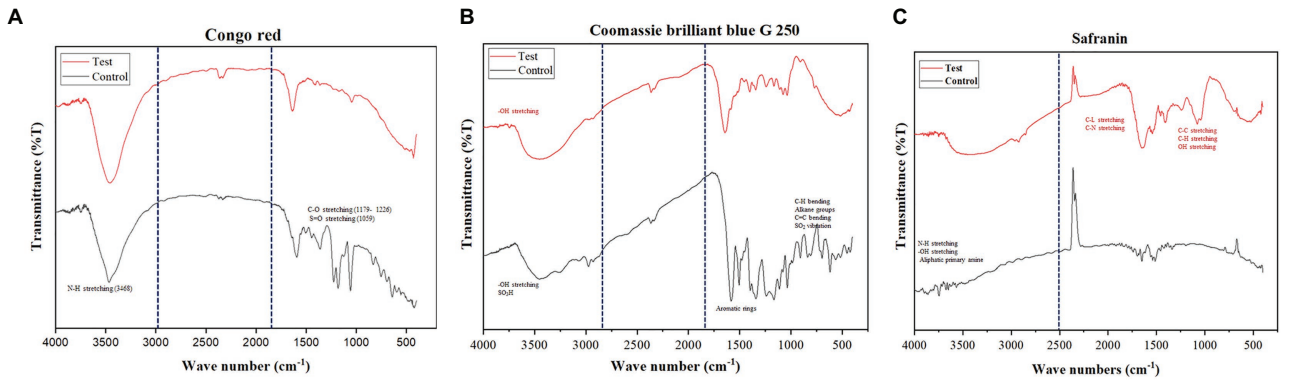


FIGURE 5 | Functional group analysis of all dyes before and after decolorization. Control indicates dye before decolorization and test indicates dye after decolorization. The important functional groups, aromatic rings, and aliphatic groups are highlighted in the control and in the test. Where, **(A)** is Congo red, **(B)** is CBB G 250, and **(C)** is Safranin.

decolorized solution. Sulfur dioxide vibration (1,000–1,200 cm^{-1}), C=C (985–618 cm^{-1}) bending, CH bending (985–618 cm^{-1}), and alkane groups (2,291–2,974 cm^{-1}) were observed in the colored solution but not in the decolorized solution, as shown in **Figure 5B** (Maity et al., 2015; Paz et al., 2017). For Safranin, the absorbance peaks observed were mainly aliphatic primary amine N–H stretching (3,000–3,560 cm^{-1}) and –OH stretching in the range of 3,589–4,000 nm in the control sample. However, in the decolorized test sample, the absorbance peaks varied: C–L stretching at 625 nm, C–N stretching 1,042–1,626 nm, C–C stretching 1,626 nm, C–H stretching 2,853–3,243 nm, and –OH stretching 3,283–3,564 nm (Babu et al., 2015; Sahu and Patel, 2015). The shift in the peaks suggests a breakdown of functional groups that may have changed the original structure of the dyes in the decolorized test samples (Chen et al., 2008; Ali et al., 2009).

Our results clearly indicate that *S. kushneri* HTSP could decolorize up to 3,000 mg/L CBB G-250; 300 mg/L for Safranin; and 800 mg/L for Congo red, which is significantly a higher concentration than previously reported. For example, Paz et al. (2017) showed that *Bacillus aryabhattai* could decolorize CBB G-250 up to 150 mg/L; Babu et al. (2013) showed that *Dietzia* sp. decolorized Congo red up to 100 mg/L. In addition, the time for decolorization was also found to be significantly less for *S. kushneri* HTSP than previously reported. Our results have shown that *S. kushneri* HTSP could decolorize a higher concentration of dyes at even a faster rate than previously reported halophilic bacteria such as *Shewanella putrefacians* and *Halomonas* sp. (Amoozegar et al., 2011; Gao et al., 2015). Previous studies on other bacteria also have reported a higher average time to decolorize the dyes used in this study even in comparatively lower concentrations (Babu et al., 2013; Paz et al., 2017).

CONCLUSION

Earlier studies have shown that *S. kushneri* HTSP has proven capacity to grow in a wide range of salinity, as well as the ability to tolerate/resist heavy metals and UV radiation (John et al., 2019), and now we report that this strain has capability to decolorize dyes in the higher concentration at a relatively faster rate. Thus, we argue that it can be potentially utilized for dye decolorization in wastewater treatment. However, further studies including cytotoxicity assays are to be conducted before using this strain at the industrial scales.

DATA AVAILABILITY STATEMENT

The data used in current study can be found in NCBI repository and all the accession numbers are provided in Materials and Methods section.

AUTHOR CONTRIBUTIONS

JJ and RD designed the research. JJ performed the decolourization experiments with KH. RD performed FTIR analysis with the help of MD and DG. JJ, RD, and AK analyzed the data and drafted the final version of the manuscript to be published. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We are grateful to the Director, CSIR-NIO, for providing the facilities necessary to carry out this study under OLP1707 and GAP2128. The computer-related studies were performed using the research computing facilities under GAP 0423: Biotechnology Information Systems project offered by the Department of Biotechnology, Govt. of India. JJ and AK would also like to thank Sathyabama Institute of Science and Technology for providing lab space and internal funding to carry out strain isolation and maintenance. The CSIR-NIO contribution number is 10295.

REFERENCES

Abbas, M., Cherfi, A., Kaddour, S., and Aksil, T. (2016). Adsorption in simple batch experiments of Coomassie blue G-250 by apricot stone activated carbon—kinetics and isotherms modelling. *Desalin. Water Treat.* 57, 15037–15048. doi: 10.1080/19443994.2015.1067871

Ali, H., Ahmad, W., and Haq, T. (2009). Decolorization and degradation of malachite green by *Aspergillus flavus* and *Alternaria solani*. *Afr. J. Biotechnol.* 8, 1574–1576.

Amoozegar, M. A., Hajighasemi, M., Hamedi, J., Asad, S., and Ventosa, A. (2011). Azo dye decolorization by halophilic and halotolerant microorganisms. *Ann. Microbiol.* 61, 217–230. doi: 10.1007/s13213-010-0144-y

Asses, N., Ayed, L., Hkiri, N., and Hamdi, M. (2018). Congo red decolorization and detoxification by *Aspergillus niger*: removal mechanisms and dye degradation pathway. *Biomed. Res. Int.* 2018:3049686. doi: 10.1155/2018/3049686

Ayed, L., Mahdhi, A., Cheref, A., and Bakhrouf, A. (2011). Decolorization and degradation of azo dye Methyl Red by an isolated *Sphingomonas paucimobilis*: biotoxicity and metabolites characterization. *Desalination* 274, 272–277. doi: 10.1016/j.desal.2011.02.024

Babu, S. S., Mohandass, C., Raj, A. V., Rajasabapathy, R., and Dhale, M. A. (2013). Multiple approaches towards decolorization and reuse of a textile dye (VB-B) by a marine bacterium *Shewanella decolorationis*. *Water Air Soil Pollut.* 224:1500. doi: 10.1007/s11270-013-1500-x

Babu, S. S., Mohandass, C., Vijayaraj, A., and Dhale, M. A. (2015). Detoxification and color removal of Congo red by a novel *Dietzia* sp. (DTS26)–a microcosm approach. *Ecotoxicol. Environ. Saf.* 114, 52–60. doi: 10.1016/j.ecoenv.2015.01.002

Bafana, A., and Chakrabarti, T. (2008). Lateral gene transfer in phylogeny of azoreductase enzyme. *Comput. Biol. Chem.* 32, 191–197. doi: 10.1016/j.compbiolchem.2008.03.003

Cao, X., Di, M., and Wang, J. (2017). Expansion of the active site of the azoreductase from *Shewanella oneidensis* MR-1. *J. Mol. Graph. and Model.* 78, 213–220. doi: 10.1016/j.jmgm.2017.10.020

Champagne, P. -P., and Ramsay, J. (2010). Dye decolorization and detoxification by laccase immobilized on porous glass beads. *Bioresour. Technol.* 101, 2230–2235. doi: 10.1016/j.biortech.2009.11.066

Chen, B. -Y., Chen, W. -M., Wu, F. -L., Chen, P. -K., and Yen, C. -Y. (2008). Revealing azo-dye decolorization of indigenous *Aeromonas hydrophila* from fountain spring in Northeast Taiwan. *J. Chin. Inst. Chem. Eng.* 39, 495–501. doi: 10.1016/j.jcice.2008.05.004

Chen, K. -C., Wu, J. -Y., Huang, C. -C., Liang, Y. -M., and Hwang, S. -C. J. (2003). Decolorization of azo dye using PVA-immobilized microorganisms. *J. Biotechnol.* 101, 241–252. doi: 10.1016/s0168-1656(02)00362-0

Dave, S. R., Patel, T. L., and Tipre, D. R. (2015). "Bacterial degradation of azo dye containing wastes" in *Microbial degradation of synthetic dyes in wastewaters*. ed. S. Singh (Cham: Springer), 57–83.

Drabik, M., Touskova, J., Hanus, J., Kobayashi, H., and Biederman, H. (2010). Properties of composite films of titania nanofibers and Safranin O dye. *Synth. Met.* 160, 2564–2572. doi: 10.1016/j.synthmet.2010.10.006

Eichlerová, I., Homolka, L., Lisá, L., and Nerud, F. (2006). The influence of extracellular H2O2 production on decolorization ability in fungi. *J. Basic Microbiol.* 46, 449–455. doi: 10.1002/jobm.200610064

Eslami, M., Amoozegar, M. A., and Asad, S. (2016). Isolation, cloning and characterization of an azoreductase from the halophilic bacterium *Halomonas elongata*. *Int. J. Biol. Macromol.* 85, 111–116. doi: 10.1016/j.ijbiomac.2015.12.065

Fang, H., Wenrong, H., and Yuezhong, L. (2004). Investigation of isolation and immobilization of a microbial consortium for decoloring of azo dye 4BS. *Water Res.* 38, 3596–3604. doi: 10.1016/j.watres.2004.05.014

Fu, Y., and Viraraghavan, T. (2001). Fungal decolorization of dye wastewaters: a review. *Bioresour. Technol.* 79, 251–262. doi: 10.1016/s0960-8524(01)00028-1

Gao, F., Ding, H., Shao, L., Xu, X., and Zhao, Y. (2015). Molecular characterization of a novel thermal stable reductase capable of decoloration of both azo and triphenylmethane dyes. *Appl. Microbiol. Biotechnol.* 99, 255–267. doi: 10.1007/s00253-014-5896-z

Georgiou, D., Metallinou, C., Aivasidis, A., Voudrias, E., and Gimouhopoulos, K. (2004). Decolorization of azo-reactive dyes and cotton-textile wastewater using anaerobic digestion and acetate-consuming bacteria. *Biochem. Eng. J.* 19, 75–79. doi: 10.1016/j.bej.2003.11.003

John, J., Siva, V., Kumari, R., Arya, A., and Kumar, A. (2020). Unveiling cultivable and uncultivable halophilic bacteria inhabiting Marakkanam saltpan, India and their potential for biotechnological applications. *Geomicrobiology* 37, 691–701. doi: 10.1080/01490451.2020.1764676

John, J., Siva, V., Richa, K., Arya, A., and Kumar, A. (2019). Life in high salt concentrations with changing environmental conditions: insights from genomic and phenotypic analysis of *Salinivibrio* sp. *Microorganisms* 7:577. doi: 10.3390/microorganisms7110577

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Kumaran, S., Ngo, A. C. R., Schultes, F. P. J., and Tischler, D. (2020). Draft genome sequence of *Kocuria indica* DP-K7, a methyl red degrading actinobacterium. *3 Biotech* 10:175. doi: 10.1007/s13205-020-2136-3

Liu, G., Zhou, J., Jin, R., Zhou, M., Wang, J., Lu, H., et al. (2008). Enhancing survival of *Escherichia coli* by expression of azoreductase AZR possessing quinone reductase activity. *Appl. Microbiol. Biotechnol.* 80, 409–416. doi: 10.1007/s00253-008-1555-6

Maity, M., Dolui, S., and Maiti, N. C. (2015). Hydrogen bonding plays a significant role in the binding of Coomassie brilliant blue-R to hemoglobin: FT-IR, fluorescence and molecular dynamics studies. *Phys. Chem. Chem. Phys.* 17, 31216–31227. doi: 10.1039/c5cp04661k

Margesin, R., and Schinner, F. (2001). Potential of halotolerant and halophilic microorganisms for biotechnology. *Extremophiles* 5, 73–83. doi: 10.1007/s007920100184

Mellado, E., and Ventosa, A. (2003). "Biotechnological potential of moderately and extremely halophilic microorganisms" in *Microorganisms for Health Care, Food and Enzyme Production*. ed. J. L. Barredo (Kerala: Research Signpost), 233–256.

Misal, S. A., and Gawai, K. R. (2018). Azoreductase: a key player of xenobiotic metabolism. *Bioresour. Bioprocess.* 5:17. doi: 10.1186/s40643-018-0206-8

Moosvi, S., Keharia, H., and Madamwar, D. (2005). Decolourization of textile dye Reactive Violet 5 by a newly isolated bacterial consortium RVM 11.1. *World J. Microb. Biot.* 21, 667–672. doi: 10.1007/s11274-004-3612-3

O'neill, C., Lopez, A., Esteves, S., Hawkes, F., Hawkes, D., and Wilcox, S. (2000). Azo-dye degradation in an anaerobic-aerobic treatment system operating on simulated textile effluent. *Appl. Microbiol. Biotechnol.* 53, 249–254. doi: 10.1007/s002530050016

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., et al. (2014). The SEED and the rapid annotation of microbial genomes using subsystems technology (RAST). *Nucleic Acids Res.* 42, D206–D214. doi: 10.1093/nar/gkt1226

Pandey, A., Singh, P., and Iyengar, L. (2007). Bacterial decolorization and degradation of azo dyes. *Int. Biodeterior. Biodegradation* 59, 73–84. doi: 10.1016/j.ibiod.2006.08.006

Paz, A., Carballo, J., Pérez, M. J., and Domínguez, J. M. (2017). Biological treatment of model dyes and textile wastewaters. *Chemosphere* 181, 168–177. doi: 10.1016/j.chemosphere.2017.04.046

Rayaroth, M. P., Aravind, U. K., and Aravindakumar, C. T. (2015). Sonochemical degradation of Coomassie brilliant blue: effect of frequency, power density, pH and various additives. *Chemosphere* 119, 848–855. doi: 10.1016/j.chemosphere.2014.08.037

Robinson, T., Mcmullan, G., Marchant, R., and Nigam, P. (2001). Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. *Bioresour. Technol.* 77, 247–255. doi: 10.1016/s0960-8524(00)00080-8

Sabnis, R. W. (2010). *Handbook of biological dyes and stains: Synthesis and industrial applications*. Hoboken: John Wiley & Sons.

Sahu, M. K., and Patel, R. K. (2015). Removal of Safranin-O dye from aqueous solution using modified red mud: kinetics and equilibrium studies. *RSC Adv.* 5, 78491–78501. doi: 10.1039/C5RA15780C

Sandhya, S., Sarayu, K., Uma, B., and Swaminathan, K. (2008). Decolorizing kinetics of a recombinant *Escherichia coli* SS125 strain harboring azoreductase gene from *Bacillus latrosporus* RRK1. *Bioresour. Technol.* 99, 2187–2191. doi: 10.1016/j.biortech.2007.05.027

Saratale, R., Saratale, G., Chang, J. -S., and Govindwar, S. P. (2009). Decolorization and biodegradation of textile dye navy blue HER by *Trichosporon beigelii* NCIM-3326. *J. Hazard. Mater.* 166, 1421–1428. doi: 10.1016/j.jhazmat.2008.12.068

Saratale, R. G., Saratale, G. D., Chang, J. -S., and Govindwar, S. P. (2011). Bacterial decolorization and degradation of azo dyes: a review. *J. Taiwan. Inst. Chem. E.* 42, 138–157. doi: 10.1016/j.jtice.2010.06.006

Shahid, A., Singh, J., Bisht, S., Teotia, P., and Kumar, V. (2013). Biodegradation of textile dyes by fungi isolated from North Indian Field soil. *Environment Asia* 6, 51–57. doi: 10.14456/ea.2013.18

Sigma-Aldrich, I. (n.d.). "Spectrum Table & Chart".

Solís, M., Solís, A., Pérez, H. I., Manjarrez, N., and Flores, M. (2012). Microbial decolouration of azo dyes: a review. *Process Biochem.* 47, 1723–1748. doi: 10.1016/j.procbio.2012.08.014

SPSS (2013). *IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0*. Armonk, NY: IBM Corp.

Stolz, A. (2001). Basic and applied aspects in the microbial degradation of azo dyes. *Appl. Microbiol. Biotechnol.* 56, 69–80. doi: 10.1007/s002530100686

Sun, J., Li, Y., Hu, Y., Hou, B., Zhang, Y., and Li, S. (2013). Understanding the degradation of Congo red and bacterial diversity in an air-cathode microbial fuel cell being evaluated for simultaneous azo dye removal from wastewater and bioelectricity generation. *Appl. Microbiol. Biotechnol.* 97, 3711–3719. doi: 10.1007/s00253-012-4180-3

Supaka, N., Juntongjin, K., Damronglerd, S., Delia, M. -L., and Strehaiano, P. (2004). Microbial decolorization of reactive azo dyes in a sequential anaerobic–aerobic system. *Chem. Eng. J.* 99, 169–176. doi: 10.1016/j.cej.2003.09.010

Suzuki, H. (2019). Remarkable diversification of bacterial azoreductases: primary sequences, structures, substrates, physiological roles, and biotechnological applications. *Appl. Microbiol. Biotechnol.* 103, 3965–3978. doi: 10.1007/s00253-019-09775-2

Vaghela, S. S., Jethva, A. D., Mehta, B. B., Dave, S. P., Adimurthy, S., and Ramachandraiah, G. (2005). Laboratory studies of electrochemical treatment of industrial azo dye effluent. *Environ. Sci. Technol.* 39, 2848–2855. doi: 10.1021/es035370c

Verma, P., Baldrian, P., and Nerud, F. (2003). Decolorization of structurally different synthetic dyes using cobalt (II)/ascorbic acid/hydrogen peroxide system. *Chemosphere* 50, 975–979. doi: 10.1016/s0045-6535(02)00705-1

Wuhrmann, K., Mechsner, K., and Kappeler, T. (1980). Investigation on rate—determining factors in the microbial reduction of azo dyes. *Eur. J. Appl. Microbiol. Biotechnol.* 9, 325–338.

Yamjala, K., Nainar, M. S., and Ramisetti, N. R. (2016). Methods for the analysis of azo dyes employed in food industry–a review. *Food Chem.* 192, 813–824. doi: 10.1016/j.foodchem.2015.07.085

Yang, X., Wang, J., Zhao, X., Wang, Q., and Xue, R. (2011). Increasing manganese peroxidase production and biodecolorization of triphenylmethane dyes by novel fungal consortium. *Bioresour. Technol.* 102, 10535–10541. doi: 10.1016/j.biortech.2011.06.034

Zhang, F., Yediler, A., Liang, X., and Kettrup, A. (2004). Effects of dye additives on the ozonation process and oxidation by-products: a comparative study using hydrolyzed C.I. *Reactive Red 120*. *Dyes Pigments* 60, 1–7. doi: 10.1016/S0143-7208(03)00111-6

Zhu, J., Zheng, W., He, B., Zhang, J., and Anpo, M. (2004). Characterization of Fe–TiO_2 photocatalysts synthesized by hydrothermal method and their photocatalytic reactivity for photodegradation of XRG dye diluted in water. *J. Mol. Catal. A Chem.* 216, 35–43. doi: 10.1016/j.molcata.2004.01.008

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 18 January 2021
doi: 10.3389/fmicb.2020.620075

Laccase Immobilization Strategies for Application as a Cathode Catalyst in Microbial Fuel Cells for Azo Dye Decolourization

Priyadharshini Mani[1]*, *V. T. Fidal*[2], *Taj Keshavarz*[1], *T. S. Chandra*[2] and *Godfrey Kyazze*[1]*

[1] School of Life Sciences, University of Westminster, London, United Kingdom, [2] Department of Biotechnology, Indian Institute of Technology (Madras), Chennai, India

Enzymatic biocathodes have the potential to replace platinum as an expensive catalyst for the oxygen reduction reaction in microbial fuel cells (MFCs). However, enzymes are fragile and prone to loss of activity with time. This could be circumvented by using suitable immobilization techniques to maintain the activity and increase longevity of the enzyme. In the present study, laccase from *Trametes versicolor* was immobilized using three different approaches, i.e., crosslinking with electropolymerized polyaniline (PANI), entrapment in copper alginate beads (Cu-Alg), and encapsulation in Nafion micelles (Nafion), in the absence of redox mediators. These laccase systems were employed in cathode chambers of MFCs for decolourization of Acid orange 7 (AO7) dye. The biocatalyst in the anode chamber was *Shewanella oneidensis MR-1* in each case. The enzyme in the immobilized states was compared with freely suspended enzyme with respect to dye decolourization at the cathode, enzyme activity retention, power production, and reusability. PANI laccase showed the highest stability and activity, producing a power density of 38 ± 1.7 mW m^{-2} compared to 25.6 ± 2.1 mW m^{-2} for Nafion laccase, 14.7 ± 1.04 mW m^{-2} for Cu-Alg laccase, and 28 ± 0.98 mW m^{-2} for the freely suspended enzyme. There was 81% enzyme activity retained after 1 cycle (5 days) for PANI laccase compared to 69% for Nafion and 61.5% activity for Cu-alginate laccase and 23.8% activity retention for the freely suspended laccase compared to initial activity. The dye decolourization was highest for freely suspended enzyme with over 85% decolourization whereas for PANI it was 75.6%, Nafion 73%, and 81% Cu-alginate systems, respectively. All the immobilized laccase systems were reusable for two more cycles. The current study explores the potential of laccase immobilized biocathode for dye decolourization in a microbial fuel cell.

Keywords: laccase, immobilization, acid orange 7, power production, decolourization

OPEN ACCESS

Edited by:
Chirayu Desai, Charotar University of Science and Technology, India

Reviewed by:
Mayur B. Kurade, Hanyang University, South Korea
S. Venkata Mohan, Indian Institute of Chemical Technology (CSIR), India

***Correspondence:**
Priyadharshini Mani
priyadharshu@gmail.com
Godfrey Kyazze
G.Kyazze@westminster.ac.uk

Specialty section:
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

Received: *21 October 2020*
Accepted: *21 December 2020*
Published: *18 January 2021*

Citation:
Mani P, Fidal VT, Keshavarz T, Chandra TS and Kyazze G (2021) Laccase Immobilization Strategies for Application as a Cathode Catalyst in Microbial Fuel Cells for Azo Dye Decolourization. Front. Microbiol. 11:620075. doi: 10.3389/fmicb.2020.620075

INTRODUCTION

Microbial Fuel cells (MFCs) have been extensively explored for treatment of dye containing wastewater and concomitant energy production (Gomaa et al., 2017; Huang et al., 2017; Sonu et al., 2020). There are several studies reporting the use of enzymes and their fungal sources in MFCs for dye decolourization (Simões et al., 2019; Liu et al., 2020). Laccase is one such enzyme used in

the textile industry for bleaching and in fuel cells for catalyzing the oxygen reduction reaction. Laccase is a multi-copper containing oxidase enzyme which is capable of one electron oxidation of other substrates and four electron reduction of O_2 to H_2O (Galhaup and Haltrich, 2001). Laccase has been employed in the cathode chamber of MFC for dye decolourization and catalysis of the oxygen reduction reaction (Savizi et al., 2012; Mani et al., 2019).

The use of enzymatic cathodes is however, limited by the short lifetime and stability of the enzymes in MFC systems due to factors such as pH gradients, salinity increases etc (Mani et al., 2017). These limitations could be overcome by immobilizing the enzymes to improve stability. Enzymes in the immobilized form are stable and resistant to environmental factors and the enzymes can be reused. When laccase was electropolymerized (120 U mg^{-1} of chitosan) on an electrode with chitosan and methylene blue and utilized in a MFC for dye decolourization, the MFC produced a maximum power density of 58.8 mWm^{-2} and a decolourization efficiency of 74% for Reactive Blue 221 dye after 120 h (Savizi et al., 2012). The different types of enzyme immobilization and their advantages and disadvantages are listed in **Table 1**.

Immobilizing enzymes on electrodes was found to provide direct electron transfer and higher power output in enzymatic biofuel cells (Cooney et al., 2008). Conducting polymers like Poly Aniline (PANI) can form an adhesive polymer on electrodes and conduct electrons (Tiwari et al., 2007). Another method of immobilizing enzymes is entrapment of the enzyme in beads. Laccase is a copper-dependent enzyme and immobilizing in copper alginate beads could retain more activity compared to other methods. Teerapatsakul and group have observed that the immobilization yield and enzyme activity was higher when $CuSO_4$ was used as crosslinking solution compared to $CaCl_2$ (Teerapatsakul et al., 2008).

A third method of immobilization involves encapsulation of laccase in Nafion polymer micelles formed by modifying the polymer with an alkyl ammonium salt such as tetrabutylammonium bromide (TBAB) (Meredith et al., 2012). The quaternary ammonium cations modify the Nafion to less acidic form by replacing the protons and counteracting the sulfonate groups. They also increase the size of micelles and channels which should result in favorable enzyme immobilization.

Platinum is the most commonly used cathode catalyst for high performance fuel cells. In recent years, because of the high cost of platinum, there has been a transition to PGM (Platinum Group Metals) free catalysts for oxygen reduction reactions with metal compounds impregnated with Nitrogen doped Carbon (N-C) serving as a good replacement for Pt. Transition metals such as Mn, Fe, Co, and Ni in their salt forms have been infused with precursor aminoantipyrine (AAPyr) and used as cathode catalysts in MFCs (Kodali et al., 2017).

In this study, laccase in the three immobilized states (Cross-linking, entrapment in beads, and micellar encapsulation) was compared with freely suspended enzyme with respect to dye decolourization, enzyme activity retention, power production, and reusability in the cathode of a microbial fuel cell. This study aimed to emphasize the effect of immobilization on laccase ability to perform as efficient cathode catalysts. The performance of the laccase electrode in terms of power generation only was also evaluated against platinum and chemical based platinum alternatives (Fe-N/C catalyst).

MATERIALS AND METHODS

Chemicals

Laccase enzyme (13.6 U/mg) from *Trametes versicolor* was obtained from Sigma Aldrich. All chemicals were analytical grade and were purchased from Sigma. *Shewanella oneidensis MR1* strain 14063 was purchased from NCIMB (UK). The Fe-N/C catalyst was obtained from Dr. J. Masa from Ruhr-University Bochum in Germany.

Laccase Immobilization

Polyaniline Laccase

Polyaniline (PANI) immobilization was carried out by electropolymerization of 0.1 M Aniline in 1 M Sulphuric acid with carbon fiber (2.5 cm^2) as working electrode, titanium wire as counter electrode and Ag/AgCl as reference using Keysight B2900A potentiostat. A current density of 4.5 mA cm^{-2} for 50 sec was used for electropolymerization of aniline onto bare carbon electrodes. After electropolymerization, PANI was functionalised using 1.25% glutaraldehyde at 37°C for 15 min. This was followed by the addition of laccase enzyme 1 U ml^{-1} (60 Units) to the solution for cross linking for 15 min. Enzyme assay of the laccase solution was carried out before and after cross linking to get an estimate of amount of laccase immobilized (Mani, 2019).

Copper Alginate Beads

The Cu-Alginate immobilization procedure was adapted from Teerapatsakul et al. (2008). A 3% w/v sodium alginate was dissolved in 40 ml of water. A 1 U ml^{-1} (60 Units) laccase was added to the alginate solution. The above mixture was passed through a 21-gauge syringe into 0.15 M cross-linker copper sulfate solution. The beads were allowed to rest for 45 min after which they were washed with and incubated in acetate buffer. Enzyme assay was performed on the laccase immobilized Cu-Alg beads and the remaining cross-linking solution to determine the immobilization yield.

Nafion Micelles Preparation

The salt modified Nafion micelles were prepared according to the method developed by Meredith et al. (2012). Two ml of 5% w/v Nafion solution (Sigma) was added to 78.3 mg of TBAB (tetrabutylammonium bromide) and vortexed at 1,500 rpm for 10 min. The solution was poured in a weighing boat and the solvent was allowed to evaporate. After 18 h a yellow transparent film was formed at the bottom of a weighing tray. The tray was then filled with 18 MΩ deionised (DI) water and soaked for 24 h to remove the excess alkyl ammonium bromide salts and HBr. The water was removed, and the polymer rinsed with DI water and allowed to dry. The resulting dry film was suspended in 2 ml ethanol.

TABLE 1 | Types of enzyme immobilization, their advantages, and disadvantages.

Types of immobilization	Description	Advantage	Disadvantage
Adsorption	Enzymes are adhered to surface of carrier matrix through ionic, hydrophobic or van der Waals interaction (Jesionowski et al., 2014)	1. Relatively simple 2. Reduces conformational changes or denaturation of enzymes 3. Suitable for wide variety of carriers (Huang and Cheng, 2008)	1. Weak bonding (Cooney et al., 2008) 2. Exposed to microenvironment (pH, Temperature) 3. Depends on affinity between enzyme and carrier matrix
Covalent bonding (Cross-linking)	Enzyme is attached to the matrix by covalent bonds (Guisan, 2006).	1. Strong Bonding 2. No leakage 3. Higher stability (Romo-Sánchez et al., 2014)	1. Enzyme loading limited by matrix functional group density (Cooney et al., 2008) 2. Structural and conformational change 3. Diffusional limitation to the active site of the enzyme
Encapsulation	Enzyme is caged micelles of polymer having hydrophobic interior and hydrophilic exterior (Moehlenbrock and Minteer, 2017)	1. Retains native enzyme structure 2. Minimal enzyme requirement 3. No chemical modification	1. Not suitable for large substrates 2. Diffusional limitation (Cooney et al., 2008) 3. Microcapsules are sensitive to surrounding medium to like pH, ionic strength etc.
Entrapment	Enzyme is caged in a porous matrix by covalent or non-covalent bonds (Datta et al., 2013)	1. Retains native enzyme structure 2. Minimal enzyme requirement 3. No chemical modification	1. There polymer used in entrapment might be charged resulting in lower activity. 2. Difficult to control the pore size 3. Enzyme leaching 4. Diffusional barrier

Immobilization of Laccase in Nafion Micelles

Laccase was dissolved in 10 ml of acetate buffer (pH 4.5) to give a concentration of 1 U ml^{-1}(60 Units). 1 ml of the ethanol-polymer suspension was added to 2 ml of the enzyme solution and vortexed. The resultant mixture was poured onto an electrode and the solvent allowed to evaporate, thus forming a film on the electrode surface.

Platinum and Fe–N/C Electrode Preparation

The cathode contained a Pt catalyst layer with a Pt loading of 0.5 mg cm^{-2}. Pt powder for the cathode was mixed with carbon black powder (Sigma Aldrich, UK) for a 10% (w/w) mixture. This mixture was suspended in Nafion solution (Sigma Aldrich) and the suspension was applied as a uniform coating on the cathode electrodes using a paint brush. The same approach was used for Fe—N/C catalyst electrode preparation.

Operation of the Microbial Fuel Cell

The MFC used in the study was the 'H'-type reactor with a working volume of 200 ml in each chamber. The electrodes were constructed from carbon fiber (non-woven) with a surface area of 25 cm^2. Cation exchange membrane CMI7000 ion exchange membrane was soaked in 5% NaCl for 12 h prior to use.

Anode Chamber Composition

The composition in the anode chamber was the same for all the reactors. The anolyte consisted of minimal salts medium containing (per liter): 0.46 g NH_4Cl, 0.22 g $(NH)_2SO_4$, 0.117 g $MgSO_4$, 7.7 g $Na_2HPO_4.7H_2O$, 2.87 g NaH_2PO_4 along with 1% (v/v) trace minerals as described by Marsili et al. (2008) and 1% (v/v) vitamin mix as described by Wolin et al. (1963). The carbon source was pyruvate at a concentration of 1 g L^{-1} and casein hydrolysate was added at 500 mg L^{-1}. The pH of the anolyte was initially adjusted to seven. The anode and cathode were connected to a resistor of 2 kΩ. The anode was inoculated with 10% v/v *S. oneidensis* MR-1 culture previously grown in Luria Bertani broth to an OD of 0.6. The anode chamber was sparged for 10 min with nitrogen gas to remove any dissolved oxygen and maintain an anaerobic environment.

Cathode Chamber Composition

The cathode chamber consisted of commercial laccase (Sigma–Aldrich) from *Trametes versicolor* (13.6 U mg^{-1}) in 100 mM sodium acetate buffer solution (pH 4.5). Free laccase was added at 0.3 U/ml (60 Units/ 200 ml) in the cathode chamber. A total of 60 Units was maintained initially for all laccase immobilized systems.

Experimental Design

Four systems were setup with laccase in the cathode chamber and one without enzyme: System 1 with Polyaniline crosslinked to laccase electrode referred to as "PANI laccase" to reduce ohmic loss; System 2 with laccase entrapped in copper alginate beads referred to as "Cu-Alg Laccase" to reduce enzyme denaturation; System 3 with laccase freely suspended in the buffer referred to as "Free laccase;" System 4 with laccase immobilized in Nafion micelles is referred to as "Nafion Laccase" to maintain activity and System 5 referred to as "Control" which consisted of dye and buffer in the absence of laccase. The immobilized and free enzymes were suspended in 200 ml of 100 mM acetate buffer (pH 4.5) with 100 mg L^{-1} of Acid Orange 7 dye. The cathode chamber was maintained in aerobic conditions by supplying air through an air stone at a rate of 200 ml air per min. Experiments were conducted at a temperature of 30°C. For platinum comparison System 1 with platinum coated electrode is referred as "Platinum" and System 2 with Fe-N/C coated electrode is referred to as "Fe-N/C." The immobilized enzymes, platinum and Fe-N/C electrode

were suspended in 200 ml of 100 mM acetate buffer (pH 4.5) in the absence of the dye. The MFC systems were connected across 2000 Ω resistor. The experiments were carried out in batch mode with one cycle in this study representing 5 days.

Analytical Procedures

Structural and Functional Characterization of the Electrodes

The morphology of the immobilized laccase was studied using Inspect-F scanning electron microscopy (SEM) equipped with EDAX at an accelerating voltage of 30 kV. The presence of PANI and Nafion functional groups was detected using Perkin Elmer Spectrum Two FTIR-ATR Spectrometer with a plain carbon electrode as the background.

Electrochemical Analysis of Laccase Crosslinked With PANI and Laccase in Nafion Micelles

The cyclic voltammetry (CV) measurements for activity of laccase was performed in a three-electrode system with the working electrode as the PANI laccase/Nafion laccase electrode, platinum as the counter, and Ag/AgCl as reference electrode. Since the enzyme was immobilized on the electrode, ABTS assay did not serve as an accurate method for enzyme activity, as the enzyme leached into the solution also accounted for the laccase activity rather than the enzyme on the electrode alone. The CV was carried out in pH 4.5 acetate buffer (100 mM) using a potentiostat Keysight B2900A by cycling between potential of −1–1.5 V at a scan rate of 20 mV s^{-1}.

Acid Orange 7 Dye Decolourization

The concentration of AO7 in the cathode for laccase systems was measured at various time intervals using a UV-visible spectrophotometer at a wavelength of 484 nm which is the maximum absorption wavelength for the dye. The decolourization efficiency was calculated by

$$\mathrm{DE}(\%) = \frac{\mathbf{Ao} - \mathbf{A_t}}{\mathbf{Ao}} \times \mathbf{100}$$

A_o and A_t are the absorbance units at the initial and each time point, respectively. A time series is plotted for the absorbance values measured.

Electrochemical Analysis

The electric potential across the system was recorded every 10 min using a data acquisition system Picolog (Pico Technology, UK).

Power and current density were normalized to the surface area of the anode electrode. To carry out polarization tests, each MFC unit was connected to various external resistances ranging from 10 Ω to 1 MΩ and the potential measured using a multimeter. Internal (ohmic) resistance was calculated from the slope of the linear portion of the polarization (V-I) curve.

Coulombic Efficiency

The CE was calculated as follows (Logan et al., 2006):

$$\mathbf{CE}(\%) = \frac{\boldsymbol{M} \int_0^t \boldsymbol{I}\, \boldsymbol{dt}}{\boldsymbol{b} * \boldsymbol{F} * \boldsymbol{V_{anode}} * \Delta \boldsymbol{COD}}$$

where M is the molecular weight of oxygen (32), I is current over a time period (A), b number of electrons exchanged per mole of oxygen, F is Faraday constant (96485C mol^{-1}), V_{anode} is working volume of anode, and COD is change in COD over time (g L^{-1}).

Enzyme Activity

The activity of free laccase and coper alginate immobilized laccase was measured using ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] as a substrate. A solution of 2 ml acetate buffer (100 mM, pH 4.5), 0.1 ml ABTS (0.5 mM), and 0.1 ml of enzyme was used for freely suspended enzyme and 300 mg for Cu-alginate beads. The oxidation of ABTS by laccase was measured by a UV spectrophotometer at 420 nm (Bakhshian et al., 2011). The enzyme activity unit (U) was defined as the amount of enzyme required to oxidize 1.0 μmol ABTS min^{-1} at 25°C (Eggert et al., 1996).

Immobilization Yield

The immobilization yield of the enzyme was calculated by:

$$\textbf{Immobilization yield}\ (\%) = \frac{\textbf{Amount of Enzyme immobilized}}{\textbf{Total Enzyme used in immobilization}}\ \textbf{x 100}$$

The amount of enzyme immobilized was calculated by the ABTS enzyme assay.

Statistical Analysis

All experimental data indicated in the text and graphs are the means of triplicate experiments unless otherwise stated. The error bars in the graphs and ± values in the text represent the standard deviation of the mean (SD).

RESULTS AND DISCUSSION

Characterization of Immobilized Laccase Biocathode Systems

The immobilized laccase biocathodes were analyzed for their functional, morphological, and electrochemical characteristics using FTIR, SEM, and cyclic voltammetry, respectively.

Functional Analysis of Laccase Biocathodes

Functional analysis was performed for polymer-based laccase immobilized biocathodes viz. PANI-laccase and Nafion-laccase. As both the immobilizations were multi-step procedures it was necessary to understand the modifications in the polymer during each step and the robustness of the laccase on immobilization.

FTIR was carried out to confirm the presence of PANI functionalization on the electrode (**Figure 1A**). The 1314 cm^{-1} is typical of PANI (emeraldine base) attributed to C-N stretch vibration of the quinoid ring. The peak at 1175 cm^{-1} indicates the vibration mode of $–NH^+$ of the charged polymer. After the glutaraldehyde cross linking, this mode disappears due to crosslinking with laccase. This mode in PANI is responsible for the delocalized electron and hence the conductivity. The peak at 882 cm^{-1} corresponds to the N-H wag of the 1° and 2° amine which disappears on cross-linking with the enzyme (**Figure 1A**). Another peak characteristic of the PANI deposited

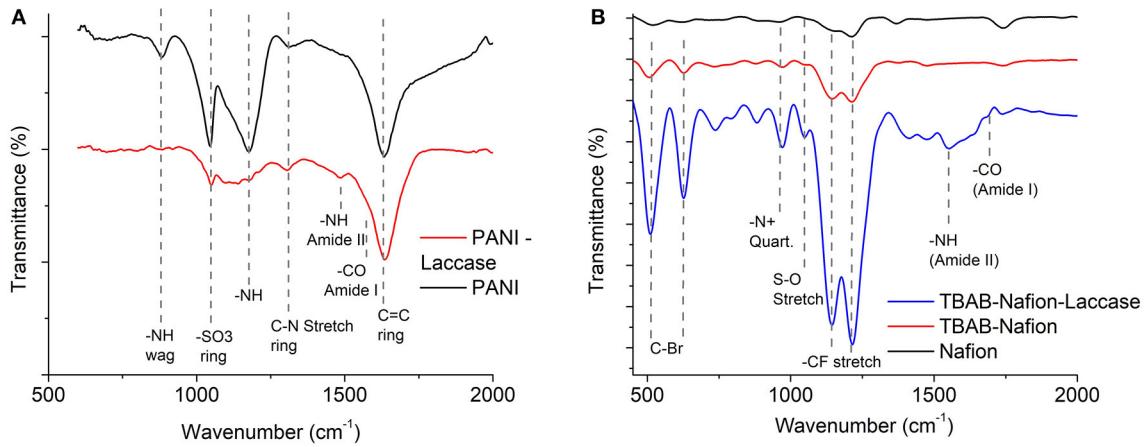


FIGURE 1 | FTIR spectra indicating the presence of functional groups of polyaniline, nafion, and immobilized laccase **(A)** PANI laccase **(B)** Nafion laccase.

in sulphuric acid is observed at 1047 cm^{-1} which is due to the sulphonation of the aniline ring due to the substitution of the SO_3^- in place of NH_3^+ (**Figure 1A**). The strong peak observed at 1627 cm^{-1} is due to the C=C in vibration within the ring (Stejskal and Gilbert, 2006).

The Nafion functional group showed a CF stretch at 1134 cm^{-1} and 1213 cm^{-1} which is characteristic of tetrafluoroethylene backbone (**Figure 1B**). The mild peak at 1048 cm^{-1} indicates the sulfonated terminal of the tetrafluoroethylene chain (Kunimatsu et al., 2010). On functionalization with TBAB, a peak appeared at 978 cm^{-1} which indicated the presence of 40 N+ embedded within the polymer (**Figure 1B**) (Hu et al., 2016). On laccase immobilization, the peak intensity increased, which might be due to the catalysis and higher vibrational modes achieved due to the charging of the polymer. The laccase was characterized by the peaks at 1559 and 1959 cm^{-1} which indicates the amine and carboxylic moiety of its amino acids (**Figure 1B**). For both PANI and Nafion immobilization, laccase was characterized by the presence of Amide I (1600–1690 cm^{-1}) and Amide II (1480–1575 cm^{-1}) for -CO and -NH stretch, respectively (Kong and Yu, 2007).

Morphological Analysis of the PANI/PANI-Laccase Biocathode

Morphological analysis was performed for PANI laccase, Cu-Alg laccase, and Nafion laccase biocathode systems. The main significance of this study was to understand the porosity of the electrode and structural changes in the polymer on immobilization of the laccase.

SEM images reveal the PANI fibers on the carbon electrode and the immobilized laccase (**Figure 2a**-Inset). PANI appears as a polymer sheath formed over the carbon fibers (**Figure 2a**-Inset). It is deposited primarily at the tight fibers of carbon due to higher charge density, with dimensions in the range of 30 × 50 μM. Crosslinking causes slight disruption of the membrane with the globular structure more prominent after laccase immobilization (**Figure 2a**).

TBAB modified Nafion was found to coalesce to form a film on the electrode surface (**Figure 2b**-Inset). Unlike PANI film, the Nafion membrane is evenly distributed over the carbon filaments. The porosity of the electrode was found to be decreased on the film formation, which might affect the charge density. A gelation of the Nafion polymer is seen on addition of TBAB. In the presence of laccase, the film appears to be a thick layer of membrane compared to bare Nafion/TBAB (**Figure 2b**). Unlike PANI-Laccase, Nafion-laccase film was seen to be restricted to the surface of the electrode. In addition, the aggregate size of the enzyme-polymer was larger in size as compared to the PANI-laccase.

Electrochemical Analysis of PANI and Nafion-Laccase Electrodes

The electrochemical analysis of the immobilized laccase biocathodes were limited to the PANI-laccase and Nafion-laccase biosystems because of the conductivity of the polymer used.

The redox property of the PANI-Laccase biocathode was analyzed by cyclic voltammetry. An oxidation peak at 0.2 V indicates the presence of polyaniline on the electrode surface (**Figure 3A**).

PANI did not display the multiple peaks usually observed in strong acids, as the electrolyte used in this study was a weak acetate buffer. Polyaniline is easily oxidized in less acidic solutions as pH increases. The electrochemical behavior of polyaniline is dependent on many parameters e.g., the choice of material and the surface area of the electrodes, composition of the electrolyte, pH and temperature etc. (Song and Choi, 2013). On cross-linking with laccase an additional peak was observed at −0.32 V which might indicate oxygen reduction reaction (Le Goff et al., 2015). With increasing scan rate the rise in cathodic peak current was proportional to the square root of scan rate, indicating an oxygen diffusion limited process (**Figure 3A**-Inset). Thus, the laccase catalytic activity was preserved on cross-linking with PANI (**Figure 3A**).

Nafion polymer alone did not show any characteristic peaks due to absence of the characteristic redox moiety. The increase

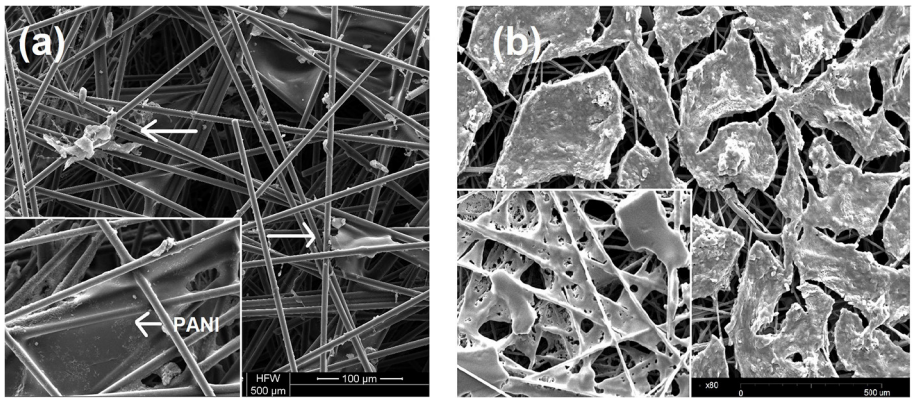


FIGURE 2 | SEM image of **(a)** PANI laccase. Arrows indicate laccase enzyme, Inset (left): PANI membrane **(b)** Nafion/TBAB/Laccase, Inset-Nafion/TBAB.

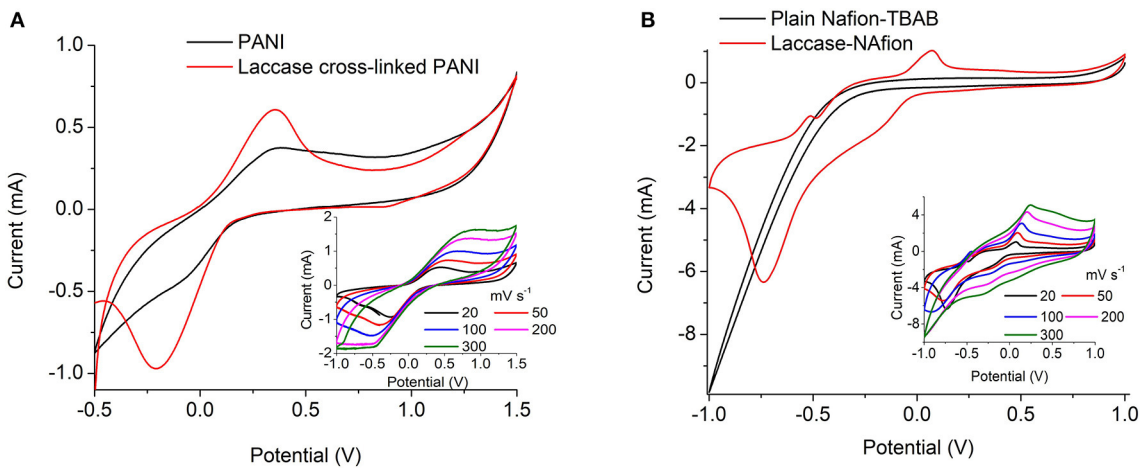


FIGURE 3 | Cyclic voltammetry at 20 mV s^{-1} of **(A)** PANI and PANI cross-linked with laccase **(B)** Nafion/TBAB and Nafion/TBAB/laccase system. Insets: Immobilized laccase at different scan rates.

in oxidation and reduction current in laccase modified electrode indicates the presence of immobilized enzyme on the surface of the electrode. Similar shifts in CV current was also observed by Luo et al. (2010) for Nafion/ABTS/laccase electrodes. In presence of laccase the ORR takes place on the surface and the reduction peak appears at −0.6 V limited by oxygen diffusion (**Figure 3B**-Inset). Nafion-laccase showed an overpotential as compared to the PANI laccase, which might be due to poor electron conductivity of the Nafion (**Figure 3B**).

Power Generation From MFCs Utilizing the Immobilized Laccase Electrodes

The maximum voltage of 480 ± 20 mV was recorded across 2 kΩ in the MFC with PANI laccase followed by freely suspended laccase (420 ± 14 mV), Nafion-laccase at 405 ± 30 mV, and Cu-Alginate laccase at 350 ± 25 mV. The higher voltage of PANI MFCs compared to those with freely suspended laccase was probably due to the decreased proximity between the catalytic sites and the electrode, thus decreasing the ohmic and mass transfer resistance. Moreover, PANI is a conducting polymer, it decreases the charge transfer resistance of the electrode thus permitting the easy electron transfer. Although Nafion is also a conducting polymer, it is known to be an ionic conductor rather than an electron conductor (Heitner-Wirguin, 1996). In this study, laccase embedded in the Nafion without any mediators was less efficient in transferring electrons from the electrode to the enzyme compared to PANI. The low voltage of Cu-Alg laccase system was probably due to the high diffusion barrier imposed by beads to both oxygen and electron transfer from the electrode. This agreed with the maximum power density which was 38 ± 1.7 mWm^{-2} for MFCs with PANI and 28 ± 0.98 mWm^{-2} for freely suspended laccase, 25.6 ± 2.08 mW m^{-2} for Nafion and 14.7 ± 1.04 mW m^{-2} for laccase entrapped in beads (**Figure 4**). A maximum power density of only 6.5 mW m^{-2} was observed by Schaetzle et al. (2009) when laccase was immobilized in hydrogels due to the reduced electron transfer of the enzyme hydrogels (Schaetzle et al., 2009). Thus, it is evident that the distance between the enzyme and the electrode is critical

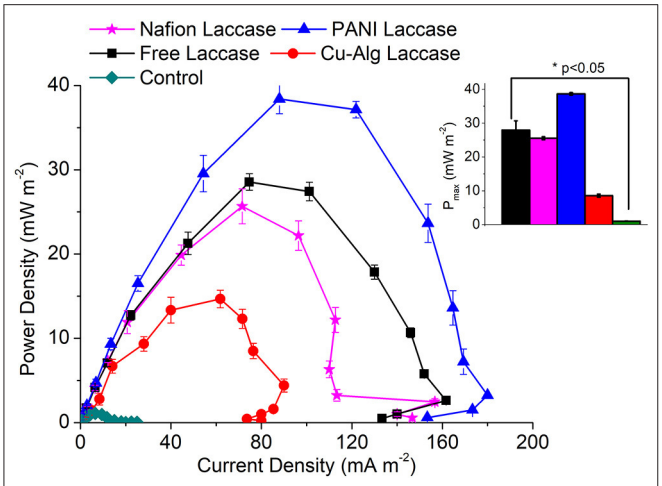


FIGURE 4 | Comparison of P_{max} (maximum power density) for the different Laccase based biocathode systems. Inset: One-way Anova of maximum power density at day 5 for each MFC systems.

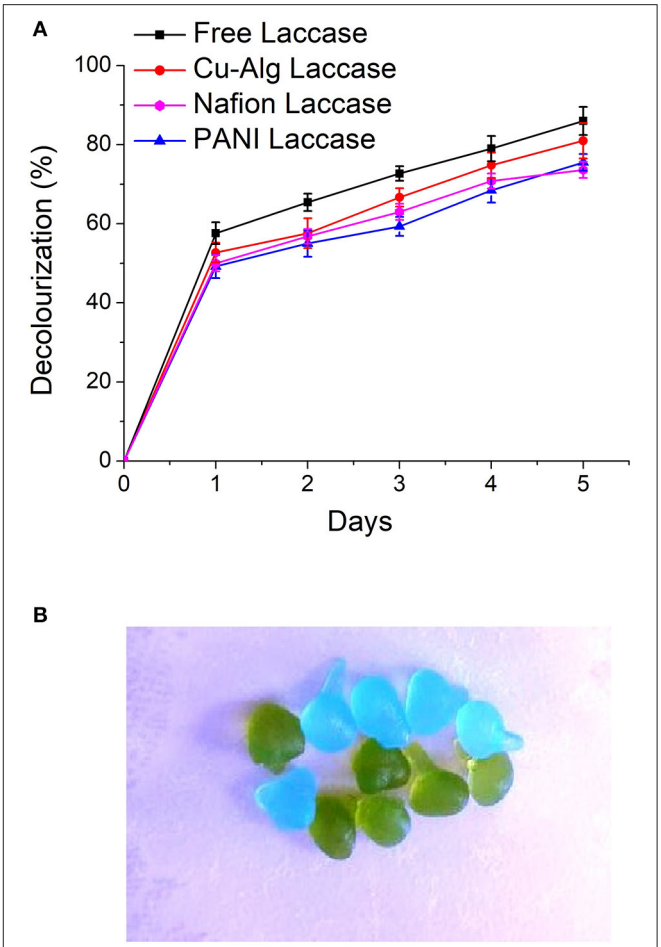


FIGURE 5 | (A) Comparison of decolourization rates of AO7 in the different MFC systems **(B)** Dye adsorption in copper alginate beads. Blue–Initial bead color, Green–On adsorption of dye.

in achieving good oxygen reduction and higher power output. PANI, Nafion and freely suspended enzyme have better contact with the electrode compared to the beads. The OCV for PANI laccase reached up to 900 ± 35 mV while for free laccase it was 700 ± 20 mV, 640 ± 48 mV for Nafion, and 500 ± 32 mV for laccase in Cu-alginate beads. The internal resistance for MFCs with PANI was 1.4 ± 0.15 kΩ which was the lowest compared to 2 ± 0.12 kΩ for free laccase and 7.5 ± 1 kΩ for the beads which was directly related to the above factors of ohmic and diffusion barrier. The internal resistance for nafion laccase was 5.3 ± 0.5 kΩ which might account for the low power output compared to PANI laccase. The coulombic efficiency was quite low for all systems with PANI-laccase highest at 4.65 ± 0.18%, Nafion with 4.23 ± 0.45%, Free laccase 3.83 ± 0 .112%, and the lowest was Cu-Alg with 2.97 ± 0.16%. In addition, PANI laccase electrodes were reusable for up to three cycles with the power and activity decreasing each cycle. Nafion and Cu-Alg laccase were reusable for two more cycles (Data in **Supplementary Tables 1**, **2**).

Dye Decolourization in MFCs Utilizing the Laccase Biocathodes

There was 85 ± 3.5% decolourization by MFC with enzyme in the freely suspended form compared to 75.6 ±2.1% for PANI laccase and 73 ± 2.04% Nafion laccase over a period of 5 days (**Figure 5A**). The decolourization in MFCs with Cu-Alg beads laccase was 81 ± 4.47%. There was >50% decolourization in the first 24 h for free and Cu-Alg laccase. Zille et al. (2003) inferred that on immobilization the protein becomes restricted to interact with the dye. Freely suspended laccase has the freedom of movement to interact with the dye and bring about better decolourization in this study. Same trend was obtained by Savizi et al., where freely suspended laccase enzyme decolourized 77% of RB 221 dye compared to 70% in immobilized laccase (Savizi et al., 2012). In addition, the amount of enzyme cross-linked on the PANI laccase was lower than the case of freely suspended enzyme due to the functional group density limitation of glutaraldehyde which also contributes to lower decolourization. Similarly, for Nafion-laccase limitation of the dye movement to the active site of the enzyme might have resulted in lower decolourization compared to free laccase and Cu-Alg laccase. The initial rapid decolourization is due to the high enzyme activity initially; the rate of decolourization decreases gradually as the enzyme activity decreases as shown in the enzyme activity graphs (**Figure 7**). The dye decolourization by laccase is probably due to asymmetric cleavage of the azo-bonds and subsequent ring cleavage to form simple aromatic compounds (Mani et al., 2019).

There was significant amount of dye adsorbed on the alginate beads which indicates that part of decolourization is due to adsorption (**Figure 5B**). Control beads without laccase showed 8 ± 0.5% decolourization of the dye.

Similar results were observed by Daâssi et al. (2014) where 34 and 24% of dyes Reactive Black and Lanset Gray, respectively was adsorbed on calcium alginate beads with laccase (Daâssi et al., 2014). The laccase beads were reusable for two more cycles with

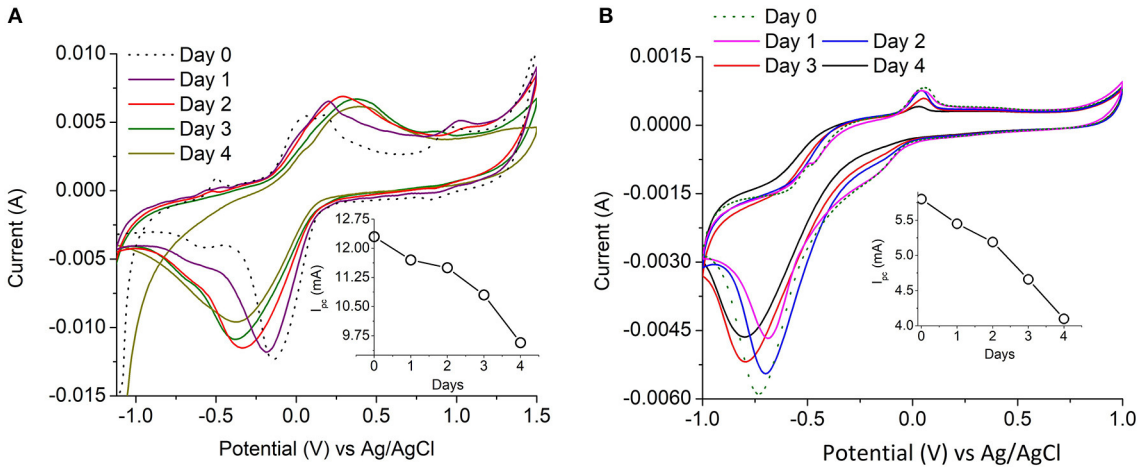


FIGURE 6 | Laccase electrochemical activity with time **(A)** PANI-Laccase **(B)** Nafion-Laccase. Inset: Cathodic peak current vs. time at 20 mV s^{-1}.

the decolourization decreasing gradually each cycle (69 and 57%). There was no further decolourization after 120 h in any systems. This decolourization pattern was also observed by (Russo et al., 2008) and they have concluded it might be due to the inhibition of laccase by the products of the dye.

Enzyme Activity of the Laccase Biocathodes in MFCs

The immobilization yield of the laccase immobilized systems was obtained by comparing the activity of laccase prior to and after the immobilization. The immobilization efficiency was highest in Cu-Alg laccase with 73 ± 8% yield followed by Nafion and PANI with 57 ± 2 and 38 ± 1%, respectively.

The enzyme activity was determined by ABTS assay for freely suspended laccase and for Cu-Alginate laccase. The relative decrease in activity for PANI-laccase and Nafion laccase on the electrodes were measured through cyclic voltammetry by comparing the cathodic peak current (Ipc) each day to the initial peak current (**Figure 6**). Cyclic Voltammetry of PANI laccase/Nafion-Laccase indicated a decrease in Ipc with the number of days. (**Figure 6**-Inset).

The relative percentage decrease in enzyme activity for each electrode compared to their initial activity is shown in **Figure 7**. PANI Laccase retained 81 ± 2.5% activity after one MFC cycle (5 days), while freely suspended enzyme retained only 23.8 ± 1.8% activity after the first MFC cycle (5 days). The rate of enzyme deactivation was highest for freely suspended laccase with about 15.2 ± 0.12% decrease in relative enzyme activity per day. The enzyme activity was also observed to be decreasing in Nafion-laccase with loss of activity at the rate of 6 ± 0.39% per day in Nafion laccase compared to PANI laccase with just 3.8 ± 0.6% per day. There was >69 ± 3.5% enzyme activity retained in Nafion-laccase after one cycle (5 days). The laccase activity was five times higher when immobilized with Nafion-TBAB compared to plain Nafion. Similar results were observed by Meredith et al. (2012) for certain enzymes immobilized with Nafion modified with TBAB. The laccase entrapped in Cu-alginate beads had an initial

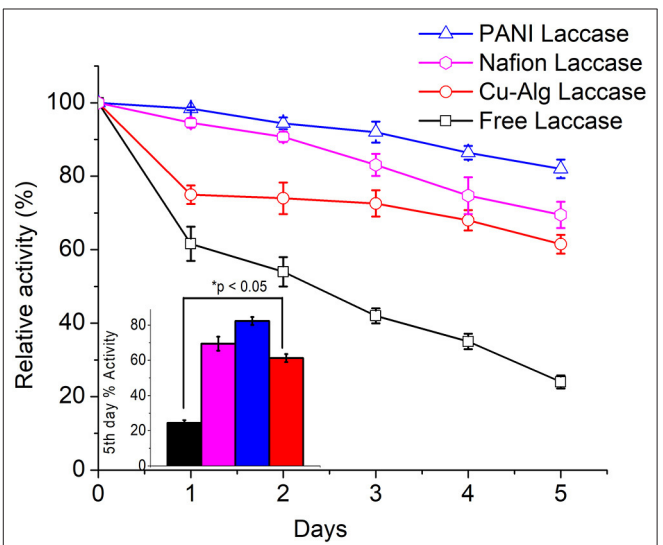


FIGURE 7 | Comparison of relative laccase activity over time for each immobilized laccase system. Inset: One-way Anova of percentage activity at day 5 for each system.

burst release of 25 ± 0.8% within the 24 h of immobilization in the catholyte of MFC; following this, per day 4 ± 0.6% for Cu-Alg Beads with retention of 61.5 ± 2.5% after 5 days. The burst release might be due to repulsion between the negatively charged alginate (−29 mV) and laccase (−6 mV) at pH of 4.5 as observed with the zeta potential. Nafion polymer laccase possibly retained better activity compared to Cu-Alg due to lower leaching of the enzyme and well-protected microenvironment in the polymer micelle. Apart from Nafion, there have been work carried out with immobilization of laccase in other ionic liquids; the immobilization efficiency was high, but the voltage and power in MFC was much lower than the Nafion used in this study (Haj Kacem et al., 2018).

TABLE 2 | Summary of the measured parameters for each system and the normalized power output.

Laccase immobilization methods	Max. power density (mW m^{-2})	Dye decolourization (%)	Relative enzyme activity after 1 cycle (%)	Coulombic efficiency (%)	Power per unit of enzyme per mg of dye decolourized (mW m^{-2} mg^{-1} U^{-1})**
PANI Lac	38 ± 1.7	75.6 ± 2.1	81 ± 2.5	4.65 ± 0.18	0.11 ± 0.003
Nafion Lac	25.6 ± 2.08	73 ± 2	69 ± 3.5	4.23 ± 0.45	0.05 ± 0.006
Cu-Alg Lac	14.7 ± 1.04	81 ± 4.47	61.5 ± 2.5	2.97 ± 0.16	0.02 ± 0.001
Free Lac	28 ± 0.98	85 ± 3.5	23.8 ± 1.8	3.83 ± 0.112	0.03 ± 0.0009

***Power density (mW m^{-2}) normalized with the amount of enzyme immobilized (U) with the amount of dye decolourized (mg) by each laccase immobilized systems.*

From the **Table 2**, PANI laccase shows the best performance of power density normalized with amount of enzyme immobilized and the amount of dye decolourized.

Comparison of the Performance of Laccase Biocathode Systems With the Conventional Pt and Fe Impregnated Catalyst (Fe-N/C) in MFCs

The laccase biocathode MFC systems above were compared with the traditional Pt and Fe impregnated N-doped carbon catalyst with regards to power generation in *Shewanella oneidensis*-based MFCs. Pt and Fe-N/C produced a power density of 80 ± 0.5 and 54.4 ± 0.84 mW m^{-2}, respectively (**Figure 8**). The highest power density produced by laccase biocathode (PANI-Laccase) (38 ± 1.7 mW m^{-2}) was much lower than that of Pt (80 ± 0.5 mW m^{-2}, **Figure 8**), but factors such as cost of the enzyme and its concomitant dye decolourization rates serve as a major advantage. The cost of platinum is 2.5 times higher than that of laccase enzyme. one gm of platinum costs £198 (Sigma Aldrich) compared to laccase at £70/gm (Sigma Aldrich). Enzyme loading in our study is much less compared to other studies (Teerapatsakul et al., 2008; Savizi et al., 2012). The normalized power output for platinum was 0.04 and 0.07 mW/£ for laccase. Laccase therefore has 1.75 times higher power output per pound compared to platinum. The use of platinum electrodes in wastewater treatment can result in biofouling of the electrode and reduced power density (An et al., 2011). Choice of enzyme or platinum need not consider cost alone; other factors such as stability and reusability of catalyst, possible leaching of catalyst in the waste stream being treated, as well as effect of environmental conditions on catalyst performance should also be considered. Enzymes require optimized conditions for their performance such as stable pH, temperature etc. but have the versatility of being engineered for better performance.

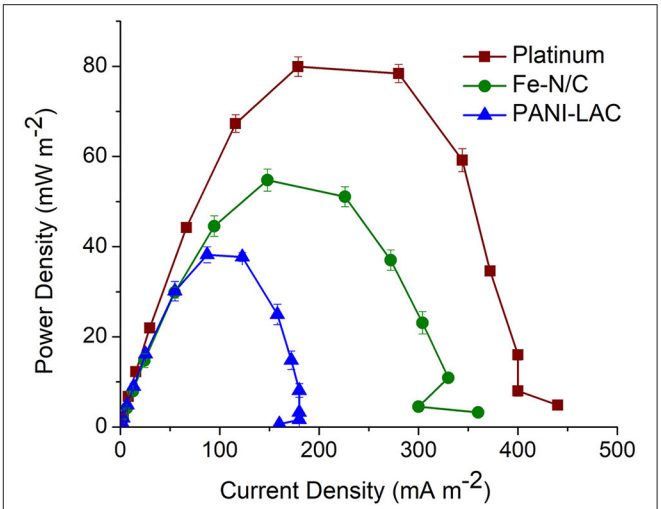


FIGURE 8 | Comparison of maximum power production for Pt catalyst, Fe-N/C, and PANI-Laccase.

CONCLUSION

In this study, different methods of immobilization of laccase i.e., cross-linking with polyaniline (PANI), entrapment in Cu alginate beads and encapsulation in Nafion micelles, were investigated with regards to their application as biocathodes in Shewanella based MFC for purposes of azo dye decolourization. Laccase cross-linked with PANI served as an effective system in striking the right balance between maintenance of enzyme activity, dye decolourization efficiency, and power output. Many studies have used large enzyme loadings of 500–2000 U ml^{-1} and mediators for achieving simultaneous dye decolourization and high-power output in MFC. In this study, we have utilized much less enzyme loadings (maximum 0.3 U ml^{-1}) in the absence of mediators to bring about decolourization of dyes and produce a good power output. The unstable nature of biological cathodes to wastewater is the major drawback for its efficiency in microbial fuel cells. Laccase has the versatility of being engineered for immobilization to extend their active lifetimes. As a result, with the advent of protein engineering laccase holds potential to be an excellent catalyst for oxygen reduction reactions to provide the comparable efficiency to that of metal catalysts.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

GK designed the experiments and proof read the manuscript. PM and VF conducted the experiments and contributed

to writing the manuscript. TK and TC helped with data analysis and proofreading of the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We acknowledged that this work is part of a Ph.D. theses titled Development of a biocathode system in microbial fuel cells for improved treatment of azo dyes by PM at University of Westminster. We have cited the theses in the references.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.620075/full#supplementary-material

REFERENCES

An, J., Jeon, H., Lee, J., and Chang, I. S. (2011). Bifunctional silver nanoparticle cathode in microbial fuel cells for microbial growth inhibition with comparable oxygen reduction reaction Activity. *Environ. Sci. Technol.* 45, 5441–5446. doi: 10.1021/es2000326

Bakhshian, S., Kariminia, H.-R., and Roshandel, R. (2011). Bioelectricity generation enhancement in a dual chamber microbial fuel cell under cathodic enzyme catalyzed dye decolorization. *Bioresour. Technol.* 102, 6761–6765. doi: 10.1016/j.biortech.2011.03.060

Cooney, M. J., Svoboda, V., Lau, C., Martin, G., and Minteer, S. D. (2008). Enzyme catalysed biofuel cells. *Energy Environ. Sci.* 1, 320–337. doi: 10.1039/b809009b

Daâssi, D., Rodríguez-Couto, S., Nasri, M., and Mechichi, T. (2014). Biodegradation of textile dyes by immobilized laccase from Coriolopsis gallica into Ca-alginate beads. *Int. Biodeterior. Biodegrad.* 90, 71–78. doi: 10.1016/j.ibiod.2014.02.006

Datta, S., Christena, L. R., and Rajaram, Y. R. S. (2013). Enzyme immobilization: an overview on techniques and support materials. *3 Biotech* 3, 1–9. doi: 10.1007/s13205-012-0071-7

Eggert, C., Temp, U., and Eriksson, K. E.,(1996). The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Appl. Environ. Microbiol* 62, 1151–1158. doi: 10.1128/AEM.62.4.1151-1158.1996

Galhaup, C., and Haltrich, D. (2001). Enhanced formation of laccase activity by the white-rot fungus *Trametes pubescens* in the presence of copper. *Appl. Microbiol. Biotechnol.* 56, 225–232. doi: 10.1007/s002530100636

Gomaa, O. M., Fapetu, S., Kyazze, G., and Keshavarz, T. (2017). The role of riboflavin in decolourisation of Congo red and bioelectricity production using *Shewanella oneidensis*-MR1 under MFC and non-MFC conditions. *World J. Microbiol. Biotechnol.* 33:56. doi: 10.1007/s11274-017-2223-8

Guisan, J. M. (2006). *Immobilization of Enzymes and Cells, 2nd Edn*. Totowa, NJ: Humana Press. doi: 10.1007/978-1-59745-053-9

Haj Kacem, S., Galai, S., Pérez de los Ríos, A., Hernández Fernández, F. J., and Smaali, I. (2018). New efficient laccase immobilization strategy using ionic liquids for biocatalysis and microbial fuel cells applications. *J. Chem. Technol. Biotechnol.* 93, 174–183. doi: 10.1002/jctb.5337

Heitner-Wirguin, C. (1996). Recent advances in perfluorinated ionomer membranes: structure, properties, and applications. *J. Memb. Sci.* 120, 1–33. doi: 10.1016/0376-7388(96)00155-X

Hu, X., Lin, X., Zhao, H., Chen, Z., Yang, J., Li, F., et al. (2016). Surface functionalization of polyethersulfone membrane with quaternary ammonium salts for contact-active antibacterial and anti-biofouling properties. *Materials (Basel)* 9, 1–12. doi: 10.3390/ma9050376

Huang, L., and Cheng, Z.-M. (2008). Immobilization of lipase on chemically modified bimodal ceramic foams for olive oil hydrolysis. *Chem. Eng. J.* 144, 103–109. doi: 10.1016/j.cej.2008.05.015

Huang, W., Chen, J., Hu, Y., Chen, J., Sun, J., and Zhang, L. (2017). Enhanced simultaneous decolorization of azo dye and electricity generation in microbial fuel cell (MFC) with redox mediator modified anode. *Int. J. Hydrog. Energy* 42, 2349–2359. doi: 10.1016/j.ijhydene.2016.09.216

Jesionowski, T., Jakub, Z., and Krajewska, B. (2014). Enzyme immobilization by adsorption: a review. *Adsorption* 20, 801–821. doi: 10.1007/s10450-014-9623-y

Kodali, M., Santoro, C., Serov, A., Kabir, S., Artyushkova, K., Matanovic, I., et al. (2017). Air Breathing cathodes for microbial fuel cell using Mn-, Fe-, Co-, and Ni-containing platinum group metal-free catalysts. *Electrochim. Acta* 231, 115–124. doi: 10.1016/j.electacta.2017.02.033

Kong, J., and Yu, S. (2007). Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochim. Biophys. Sin. (Shanghai)* 39, 549–559. doi: 10.1111/j.1745-7270.2007.00320.x

Kunimatsu, K., Yoda, T., Tryk, D. A., Uchida, H., and Watanabe, M. (2010). *In situ* ATR-FTIR study of oxygenreduction at the Pt/Nafion interface. *Phys. Chem. Chem. Phys.* 12, 621–629. doi: 10.1039/B917306D

Le Goff, A., Holzinger, M., and Cosnier, S. (2015). Recent progress in oxygen-reducing laccase biocathodes for enzymatic biofuel cells. *Cell. Mol. Life Sci.* 72, 941–952. doi: 10.1007/s00018-014-1828-4

Liu, S. H., Tsai, S. L., Guo, P. Y., and Lin, C. W. (2020). Inducing laccase activity in white rot fungi using copper ions and improving the efficiency of azo dye treatment with electricity generation using microbial fuel cells. *Chemosphere* 243:125304. doi: 10.1016/j.chemosphere.2019.125304

Logan, B. E., Hamelers, B., Rozendal, R., Schroder, U., Keller, J., Freguia, S., et al. (2006). Microbial fuel cells: methodology and technology. *Environ. Sci. Technol.* 40, 5181–5192. doi: 10.1021/es0605016

Luo, H., Jin, S., Fallgren, P. H., Park, H. J., and Johnson, P. A. (2010). A novel laccase-catalyzed cathode for microbial fuel cells. *Chem. Eng. J.* 165, 524–528. doi: 10.1016/j.cej.2010.09.061

Mani, P. (2019). *Development of a biocathode system in microbial fuel cells for treatment of azo dyes* (Ph.D. theses). University of Westminster, London, United Kingdom.

Mani, P., Fidal, V. T., Bowman, K., Breheny, M., Chandra, T. S., Keshavarz, T., et al. (2019). Degradation of azo dye (acid orange 7) in a Microbial fuel cell: comparison between anodic microbial-mediated reduction and cathodic laccase-mediated oxidation. *Front. Energy Res.* 7:101. doi: 10.3389/fenrg.2019.00101

Mani, P., Keshavarz, T., Chandra, T. S., and Kyazze, G. (2017). Decolourisation of acid orange 7 in a microbial fuel cell with a laccase-based biocathode: influence of mitigating pH changes in the cathode chamber. *Enzyme Microb. Technol.* 96, 170–176. doi: 10.1016/j.enzmictec.2016.10.012

Marsili, E., Baron, D. B., Shikhare, I. D., Coursolle, D., Gralnick, J. A., and Bond, D. R. (2008). Shewanella secretes flavins that mediate extracellular electron transfer. *Proc. Natl. Acad. Sci. U.S.A.* 105, 6–11. doi: 10.1073/pnas.0710525105

Meredith, S., Xu, S., Meredith, M. T., and Minteer, S. D. (2012). Hydrophobic salt-modified Nafion for enzyme immobilization and stabilization. *J. Vis. Exp.* 65, 1–6. doi: 10.3791/3949

Moehlenbrock, M. J., and Minteer, S. D. (2017). "Introduction to the field of enzyme immobilization and stabilization," in *Enzyme Stabilization and Immobilization. Methods in Molecular Biology*, ed S. Minteer (New York, NY: Humana Press), 1–7. doi: 10.1007/978-1-4939-6499-4_1

Romo-Sánchez, S., Camacho, C., Ramirez, H. L., and Arévalo-Villena, M. (2014). Immobilization of commercial cellulase and xylanase by different methods using two polymeric supports. *Adv. Biosci. Biotechnol.* 05, 517–526. doi: 10.4236/abb.2014.56062

Russo, M. E., Giardina, P., Marzocchella, A., Salatino, P., and Sannia, G. (2008). Assessment of anthraquinone-dye conversion by free and immobilized crude laccase mixtures. *Enzyme Microb. Technol.* 42, 521–530. doi: 10.1016/j.enzmictec.2008.01.011

Savizi, I. S. P., Kariminia, H. R., and Bakhshian, S. (2012). Simultaneous decolorization and bioelectricity generation in a dual chamber microbial fuel cell using electropolymerized-enzymatic cathode. *Environ. Sci. Technol.* 46, 6584–6593. doi: 10.1021/es300367h

Schaetzle, O., Barrière, F., and Schröder, U. (2009). An improved microbial fuel cell with laccase as the oxygen reduction catalyst. *Energy Environ. Sci.* 2, 96–99. doi: 10.1039/B815331K

Simões, M. F., Maiorano, A. E., dos Santos, J. G., Peixoto, L., de Souza, R. F. B., Neto, A. O., et al. (2019). Microbial fuel cell-induced production of fungal laccase to degrade the anthraquinone dye Remazol Brilliant Blue R. *Environ. Chem. Lett.* 17, 1–8. doi: 10.1007/s10311-019-00876-y

Song, E., and Choi, J.-W. (2013). Conducting polyaniline nanowire and its applications in chemiresistive sensing. *Nanomaterials* 3, 498–523. doi: 10.3390/nano3030498

Sonu, K., Syed, Z., and Sogani, M. (2020). Up-scaling microbial fuel cell systems for the treatment of real textile dye wastewater and bioelectricity recovery. *Int. J. Environ. Stud.* 77, 692–702. doi: 10.1080/00207233.2020.1736438

Stejskal, J., and Gilbert, R. G. (2006). Polyaniline. Preparation of a conducting polymer (IUPAC technical report). *Pure Appl. Chem.* 74, 857–867. doi: 10.1351/pac200274050857

Teerapatsakul, C., Bucke, C., Parra, R., Keshavarz, T., and Chitradon, L. (2008). Dye decolorisation by laccase entrapped in copper alginate. *World J. Microbiol. Biotechnol.* 24, 1367–1374. doi: 10.1007/s11274-007-9617-y

Tiwari, D. C., Jain, R., and Sharma, S. (2007). Electrochemically deposited polyaniline/polypyrrole polymer film modified electrodes for determination of furazolidone drug. *J. Sci. Ind. Res.* 66, 1011–1018.

Wolin, E. A., Wolin, M. J., and Wolfe, R. S. (1963). Formation of methane by bacterial extracts. *J. Biol. Chem.* 238, 2882–2886.

Zille, A., Tzanov, T., Gübitz, G. M., and Cavaco-Paulo, A. (2003). Immobilized laccase for decolourization of reactive Black 5 dyeing effluent. *Biotechnol. Lett.* 25, 1473–1477. doi: 10.1023/A:1025032323517

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 17 March 2021
doi: 10.3389/fmicb.2021.634753

Uncovering Competitive and Restorative Effects of Macro- and Micronutrients on Sodium Benzoate Biodegradation

*Purvi Zaveri, Aishwarya Raghu Iyer, Rushika Patel and Nasreen Shakil Munshi**

Institute of Science, Nirma University, Ahmedabad, India

A model aromatic compound, sodium benzoate, is generally used for simulating aromatic pollutants present in textile effluents. Bioremediation of sodium benzoate was studied using the most abundant bacteria, *Pseudomonas citronellolis*, isolated from the effluent treatment plants of South Gujarat, India. Multiple nutrients constituting the effluent in actual conditions are proposed to have interactive effects on biodegradation which needs to be analyzed strategically for successful field application of developed bioremediation process. Two explicitly different sets of fractional factorial designs were used to investigate the interactive influence of alternative carbon, nitrogen sources, and inorganic micronutrients on sodium benzoate degradation. The process was negatively influenced by the co-existence of other carbon sources and higher concentration of KH_2PO_4 whereas NH_4Cl and $MgSO_4$ exhibited positive effects. Optimized concentrations of NH_4Cl, $MgSO_4$, and KH_2PO_4 were found to be 0.35, 1.056, and 0.3 mg L^{-1} respectively by central composite designing. The negative effect of high amount of KH_2PO_4 could be ameliorated by increasing the amount of NH_4Cl in the biodegradation milieu indicating the possibility of restoration of the degradation capability for sodium benzoate degradation in the presence of higher phosphate concentration.

Keywords: textile effluents, macronutrients, micronutrients, sodium benzoate biodegradation, *Pseudomonas citronellolis*, response surface methodology

OPEN ACCESS

Edited by:
Eric D. van Hullebusch, Université de Paris, France

Reviewed by:
Manikant Tripathi, Avadh University, India
Muhammad Arslan, University of Alberta, Canada

***Correspondence:**
Nasreen Shakil Munshi
nasreen.munshi@nirmauni.ac.in

Specialty section:
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

Received: *28 November 2020*
Accepted: *01 February 2021*
Published: *17 March 2021*

Citation:
Zaveri P, Iyer AR, Patel R and Munshi NS (2021) Uncovering Competitive and Restorative Effects of Macro- and Micronutrients on Sodium Benzoate Biodegradation. Front. Microbiol. 12:634753. doi: 10.3389/fmicb.2021.634753

INTRODUCTION

India is the second largest textile product-exporting country worldwide, and textile sector plays a major role in the employment, economic growth, and financial empowerment to millions of Indians from smll handicraft units to large apparel industries. Gujarat is a major textile-manufacturing hub in the country. Textile industries utilize huge amount of fresh water for production of finished goods. Effluents generated from fabric printing, yarn printing, and dyeing can cause considerable amount of damage to the environment due to the presence of colors, salts, and a variety of recalcitrant compounds (Ayed et al., 2012). Years of research has indicated the importance and cost effectiveness of biological treatment over physicochemical method for textile wastewater treatment. However, bioremediation as a bioprocess when applied to field becomes one of the

most dynamic and complex interplay of pollutants, nutrients, and microorganisms. Microbial community present in contaminated environment (e.g., soil, water, wastewater) generally has efficient microbial degraders like *Pseudomonas* species in adequate numbers (Rossello-mora et al., 1994; Junca and Pieper, 2004). Such highly versatile organisms are found to have an ability to metabolize complex/toxic hydrocarbons like phenol, toluene, and phenanthrene under laboratory conditions (Lakshmi et al., 2013). The question arises, with so diverse metabolic capability why such efficient microbial community fails to generate the laboratory-evident degradation rate under field conditions? The limitation lies in our understanding to generate conducive environment, interplay of nutrients, translation of bioremediation to field, and strategic optimization of bioprocess developed in laboratory. To understand the interaction and interference of various macro- and micronutrient on biodegradation, statistical optimization was employed using a simplest model hydrocarbon such as sodium benzoate (SB) which was used to mimic aromatics in textile wastewater (Yaseen and Scholz, 2019), by *P. citronellolis* (GenBank accession number: KM871063) (Zaveri et al., 2015). Under various environmental conditions, sodium benzoate has been reported to travel through route of wastewaters and end up in sludge (Wibbertmann et al., 2005). Though it is easily biodegradable under controlled laboratory conditions (Wibbertmann et al., 2005), reports also suggest that the presence of preferable carbon and/or nitrogen sources (glucose, acetate, amino acids, etc.) result in either increase or decrease in hydrocarbon biodegradation efficiency (Chun et al., 2004; Jonsson et al., 2007; Das and Chandran, 2011). Hence, in the presence of easily assimilable carbon sources, hydrocarbon compounds will be least preferred and may not be degraded. Similarly, even if the organisms have the capacity to degrade such hydrocarbon compounds, but in absence of suitable nitrogen sources, the compounds remain undegraded.

It is known that deficiency of required inorganic nutrients may lead to lower biodegradation rates which may be a result of nutrient starvation leading to degradation of cellular proteins (John and Goldberg, 1980; Walworth et al., 2007). However, there are reports which also suggest that supplementing the depleted inorganic nutrients may not be the stimulus each time as these metabolic processes are dependent on interplay of compounds and/or organisms (Swindoll et al., 1988; Steffensen and Alexander, 1995). Thus, optimization of bioprocess turns out to be the key factor for processes like enzyme production as well as biodegradation to achieve high biodegradation efficiency (Dibblef and Bartha, 1979; Leahy and Colwell, 1990; Yan et al., 2014; Priyadharshini and Bakthavatsalam, 2016; Hashem et al., 2018).

With increased access to sophisticated statistical software and computing, research field witnesses large application of Design of Experiment (DOE) mathematical tool for bioprocess engineering. Statistical designing of experiments has been shown to be a very important tool in optimizing various media components for industrial scale processes such as production of enzymes (Abdel-Fattah et al., 2005; Yuan et al., 2008; Demir et al., 2015), biodegradation of various toxic compounds (Östberg et al., 2007; de Guillén-Jiménez et al., 2012; Ibn Abubaker et al., 2012; Lakshmi et al., 2013; Su and Lin, 2013), biodegradation in marine environment (Zahed et al., 2010), bacterial biomass production (Gutierrez-Rojas et al., 2011; Zhang et al., 2014; Chen et al., 2015), etc. As these tools have the capability to display interactive influential effect of variables, they have replaced conventional one-variable-at-a-time approach (OVAT) for multivariant experiments. Plackett-Burman design (PBD), a fractional factorial design, determines critical/significant variables affecting the processes in a limited number of experiments (Burman and Plackett, 1946). This design helps to illustrate and identify the effect of each parameter on the bioprocess and their interaction with each other (El-Hamid et al., 2018). It has also been found to be helpful in attaining increased gene expression and enzyme production (Abdel-Fattah et al., 2007; Halder et al., 2013; Meng et al., 2015). The screened variables/components significantly affecting the biodegradation process can then be further optimized using response surface methodology (RSM) (Darvishmotevalli et al., 2019). For a generation of response plots, various designs like central composite design (CCD), Box-Behnken design, etc. are widely applied.

In the present study, field condition was simulated for degradation of single-ring hydrocarbon with the help of statistical tools for characterization of main effect of variables, i.e., nutrients, on SB biodegradation process by *Pseudomonas citronellolis*. Initially, in our studies on sodium benzoate degradation by *P. citronellolis*, few variables were optimized using OVAT approach including pH, substrate concentration, temperature, aeration-agitation, and nitrogen source. Furthermore, 9 variables were identified which were supposed to influence SB degradation. To reduce the number of experiments required for optimizing the rest of the 9 parameters for SB degradation, experiments were designed using fractional factorial design of Plackett-Burman. Out of 11 variables selected in the PB design, two variables were having known effects, i.e., substrate concentration and pH obtained from independent studies using OVAT approach. In two explicitly different sets of PBD for studying the interaction of carbon and nitrogen sources in degradation experiments, the importance of the presence of micronutrients was realized. In addition, a detailed analysis of desirable concentration and influence of variables on each other is described using 3D response plots. Such detailed approach can be used as platform for interactive analysis of other combinations of nutrients, as the robustness of the design helps the researcher to conclude the most influential variable easily. The results obtained indicate the obstacles with degradation of target hydrocarbon in the presence of easily degradable substrates which might be available in surrounding environment. The primary aim of using PBD was to evaluate the influence of additional "C" source selected arbitrarily as supplementary nutrients for growth of bacterial cells used for bioremediation or various "N" sources for screening their effects on SB degradation and interaction with other media constituents. The study deals with the nutrient interplay involved in sodium benzoate degradation and does not focus on the mass balance and mineralization thus stoichiometry of the benzoate degradation process and pathways are not in the scope of the present study.

Moreover, no simulation software/AI was used to generate any biological data, and the results are outcome of SB degradation experiments conducted under laboratory conditions.

MATERIALS AND METHODS

Materials

All chemicals used in this study were of the highest purity grade purchased from Sigma-Aldrich, Merck-India, or Himedia-India.

Microorganism and Culture Activation

The bacterial strain used in this study, *Pseudomonas citronellolis* was isolated from common industrial effluents of South Gujarat, India and was identified as the most abundant bacteria in effluent stream in our previous work (Zaveri et al., 2015). Activated culture grown on nutrient broth (OD_{560} □0.8) was harvested at 7,500 rpm at 27 ± 2°C temperature (Eppendorf Centrifuge Model No. 5430 R, Germany). To ensure removal of organic matter, activated culture was washed twice with sterile normal saline before inoculation (5%, *v*/*v*) in autoclaved media prepared for PBD and RSM experiments as described in the section "Statistical Design of Experiments."

Statistical Design of Experiments

The objective of applying statistical techniques was to reduce the number of optimization experiments to deduce the most optimal conditions for SB degradation using limited number of experiments with statistical validity.

Plackett-Burman Design: Fractional Factorial Experiment

The effects of carbon and nitrogen sources and their interactions with micronutrients on SB degradation were assessed independently in two experimental set ups designed using PBD. Design Expert (ver. 10) was used to prepare run combinations for all experiments described here. Design A was formulated for evaluation of the presence of more than one *carbon sources* and design B was a representation of condition where the presence of more than one *nitrogen sources* was evaluated. Essential micronutrients (KH_2PO_4, Na_2HPO_4, $MgSO_4$, and $CaCl_2$) were kept as common variables in both the designs although the "−1" concentrations were kept intentionally different (with difference of one level) so that they do not become the limiting factor in runs with longer incubation period. Concentration of all other variables in both the designs were kept at "−1" (low) and " + 1" (high) levels. The concentrations and the details of the variables used are presented in **Table 1**. The two levels of all factors (concentration of " + 1" and "−1") were selected in such a way as to match either the concentration of ingredients of minimal salt medium (MSM) (Atlas, 2005) or which helps us to further select the concentrations to be used in five-level experimental design of response surface methodology using CCD.

The code designations of variables were kept random to avoid any bias in design. A total of 11 variables were considered for investigation, where 9 were independent variables and some were used as dummy variables (unassigned variables) viz. SB concentration and pH in design A and only SB concentration in design B. They were screened in 12 combinations/runs according to the design. In design A, factors 1–5 were ingredients of modified MSM [Na_2HPO_4,600 mg L^{-1}; KH_2PO_4, 300 mg L^{-1}; NH_4Cl, 100 mg L^{-1}; NaCl, 50 mg L^{-1}; $MgSO_4$, 24.6 mg L^{-1}; $CaCl_2$, 1.4 mg L^{-1}; and SB, 72 mg L^{-1} (5 mM)] selected for hydrocarbon degradation assay. Factors 6, 7, and 8 were selected as additional substrates/"C" source to investigate their role as supplementary/co-existing substances to investigate whether they may support growth of more number of cells and thus may potentially enhance SB degradation. Also, whether they are interacting with other constituents of medium could become clear in such experiments. Similarly, in the case of design B, factors 1–5 were the constituents of modified minimal salt basal medium and factors 6–9 were the nitrogen sources selected to be screened (**Table 1**). All the runs were performed in triplicates with sufficient negative controls. According to described concentrations, 100 ml media were prepared and inoculated with active, washed culture (5%, *v*/*v*) of *P. citronellolis* and incubated at 30 ± 2°C under shaking conditions (80 rpm) (Remi CIS-24 plus, India). Initial pH of the medium was adjusted using 0.1 N HCl or NaOH. Responses were calculated in terms of percent SB degradation from average values of replicates in each run (Rajendran et al., 2007; Patil and Jena, 2015; El-Hamid et al., 2018).

The main effect plot was prepared, and using Pareto chart, the influential factors were identified as those exhibiting values above "t-critical' and Bonferroni's limit.

Central Composite Design: Response Surface Methodology

As from the results of designs "A" and "B," micronutrients viz. NH_4Cl, $MgSO_4$, and KH_2PO_4 were found to have a significant effect on SB degradation bioprocess; they were then further considered for model development using CCD through RSM. A three-factor, five-level CCD with 20 runs was employed (Safa et al., 2017). The variables NH_4Cl, $MgSO_4$, and KH_2PO_4 were denoted as factors 1, 2, and 3, respectively, and each of them were assessed at five different levels, combining factorial points (−1, + 1), axial points (−α, + α), and central point (0). The concentration used for matrix designing using CCD is projected in **Table 2** (Priyadharshini and Bakthavatsalam, 2016). Twenty runs were required according to Design Expert (ver. 10.0), and run details are presented as **Supplementary Material**. According to levels of all three factors, with respective concentrations of these ingredients, media were prepared in 100 ml volumes, sterilized, and inoculated with 5% (*v*/*v*) *P. citronellolis* culture. Responses were calculated as SB degradation percentages obtained from experimental sets performed in triplicates. The rest of all the components of modified MSM were kept to their original concentrations including 5 mM of sodium benzoate as sole source of carbon.

The predicted values as obtained through modeling were compared with actual values obtained through the experiment. Three-dimensional response plots and their respective contour plots for all three micronutrients were prepared using Design

TABLE 1 | Details of variables used for Plackett-Burman designing for optimization of sodium benzoate degradation.

Nutrient type	Sr. No.	Design A			Design B		
		Factor (code)	Concentration levels (mg)		Factor (code)	Concentration levels (mg)	
			−1	+1		−1	+1
Micronutrients	1	KH_2PO_4 (H)	300	400	KH_2PO_4 (A)	200	400
	2	Na_2HPO_4 (G)	1,200	1,800	Na_2HPO_4 (B)	600	1,800
	3	NaCl (J)	800	1,000	NaCl (C)	600	1,000
	4	$MgSO_4$ (B)	1.23	1.56	$MgSO_4$ (D)	0.738	1.722
	5	$CaCl_2$ (C)	0.0735	0.1029	$CaCl_2$ (E)	0.041	0.102
Macronutrients	6	Succinate (D)	500	700	Glycine (G)	75	125
	7	Acetate (E)	500	700	Proline (H)	75	125
	8	Glucose (F)	500	700	Cysteine (J)	75	125
	9	NH_4Cl (A)	500	700	Ammonium tartrate (L)	100	300
	10	pH (L)	6.5	7	Paranitrophenol (K)	1.5	2.5
	11	Sodium benzoate (K)	5 mM	7 mM	Sodium benzoate (F)	5 mM	7 mM

TABLE 2 | Selected levels and concentrations of variables used in central composite design for applying response surface methodology.

Factors	Levels				
	−α	−1	0	+ 1	+α
KH_2PO_4	0.131	0.200	0.300	0.4	0.468179
NH_4Cl	0.163	0.300	0.500	0.7	0.83636
$MgSO_4$	0.402	0.738	1.230	1.722	2.05744

Expert (Ver. 10.0) and analyzed to obtain the desirable concentration for better SB remediation.

Monitoring of SB Degradation

All flasks were incubated at 30 ± 2°C in an incubator shaker at 80 rpm. At regular intervals, 1 ml sample was aseptically withdrawn to determine growth of organism and degradation of sodium benzoate. Changes in pH values were observed using universal pH indicator (Himedia, India product code-I013); however, it was found to be negligible. Remaining sodium benzoate concentration was determined spectrophotometrically at 230 nm (Agilent Technologies Carry 60 UV-Vis, United States) (Zaveri et al., 2015). Abiotic loss of SB during incubation was less than 2% and thus, all the degradation values were normalized accordingly. Percent SB degradation was calculated using the following formula:

$$SB\ degradation\ (\%) = \left[(Initial\ SB\ concentration - Final\ SB\ concentration)/Initial\ SB\ concentration\right] \times 100 \quad (1)$$

All the degradation experiments were performed in triplicates, and the results presented are average of three data sets.

RESULTS

Initial studies for sodium benzoate degradation were conducted to optimize variables over a possible range for individual parameter. This data contributed to the basic understanding of degradation pattern and characteristic of organism under variously stressed conditions. The "One Variable at a Time" approach was used for basic parameters like temperature (15, 20, 25, 30, and 40°C), pH (4, 5, 6, 7, 8, 9, and 10), agitation (0, 80, and 150 rpm), nitrogen source (NH_4Cl and KNO_3), and substrate concentrations (ranging from 1 to 100 mM). The results obtained indicated that organism was able to optimally utilize 1–50 mM sodium benzoate using NH_4Cl as source of nitrogen at 30°C and at 80 rpm. The organism could not withstand pH 4 and 10 and substrate concentrations above 50 mM. The organism was able to degrade substrate under both static and agitating conditions; however, agitation was supporting faster degradation as compared with static conditions. From the range of substrate concentrations, 5 and 7 mM were selected for factorial designing because of the least possible degradation time required and the lowest sensitive detection with spectrophotometric method.

Statistical methods were used only to design the experimental combinations of nutrients, based on which flask-level experiments were conducted. No data reported in the present manuscript is software derived. All the data for SB degradation were obtained after conducting wet lab experiments at flask level.

Identification of Major Effect in Two Independent Plackett-Burman Experiments

The experimental response obtained in the form of SB degradation through both the designs according to the change in the medium composition is depicted in **Table 3**. In the modified MSM medium, *Pseudomonas citronellolis* could degrade SB by more than 95% within 24 h of incubation. With the change in the composition of the medium, degradation percentage ranged from 0 to 68% (incubation till 18 days) in design A and 40–87%

TABLE 3 | Response of SB degradation by *Pseudomonas citronellolis* in two PB designs for medium constituents.

Run number	SB degradation (%) Design A	SB degradation (%) Design B
1	69 ± 6	85 ± 2
2	65 ± 2	78 ± 2
3	56 ± 8	77 ± 2
4	59 ± 9	76 ± 1
5	57 ± 3	83.6
6	59 ± 2	78 ± 2
7	0.0	82 ± 1
8	39 ± 1	40 ± 1
9	63 ± 5	55 ± 4
10	0.0	85 ± 1
11	6 ± 6	86 ± 6
12	0	83 ± 1

degradation in design B (incubation till 6 days). The wide range of values obtained indicates prominent effect of multiple carbon and nitrogen sources in the medium and interaction of variables on degradation of sodium benzoate.

Table 3 depicts that levels of factors in run number 1 in design A and run number 11 in design B resulted in highest SB degradation efficiency of 69 and 86%, respectively. The significance of the experimental data generated was calculated using the Design Expert software considering the degradation percentage obtained from various wet lab experimental sets as an input parameter. Based on these experimental degradation values, the software calculates the effectiveness of different combinations of nutrients and their concentrations and generates the graphical presentation of positive and negative effects (**Figures 1**–**3**). The positive influence of factor here is described in terms of higher SB degradation achieved with increasing concentration of variable factor and *vice-a-versa* for negatively influencing factors. The Pareto charts highlighted the statistically significant factors having either positive or negative impact on SB degradation in decreasing order of influence and indicate the main effect factors by displaying them above "t critical" and Bonferroni limits (Design expert Ver. 10.0) (**Figures 1A,B**).

The variables with negative and positive effects on SB degradation are displayed in different colors. Out of all the parameters analyzed for study, $MgSO_4$, pH, and substrate concentrations (SB) were shown to have a positive impact, of which the first two were significant for SB degradation in design A whereas $MgSO_4$, $CaCl_2$, ammonium tartrate, and substrate concentration (SB) in design B were shown to have a positive impact on SB degradation, among which the first two were found to be significant. On the other hand, KH_2PO_4, Na_2HPO_4, and NaCl exhibited negative effect on SB degradation in both the designs. It is important to realize that change in the selection of macronutrients did not affect the inorganic nutrients' requirement.

The data displayed in Pareto chart became more defined in graphs presented in **Figures 2**, **3** when individual factors were analyzed. As seen in **Figures 2**, **3**, the *x*- and *y*-axis

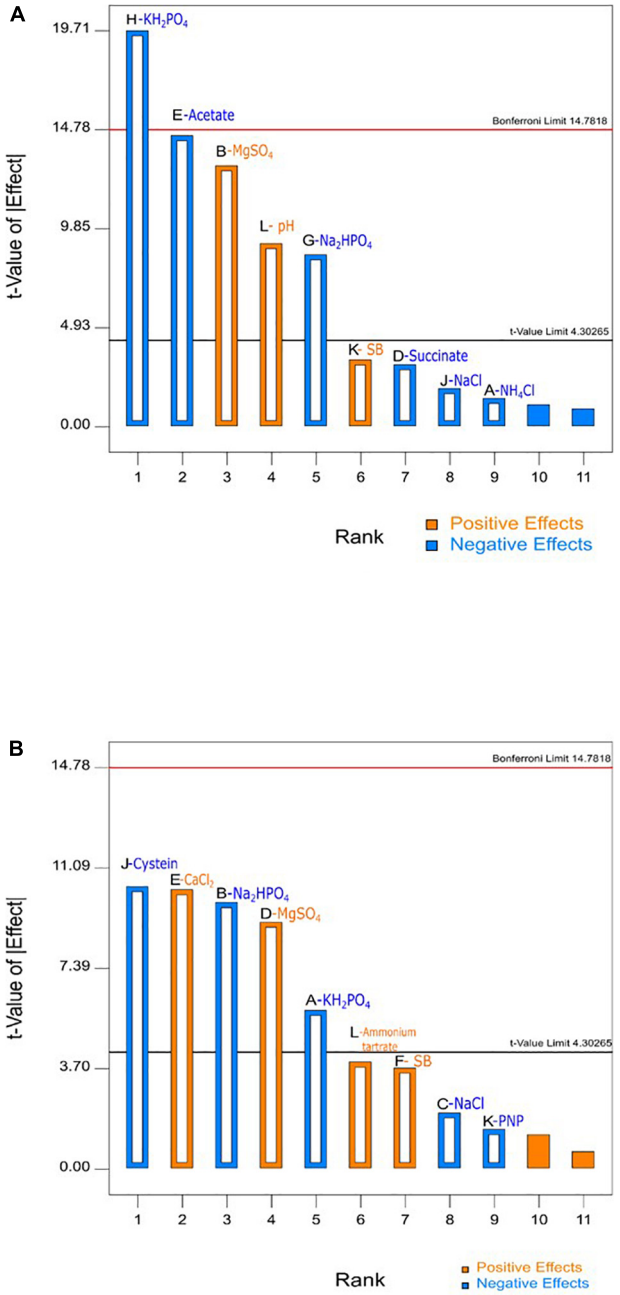


FIGURE 1 | Pareto chart for obtaining main effects using fractional factorial **(A)** design A and **(B)** design B conducted for sodium benzoate degradation by *P. citronellolis*.

in each graph represent the concentration of the factor in milligrams and percent SB degradation, respectively. The line in the graph represents the effect of each factor on SB degradation at its variable concentration (+ 1 or −1), and the slope of the line indicates relationship of degradation percentage with concentration of the variables selected for these experiments.

Likewise, in the case of design B (**Figure 3**), compounds like KH_2PO_4, Na_2HPO_4, NaCl, and cysteine had negative

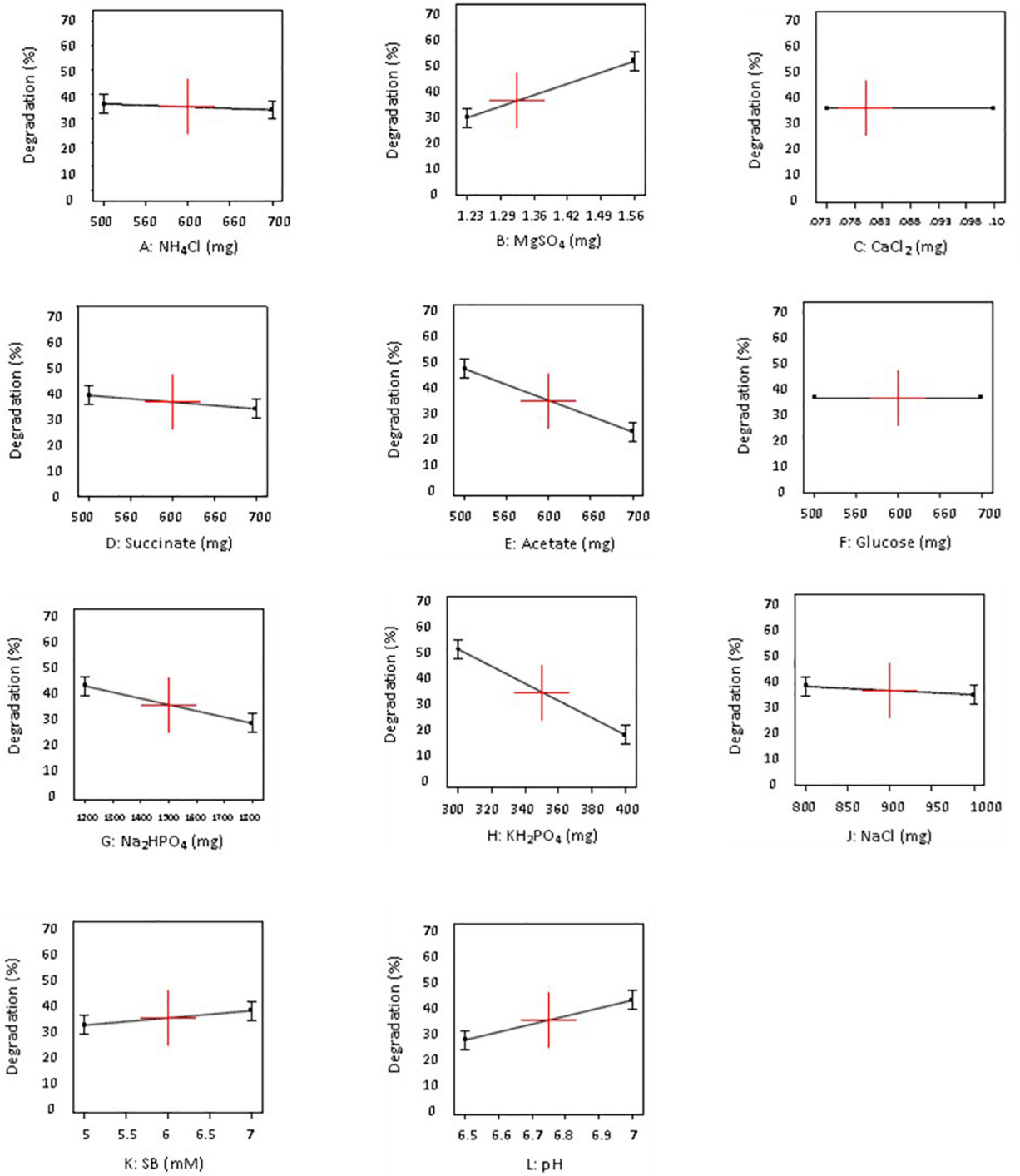


FIGURE 2 | Influence of different concentrations (−1 and +1 levels) on degradation of sodium benzoate by *P. citronellolis* (design A).

effect on degradation. On the other hand, higher levels of ammonium tartrate, $MgSO_4$, $CaCl_2$, and substrate concentration had positive influence on SB degradation efficiency. Whereas, in the case of glycine, proline, and PNP, in both their variable concentrations, they did not show much significant effect on SB degradation.

The ANOVA analysis of the model is represented in **Table 4**. The *F* value of model A was calculated as 105.48 (p = 0.0094) which indicated significance of the developed model. Similarly, model B was also found to be significant with p value of 0.0192.

Considering the coefficient values, "effect equation" for degradation of sodium benzoate was possible to be derived. The

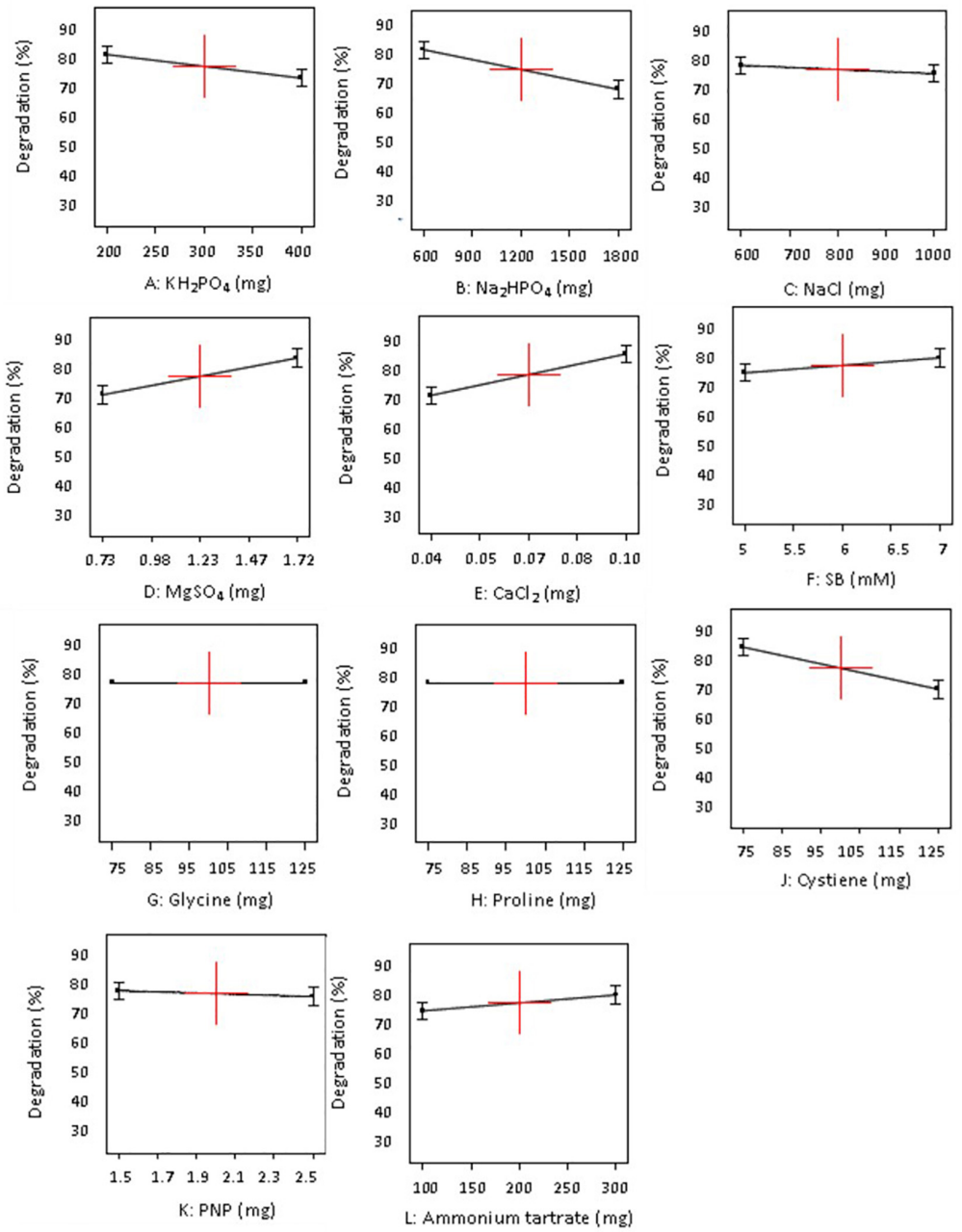


FIGURE 3 | Influence of different concentrations (+ 1 and −1) of individual 11 variables on degradation of sodium benzoate by *P. citronellolis* (design B).

model equation for sodium benzoate degradation (Y) could be written as:

For design A:

$$Degradation\ (Y, designA) = 39.40 - 1.27 \times A + 11.68 \times B - 2.68 \times D - 13.03 \times E - 7.70 \times G - 17.73 \times H - 1.71 \times J + 2.99 \times K + 8.20 \times L. \quad (2)$$

For design B:

$$Degradation\ (Y, DesignB) = 75.72 - 3.65 \times A + 6.31 \times B - 1.29 \times C + 5.68 \times D + 6.42 \times E + 2.31 \times F - 6.49 \times J - 0.91 \times K + 2.46 \times L. \quad (3)$$

The predicted values of sodium benzoate degradation were calculated using first-order model using Design Expert (ver. 10.0)

TABLE 4 | Analysis of variance for Plackett-Burman designs A and B developed for sodium benzoate degradation.

Source	Sum of squares	*df*	Mean square	*F*-value	*P*-value	Significance
Model A	9,221.39	9	1,024.60	105.48	0.0094	Significant
Model B	2,164.91	9	240.55	51.38	0.0192	Significant

and compared with values that were determined experimentally for each run (refer to **Supplementary Material**). Similarity least variation observed for degradation percentage between actual and predicted values demonstrated accurate model prediction.

Optimization of Concentrations of Major Influential Factors Using Central Composite Design and Response Surface Methodology

Steepest ascent is an experimental approach which leads us toward optimal increase in the response. The objective of such optimization processes involves the use of higher or lower concentration of significant factors to reach a maximum degradation (Montgomery, 2017). Results of both PBD contributed to narrowing down the main effect factors toward inorganic micronutrients. In both designs, KH_2PO_4 and Na_2HPO_4 displayed a negative effect. Out of these two sources of phosphate, as KH_2PO_4 which was proved to be above Bonferroni limit was then selected for further optimization. In the case of $MgSO_4$, in both the designs, it came up to be highly positively influencing factor and thus was processed for optimization *via* RSM. Hydrogen ion concentration being the dummy factor was excluded out of the next level of experimental designing. Taking in consideration the former experiments on SB degradation in our lab and reports on NH_4Cl being the best N source for degradation (Zaveri et al., 2015), and thus even if it was not indicated as an influencing factor, it was further selected in the CCD design. Response obtained in terms of SB degradation was analyzed, and percent degradation data is presented in **Table 5**.

Sets for all runs were performed in triplicates, and degradation percentages indicated in **Table 5** represents mean values obtained. With increase in incubation time, there was an apparent increase in the degradation percentage. However, run number 19 had no change in initial sodium benzoate concentration displaying no degradation. Almost complete degradation was achieved in the rest of all sets of experiments performed.

Response surface graphs were obtained after analysis of data. Surface plots and contour plots obtained depicted interaction of selected variables for the design (**Figures 4A–C**). These figures indicate responses at 24 h of incubation keeping all other parameters constant. In the case of $MgSO_4$ and NH_4Cl, all five concentrations selected were found to fall in optimally required range of concentrations for higher SB degradation. Mid-point with 100% degradation was indicated by software with relative upward boundaries of responses.

Figure 4B was found to be very informative as it clearly indicates lower concentrations of phosphate being optimal for SB degradation ($\leq$3 mg L^{-1}). **Figure 4B** presents interaction of NH_4Cl and KH_2PO_4 at 24 h of incubation. With increase in incubation time, decline in the degradation percentage at higher concentration of KH_2PO_4 was observed.

TABLE 5 | SB Degradation percentage achieved with CCD design using three inorganic nutrient variables.

Run	SB degradation (%)	Run	SB degradation (%)
1	96	11	97
2	97	12	97
3	97	13	97
4	97	14	98
5	97	15	97
6	97	16	98
7	97	17	98
8	97	18	98
9	98	19	0.00
10	98	20	88

Calculated values of sum of squares, *F* values, and *p* values are summarized in ANOVA table (**Table 6**).

F-value of 42.57 and $p < 0.0003$ proved the statistical significance of the model developed for sodium benzoate degradation by CCD design. In addition to the significance of the model, variable C was found to have a *p* value of less than 0.0001, indicating a significant importance of factor in design development and degradation of sodium benzoate. The calculated desirable concentration for NH_4Cl, $MgSO_4$, and KH_2PO_4 was found to be 0.35, 1.056, and 0.3 mg L^{-1}, respectively.

DISCUSSION

A range of *Pseudomonas* spp. possesses a vital role in bioremediation process of textile effluents and aromatic hydrocarbon compounds. Taking the advantage of PBD design, study started with the crucial interaction of multiple carbon sources such as, acetate, succinate, and glucose and effect of micronutrients on SB degradation by *P. citronellolis*, a promising strain (Zaveri et al., 2015). The initial investigation involved the execution of 12 different run combinations for 11 variables (design A). The result of the study was remarkable and indicated a clear decrease in degradation of SB in the presence of additional carbon sources when compared with results of SB degradation where SB was present as a sole carbon source. Influence of inorganic micronutrients was also evident by results of design A performed in the presence of additional carbon sources. Results of the first PB design provoked the curiosity to understand the cumulative or individual effect of

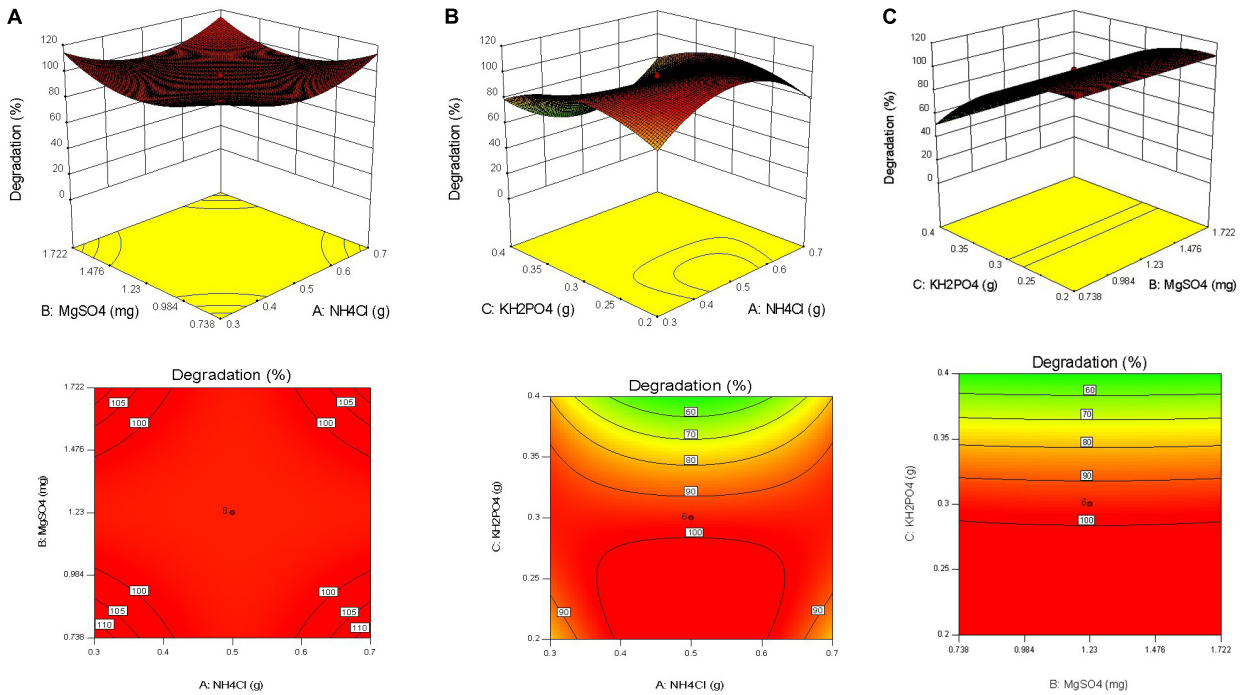


FIGURE 4 | (A) Response surface plots and contour plots for NH_4Cl and $MgSO_4$ for sodium benzoate degradation by *Pseudomonas citronellolis*. **(B)** Response surface plots and contour plot for NH_4Cl and KH_2PO_4 for sodium benzoate degradation by *Pseudomonas citronellolis*. **(C)** Response surface plots and contour plots for $MgSO_4$ and KH_2PO_4 for sodium benzoate degradation by *Pseudomonas citronellolis*.

TABLE 6 | ANOVA table for central composite design for analysis of response surface obtained.

Source	Sum of squares	*df*	Mean square	*F*-value	*p*-value	Significance
Model	8,969.24	14	640.66	42.57	0.0003	Significant
A-NH_4Cl	3.60×10^{-4}	1	3.60×10^{-4}	2.40×10^{-5}	0.9963	
B-$MgSO_4$	4.40×10^{-3}	1	4.40×10^{-3}	2.93×10^{-3}	0.9589	
C-KH_2PO_4	4.75×10^{3}	1	4.75×10^{3}	316.17	<0.0001	

various nitrogen sources on the degradation efficiency. Another PB experiment involving 11 independent variables in 12 runs was designed (design B) using possible alternate nitrogen sources, and similar micronutrients were used as in the former design. After confirming the main effects from Pareto chart and coefficient table for significance, the concentrations of variables were further optimized by RSM.

Effect of Alternative Carbon and Nitrogen Sources on SB Degradation—Analysis of Fractional Factorial Experiments

The result of the very first effort for understanding the interaction and influence of the presence of multiple easily assimilable carbon sources indicated an obvious negative impact on sodium benzoate degradation (**Table 3**). It was observed that when NH_4Cl, $MgSO_4$, $CaCl_2$, Na_2HPO_4, and NaCl salts were at + 1 concentrations, and all the three additional carbon sources were at −1 concentrations, SB degradation achieved was maximum with a value around 69% (run number 1 in **Table 3**) in design A (the experimental case with carbon sources). Similar effect of biostimulation using inorganic nutrient (addition of N and P source) has been observed in case of removal of solvents from textile manufacturing wastewater (Freedman et al., 2005). Under critical environmental conditions like hypersalinity, addition of yeast extract, glucose, KCl, and four mineral nutrients (solutions of phosphate buffer, calcium chloride, magnesium sulfate, ferric chloride, ammonium sulfate) was reported to enhance phenol degradation capacity (Li et al., 2010).

It was observed that the presence of multiple carbon sources prolonged the lag phase for hydrocarbon degradation (Wigginst and Alexander, 1988). This, however, led to generation of considerable biomass but was not able to exhibit effects like co-metabolism as indicated by Schmidt and Alexander (1985). The delay as well as reduction in the efficiency of SB degradation can be attributed to the presence of additional "C" source (s) which might have been used easily before the commencement of SB degradation thus affecting the overall

activity. In this experiment, the maximum SB degradation achieved was only 68.5% (**Table 3**), which did not support the hypothesis of faster degradation of SB as a result of higher biomass production achieved through utilization of certain easily assimilable C source.

Similarly, textile dye Remazol black B biodegradation process was optimized using the multifactorial Plackett-Burman design by Hashem et al. (2018), where 11 independent factors were included. Out of the which, eight factors were media compositions (such as glucose concentration, yeast extract, sodium acetate, sodium nitrate, EDTA, iron concentration, magnesium concentration, and NaCl) and other two were environmental conditions (temperature and pH) in addition to dye concentration. A significant increase in biodegradation rates was observed in the presence of iron, magnesium, and yeast extract and at high pH value.

Discoloration of reactive orange 4 dye was also optimized by RSM-Box-Behnken design under different cultural and nutritional conditions using *Pseudomonas putida* SKG-1 strain. Such experimental design allowed a 97.8% discoloration of dye in 72 h of incubation period. Furthermore, dye discoloration was also studied in bioreactor which gave 98% efficiency in 60 h of incubation period (Garg et al., 2015).

These observations contribute significantly to the understanding of field conditions for degradation of sodium benzoate. It is believed that degradable hydrocarbon would be taken care by the microbial community present in either effluent stream, wastewater treatment plant, soil, or sludge. The results obtained strongly stand for the organism's behavior of selecting preferential carbon sources over hydrocarbons, and thus, leading to delayed initiation or negligible SB degradation. This indicates that the given resident time in the treatment plant or at any contaminated site may not always result in the removal of even degradable fraction of hydrocarbons or pollutants. After realization of negative influence of the presence of multiple C sources on SB degradation, another PBD experiment was designed to investigate the effects of diverse nitrogen sources (design B).

Treatment of contaminants with preferable nitrogen source could decrease lag phase and increase cell growth and bioremediation activities. Nitrogen is most often the limiting nutrient affecting biodegradation of hydrocarbon. However, it was also reported that excess amount of nitrogen source inhibits biodegradation rate in polluted soil due to osmotic soil water potential depression (Walworth et al., 2007). In the present study, it was observed that with nitrogen and minerals, lower concentration level (−1) of NaCl, Na_2HPO_4, cysteine, and $MgSO_4$ resulted in maximum SB degradation, i.e., 87% (run number 11 in **Table 3**). In contrast to the observation with C sources, in the case of the second set of experiments, occurrence of multiple N sources did not influence SB degradation negatively. This study did not reveal the preferential utilization of any N source over others for SB degradation. Another observation was the absence of delay/lag in the utilization of SB as seen in the case of the first PBD design and could be achieved within 24 h optimum SB degradation.

Similar to the first PBD design, the role of inorganic micronutrients also came out to be statistically significant for SB degradation in the presence of various N sources.

Interaction of Concentrations of Micronutrients Revealed by CCD

For next level of steep ascent focusing, two factors KH_2PO_4 and $MgSO_4$ exhibiting main effects in Plackett-Burman design and NH_4Cl were taken for CCD development. These experiments were aimed to meticulously optimize the addition of these nutrients for sodium benzoate degradation. Lakshmi et al. (2013) presented reports comprising optimization of phenanthrene degradation where, in addition to substrate concentration as a variable, Na_2HPO_4, $MgSO_4$, and $FeSO_4$ were found to be the most important factors for degradation. Similarly, importance and optimization of micronutrients has been emphasized in several other degradation studies using statistical tools (Mohajeri et al., 2010). Likewise, in a study focused on increasing extracellular nuclease, *Nuc*B, from *Bacillus licheniformis*, addition of manganese had a stimulating effect with a 10-fold increase in *Nuc*B activity. On the other hand, phosphate availability had an inhibitory effect on *Nuc*B synthesis (Rajarajan et al., 2013). The importance of micronutrients, magnesium and phosphate, has been emphasized for various enzyme metabolisms like enhancing the expression of amylase enzyme in *A. oryzae* CBS 819.72 (Kammoun et al., 2008) and shikimic acid production in *Citrobacter freundii* GR-21 (KC466031) (Rawat et al., 2013).

In one of the major observations, it was revealed that higher NH_4Cl concentrations could ameliorate inhibitory effect of higher concentration of phosphate. Similar inhibitory effect of phosphate is observed in **Figure 4C**. Looking to the steepness of the curve at high concentration of phosphate, addition of even high concentration of $MgSO_4$ may not help to increase sodium benzoate degradation. Farag et al. (2018) investigated the role of $MgSO_4$ in crude oil biodegradation process by *Pseudomonas* sp. sp48 using response surface method (Box-Behnken design). This study revealed that the lower concentration of $MgSO_4$ was the best for the optimal crude oil biodegradation process.

Contour of response displayed a step-wise change in colors toward green, indicating a decrease in the degradation percentage with increase in KH_2PO_4 concentrations. Similarly, diesel oil degradation was optimized using PB and RSM (CCD) where three parameters, i.e., P source (KH_2PO_4), pH, and N source ($NaNO_3$) were major influencers affecting oil degradation. It was observed that middle range of KH_2PO_4 concentration (0.022 g) was sufficient for the ideal diesel biodegradation. The optimal diesel oil biodegradation (total petroleum hydrocarbons 125 mg L^{-1}) was obtained with 0.143 g of $NaNO_3$, 0.22 g of KH_2PO_4, and at 7.4 pH. These results demonstrated the cellular levels of C, N, and P ratios required for the significant biodegradation rate (Xia et al., 2012). This observation would help in modulating the concentration of required nutrient in field conditions while facing shock loads of phosphate. Such detailed analysis of bioprocess and optimization may lead to unfolding of more interaction of nutrient and may help in improving the performance of laboratory-optimized studies in the field.

The next question arises for the type of pathway and the mechanism involved for sodium benzoate degradation available with *P. citronellolis* which may enhance our understanding about the complete role of nutrients in the regulation of the degradation process. The molecular details of generalized pathway utilized by aerobic bacteria are widely known and are available with KEGG database. However, the associated mechanism for sodium benzoate degradation by *P. citronellolis* is being explored by studying specific enzymatic reactions, transcriptome analysis, and gene expression studies.

CONCLUSION

Statistical methods, PB and RSM enabled to understand the interplay of additional carbon and nitrogen sources as well as micronutrients on sodium benzoate degradation by *P. citronellolis.* Being vulnerable to biological degradation, even sodium benzoate degradation faced severe competition due to the co-existence of other carbon sources. Micronutrients turned out to be the major influencing factors, of which NH_4Cl and $MgSO_4$ were positively influencing whereas KH_2PO_4 was negatively influencing sodium benzoate degradation. However, the negative effects of KH_2PO_4 could be nullified by higher amount of NH_4Cl. Statistically significant model was developed for SB degradation where the desirable concentrations of micronutrients were derived. With the ability to quantitatively describe the interaction effects of multiple nutrients on the system response, DOE and statistical modeling helps in strategically designing bioprocesses for successful full-scale application of bioremediation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

NM and PZ conceptualized the research work. PZ executed in laboratory, prepared the draft of manuscript. NM edited the manuscript. RP and AI helped in performing the statistical and data analysis as well as in preparation of manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was partly supported by the Department of Biotechnology (DBT), Govt. of India, Delhi through a project (Grant NO. BT/PR-13691/BCE/08/797/2010 dated 14 Jun 2011).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.634753/full#supplementary-material

REFERENCES

Abdel-Fattah, Y. R., Saeed, H. M., Gohar, Y. M., and El-baz, M. A. (2005). Improved production of *Pseudomonas aeruginosa* uricase by optimization of process parameters through statistical experimental designs. *Proc. Biochem.* 40, 1707–1714. doi: 10.1016/j.procbio.2004.06.048

Abdel-Fattah, Y. R., Soliman, N. A., and Berekaa, M. M. (2007). Application of box-behnken design for optimization of poly-(-glutamic acid production by *Bacillus licheniformis* SAB-26. *Res. J. Microbiol.* 2, 664–670.

Atlas, R. M. (2005). *Handbook of Media for Environmental Microbiology*. Boca Raton, FL: Taylor and Francis, doi: 10.1201/9781420037487

Ayed, L., Ksibi, I., Cheref, A., and Bakhrouf, A. (2012). Response surface methodology for optimization of the treatment of textile wastewater by a novel bacterial consortium: Enzymes and metabolites characterization. *Afr. J. Biotechnol.* 11, 12339–12355. doi: 10.5897/ajb11.3506

Burman, R. L., and Plackett, J. P. (1946). The design of optimum multifactorial experiments. *Biometrika* 33, 305–325. doi: 10.2307/2332195

Chen, H., Niu, J., Qin, T., Ma, Q., Wang, L., and Shu, G. (2015). Optimization of the medium for *Lactobacillus acidophilus* by plackett-burman and steepest ascent experiment. *Acta Scientiarum/ Polonorum Technol. Aliment* 14, 227–232. doi: 10.17306/J.AFS.2015.3.24

Chun, M., Chngchun, S., Yanghao, G., Xian, S., Jianfeng, C., and Fen, Y. (2004). Study on characteristics of biocometabolic removal of omethoate by the *Aspergillus spp. Water Res.* 38, 1139–1146. doi: 10.1016/j.watres.2003.11.026

Darvishmotevalli, M., Zarei, A., Moradnia, M., Noorisepehr, M., and Mohammadi, H. (2019). Optimization of saline wastewater treatment using electrochemical oxidation process: prediction by RSM method. *MethodsX* 6, 1101–1113. doi: 10.1016/j.mex.2019.03.015

Das, N., and Chandran, P. (2011). Microbial degradation of petroleum hydrocarbon contaminants: an overview. *Biotechnol. Res. Int.* 2011, 1–13. doi: 10.4061/2011/941810

de Guillén-Jiménez, F. M., Cristiani-Urbina, E., Cancino-Díaz, J. C., Flores-Moreno, J. L., and Barragán-Huerta, B. E. (2012). Lindane biodegradation by the *Fusarium verticillioides* AT-100 strain, isolated from *Agave tequilana* leaves: kinetic study and identification of metabolites. *Int. Biodeterior. Biodegradation* 74, 36–47. doi: 10.1016/j.ibiod.2012.04.020

Demir, T., Hameş, E. E., Öncel, S. S., and Vardar-Sukan, F. (2015). An optimization approach to scale up keratinase production by *Streptomyces sp*. 2M21 by utilizing chicken feather. *Int. Biodeterior. Biodegradation* 103, 134–140. doi: 10.1016/j.ibiod.2015.04.025

Dibblef, J. T., and Bartha, R. (1979). Effect of environmental parameters on the biodegradation of oil sludge. *Appl. Environ. Microbiol.* 37, 729–739.

El-Hamid, H. T. A., Al-Prol, A. E., and Hafiz, M. A. (2018). Plackett-Burman and response surface methodology for optimization of oily wastewater bioremediation by *Aspergillus sp. South Asian J. Res. Microbiol.* 2, 1–9. doi: 10.9734/sajrm/2018/v2i129242

Farag, S., Soliman, N. A., and Abdel-Fattah, Y. R. (2018). Statistical optimization of crude oil bio-degradation by a local marine bacterium isolate *Pseudomonas sp*. sp48. *J. Genet Eng. Biotechnol.* 16, 409–420.

Freedman, D. L., Payauys, A. M., and Karanfil, T. (2005). The effect of nutrient deficiency on removal of organic solvents from textile manufacturing wastewater during activated sludge treatment. *Environ. Technol.* 26, 179–188. doi: 10.1080/09593332608618570

Garg, S. K., Tripathi, M., and Lal, N. (2015). Response surface methodology for optimization of process variable for reactive orange 4 dye discoloration by *Pseudomonas* putida SKG-1 strain and bioreactor trial for its possible use in large-scale bioremediation. *Desalinat. Water Treatment* 54, 3122–3133. doi: 10.1080/19443994.2014.905975

Gutierrez-Rojas, I., Torres-Geraldo, A. B., and Moreno-Sarmiento, N. (2011). Optimizing carbon and nitrogen sources for *Azotobacter chroococcum* growth. *Afr. J. Biotechnol.* 10, 2951–2958. doi: 10.5897/AJB10.1484

Halder, S. K., Maity, C., Jana, A., Das, A., Paul, T., Das Mohapatra, P. K., et al. (2013). Proficient biodegradation of shrimp shell waste by *Aeromonas hydrophila* SBK1 for the concomitant production of antifungal chitinase and antioxidant chitosaccharides. *Int. Biodeterior. Biodegradation* 79, 88–97. doi: 10.1016/j.ibiod.2013.01.011

Hashem, R. A., Samir, R., Essam, T. M., Ali, A. E., and Amin, M. A. (2018). Optimization and enhancement of textile reactive Remazol black B decolorization and detoxification by environmentally isolated pH tolerant *Pseudomonas aeruginosa* KY284155. *AMB Express* 8:83. doi: 10.1186/s13568-018-0616-1

Ibn Abubaker, B. S. U., Abdullah, N., Idris, A., Zakaria, M. P., and Abdul Shokur, M. Y. (2012). Optimization of pyrene removal by a mixed culture in a soil-slurry bioreactor using Response Surface Methodology (RSM). *Procedia Eng.* 50, 785–799. doi: 10.1016/j.proeng.2012.10.087

John, A. C. St, and Goldberg, A. L. (1980). Effects of starvation for potassium and other inorganic ions on protein degradation and Ribonucleic Acid synthesis in *Escherichia coli*. *J. Bacteriol.* 143, 1223–1233.

Jonsson, A. P., Bylund, D., Lundstro, U. S., and Tomas, L. O. (2007). The effects of carbon sources and micronutrients in fermented whey on the biodegradation of n-hexadecane in diesel fuel contaminated soil. *Int. Biodeterior. Biodegradation* 60, 334–341.

Junca, H., and Pieper, D. H. (2004). Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries. *Environ. Microbiol.* 6, 95–110. doi: 10.1046/j.1462-2920.2003.00541.x

Kammoun, R., Naili, B., and Bejar, S. (2008). Application of a statistical design to the optimization of parameters and culture medium for (-amylase production by *Aspergillus oryzae* CBS 819.72 grown on gruel (wheat grinding by-product). *Bioresour. Technol.* 99, 5602–5609. doi: 10.1016/j.biortech.2007.10.045

Lakshmi, M. B., Muthukumar, K., Velan, M., and Patel, S. (2013). Optimization of minimal salt medium for efficient phenanthrene biodegradation by *Mycoplana sp*. MVMB2 isolated from petroleum contaminated soil using factorial design experiments. *Clean Soil Air Water* 41, 51–59. doi: 10.1002/clen.201000208

Leahy, J. G., and Colwell, R. R. (1990). Microbial degradation of hydrocarbons in the environment. *Microbiol. Rev.* 54, 305–315.

Li, Y., Lei, Z., Zhang, Z., and Sugiura, N. (2010). Effects of nutrient addition on phenol biodegradation rate in biofilm reactors for hypersaline wastewater treatment. *Environ. Technol.* 27, 511–520. doi: 10.1080/09593332808618662

Meng, C., He, Q., Huang, J. W., Cao, Q., Yan, X., Li, S. P., et al. (2015). Degradation of chlorothalonil through a hydrolytic dehalogenase secreted from *Bacillus subtilis* WB800. *Int. Biodeteriorat. Biodegradation* 104, 97–104. doi: 10.1016/j.ibiod.2015.05.017

Mohajeri, L., Abdul, H., Hasnain, M., and Ali, M. (2010). A statistical experiment design approach for optimizing biodegradation of weathered crude oil in coastal sediments. *Bioresour. Technol.* 101, 893–900. doi: 10.1016/j.biortech.2009.09.013

Montgomery, D. C. (2017). *Design and Analysis of Experiments*. New York, NY: John Wiley and Sons.

Östberg, T. L., Jonsson, A. P., Bylund, D., and Lundström, U. S. (2007). The effects of carbon sources and micronutrients in fermented whey on the biodegradation of n-hexadecane in diesel fuel contaminated soil. *Int. Biodeteriorat. Biodegradation* 60, 334–341. doi: 10.1016/j.ibiod.2007.05.007

Patil, S. S., and Jena, H. M. (2015). Statistical optimization of phenol degradation by *Bacillus pumilus* OS1 using plackett–burman design and response surface methodology. *Arab. J. Sci. Eng.* 40, 2141–2151. doi: 10.1007/s13369-015-1765-z

Priyadharshini, D. S., and Bakthavatsalam, A. K. (2016). Optimization of phenol degradation by the microalga *Chlorella pyrenoidosa* using plackett-burman design and response surface methodology. *Bioresour. Technol.* 207, 150–156. doi: 10.1016/j.biortech.2016.01.138

Rajarajan, N., Ward, A. C., Burgess, J. G., and Glassey, J. (2013). Use of physiological information and process optimisation enhances production of extracellular nuclease by a marine strain of *Bacillus licheniformis*. *Bioresour. Technol.* 130, 552–558. doi: 10.1016/j.biortech.2012.12.064

Rajendran, A., Sundaramurthy, A. B., and Thangavelu, V. (2007). Statistical evaluation of medium components using Plackett-burman experimental design and kinetic modelling of lipase production by *Bacillus sphaericus*. *Ind. J. Biotechnol.* 21, 181–188.

Rawat, G., Tripathi, P., Yadav, S., and Saxena, R. K. (2013). An interactive study of influential parameters for shikimic acid production using statistical approach, scale up and its inhibitory action on different lipases. *Bioresour. Technol.* 144, 675–679. doi: 10.1016/j.biortech.2013.06.113

Rossello-mora, R. A., Lalucat, J., and Garcia-valdes, E. (1994). Comparative biochemical and genetic analysis of naphthalene degradation among *Pseudomonas stutzeri* strains. *Appl. Environ. Microbiol.* 60, 966–972.

Safa, Z. J., Aminzadeh, S., Zamani, M., and Motallebi, M. (2017). Significant increase in cyanide degradation by *Bacillus sp*. M01 PTCC 1908 with response surface methodology optimization. *AMB Express* 7:200. doi: 10.1186/s13568-017-0502-2

Schmidt, S. K., and Alexander, M. (1985). Effects of dissolved organic carbon and second substrates on the biodegradation of organic compounds at low concentrations. *Appl. Environ. Microbiol.* 49, 822–857.

Steffensen, W. S., and Alexander, M. (1995). Role of competition for inorganic nutrients in the biodegradation of mixtures of substrates. *Appl. Environ. Microbiol.* 61, 2859–2862.

Su, W. T., and Lin, C. H. (2013). Fungal-bacterial synergism enhanced decolorization of reactive red 120 by response surface methodology. *Int. Biodeterior. Biodegradation* 82, 1–8. doi: 10.1016/j.ibiod.2013.02.008

Swindoll, C. M., Aelion, C. M., and Pfaender, F. K. (1988). Influence of inorganic and organic nutrients on aerobic biodegradation and on the adaptation response of subsurface microbial communities. *Appl. Environ. Microbiol.* 54, 212–217.

Walworth, J., Pond, A., Snape, I., Rayner, J., Ferguson, S., and Harvey, P. (2007). Nitrogen requirements for maximizing petroleum bioremediation in a sub-Antarctic soil. *Cold Reg. Sci. Technol.* 48, 84–91. doi: 10.1016/j.coldregions.2006.07.001

Wibbertmann, A., Kielhorn, G., Koennecker, J., Mangelsdorf, I., and Melber, C. (2005). *Benzoic Acid and Sodium Benzoate*. Geneva: Concise International Chemical Assessment Document.

Wigginst, B. A., and Alexander, M. (1988). Role of chemical concentration and second carbon sources in acclimation of microbial communities for biodegradation. *Appl. Environ. Microbiol.* 54, 2803–2807.

Xia, W., Li, J., Xia, Y., Song, Z., and Zhou, J. (2012). Optimization of diesel oil biodegradation in seawater using statistical experimental methodology Water Sci. *Technology* 66, 1301–1309.

Yan, J., Chen, D., Yang, E., Niu, J., Chen, Y., and Chagan, I. (2014). Purification and characterization of a thermotolerant laccase isoform in *Trametes trogii* strain and its potential in dye decolorization. *Int. Biodeterior. Biodegradation* 93, 186–194. doi: 10.1016/j.ibiod.2014.06.001

Yaseen, D. A., and Scholz, M. (2019). Textile dye wastewater characteristics and constituents of synthetic effluents: a critical review. *Int. J. Environ. Sci. Technol.* 16:1193–1226. doi: 10.1007/s13762-018-2130-z

Yuan, L., Li, Y., Wang, Y., Zhang, X., and Xu, Y. (2008). Optimization of critical medium components using response surface methodology for phenazine-1-carboxylic acid production by *Pseudomonas sp*. M-18O. *J. Biosci. Bioeng.* 105, 232–237. doi: 10.1263/jbb.105.232

Zahed, A. M., Aziz, A. H., Mohajeri, L., Mohajeri, S., Kutty, R. S. M., and Isa, H. M. (2010). Application of statistical experimental methodology to optimize bioremediation of n-alkanes in aquatic environment. *J. Hazardous Mater.* 184, 350–356. doi: 10.1016/j.jhazmat.2010.08.043

Zaveri, P., Munshi, N., Vaidya, A., Jha, S., and Naresh Kumar, G. (2015). Functional microbial diversity dynamics in common effluent treatment plants of South Gujarat and hydrocarbon degradation. *Can. J. Microbiol.* 61, 389–395. doi: 10,1139/cjm2014-0700

Zhang, C., Zhang, Y., Zhuang, B., and Zhou, X. (2014). Strategic enhancement of algal biomass, nutrient uptake and lipid through statistical optimization of nutrient supplementation in coupling Scenedesmus obliquus-like microalgae cultivation and municipal wastewater treatment. *Bioresour. Technol.* 171, 71–79. doi: 10.1016/j.biortech.2014.07.060

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

MINI REVIEW
published: 25 March 2021
doi: 10.3389/fmicb.2021.651025

Current Trends on Role of Biological Treatment in Integrated Treatment Technologies of Textile Wastewater

Maria Belen Ceretta[1,2], Débora Nercessian[3] and Erika A. Wolski[1,2]*

[1] Biochemical Engineering Group, Institute of Science and Technology of Food and Environment (INCITAA), Faculty of Engineering, National University of Mar del Plata, Mar del Plata, Argentina, [2] National Scientific and Technical Research Council (CONICET), Ministry of Science, Technology and Innovation, Buenos Aires, Argentina, [3] Institute of Biological Research (IIB), CONICET, National University of Mar del Plata, Mar del Plata, Argentina

Wastewater discharge is a matter of concern as it is the primary source of water pollution. Consequently, wastewater treatment plays a key role in reducing the negative impact that wastewater discharge produce into the environment. Particularly, the effluents produced by textile industry are composed of high concentration of hazardous compounds such as dyes, as well as having high levels of chemical and biological oxygen demand, suspended solids, variable pH, and high concentration of salt. Main efforts have been focused on the development of methods consuming less water or reusing it, and also on the development of dyes with a better fixation capacity. However, the problem of how to treat these harmful effluents is still pending. Different treatment technologies have been developed, such as coagulation-flocculation, adsorption, membrane filtration, reverse osmosis, advanced oxidation, and biological processes (activated sludge, anaerobic-aerobic treatment, and membrane bioreactor). Concerning to biological treatments, even though they are considered as the most environmentally friendly and economic methods, their industrial application is still uncertain. On the one hand, this is due to the costs of treatment plants installation and, on the other, to the fact that most of the studies are carried out with simulated or diluted effluents that do not represent what really happens in the industries. Integrated treatment technologies by combining the efficiency two or more methodologies used to be more efficient for the decontamination of textile wastewater, than treatments used separately. The elimination of hazardous compounds had been reported using combination of physical, chemical, and biological processes. On this way, as degradation products can sometimes be even more toxic than the parent compounds, effluent toxicity assessment is an essential feature in the development of these alternatives. This article provides a critical view on the state of art of biological treatment, the degree of advancement and the prospects for their application, also discussing the concept of integrated treatment and the importance of including toxicity assays to reach an integral approach to wastewater treatment.

Keywords: textile wastewater, biological treatment, simulated effluents, real effluents, toxicity

OPEN ACCESS

Edited by:
Eric D. van Hullebusch, Université de Paris, France

Reviewed by:
Marta Gmurek, Łódź University of Technology, Poland
Gaurav Saxena, Jawaharlal Nehru University, India

****Correspondence:***
Maria Belen Ceretta
mbceretta@mdp.edu.ar

Specialty section:
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

Received: *08 January 2021*
Accepted: *22 February 2021*
Published: *25 March 2021*

Citation:
Ceretta MB, Nercessian D and Wolski EA (2021) Current Trends on Role of Biological Treatment in Integrated Treatment Technologies of Textile Wastewater. Front. Microbiol. 12:651025. doi: 10.3389/fmicb.2021.651025

INTRODUCTION

There is an increasing awareness of how our actions impact the environment and that is why in the last decades a great number of environmentally friendly strategies and technologies have been developed. In addition, and as a consequence of social demand, environmental protection policies have been turning more rigorous. Industrial activity is not exempt and it is changing toward sustainable production models. Faced with this new paradigm, textile industry represents a hot spot since it is not only responsible for generating large volumes of effluents, but being also highly toxic. Among the physicochemical characteristics, the composition of the effluents is highly variable; being the concentration of the parameters very different between the studies available in literature. This type of effluent are characterized for having a wide range of pH between values of 5 and 12 (Singh and Singh, 2017; Yaseen and Scholz, 2019; Paździor and Bilińska, 2020; Samuchiwal et al., 2021), elevated chemical oxygen demand (COD) from 10 to 2250 $mgO_2\ L^{-1}$ (Ghaly et al., 2014; Singh and Singh, 2017; Paździor and Bilińska, 2020; Samuchiwal et al., 2021), biochemical oxygen demand (BOD) among 100 and 3000 $mgO_2\ L^{-1}$ (Işik and Sponza, 2004; Paździor and Bilińska, 2020), suspended solids, heavy metals and salts (Saratale et al., 2011; Singh and Singh, 2017; Vikrant et al., 2018; Yaseen and Scholz, 2019). Regarding to dye concentration, they are highly colored. For example, concentration of dyes varies from 10 to 250 mg L^{-1} (Ghaly et al., 2014), however, concentrations up to 600–800 mg L^{-1} have been found, and the same variability is usually found for COD and BOD (Yaseen and Scholz, 2019). This variability is a matter of concern, since investigations for the development of treatment technologies are mostly carried out with simulated effluents. Therefore, this represents an obstacle when the treatments developed at laboratory scale with simulated effluents must be applied since there is no specific composition of textile wastewater.

Otherwise, textile production must overcome a series of challenges including the development of dyes with better fixation capacity, reduction of water use, reuse of water, reduction of costs, improvement of the finish of garments and treatment processes, all of them tending to achieve a more sustainable process. Consequently, the approach to the problem must be multifactorial if we are looking to solve the impact that textile discharges cause on the environment. Not only the methodologies and models used in research must be adapted, but there must be a decision on the industrial sector, accompanied by the creation of public policies, that promote the development of these technologies.

Reasons for the change are more than enough since the negative effects in the environment are widely known. The degree of damage depends on the volume, composition and duration of the discharged wastewater. This can modify the penetration of sunlight into water bodies and change solubility of atmospheric gases, altering photosynthesis and therefore the entire aquatic ecosystem (O'Neill et al., 1999; Saratale et al., 2011; Gonçalves et al., 2017; Rather et al., 2018). In addition, the toxic, mutagenic and carcinogenic effect of several compounds present in the effluents, such as dyes, have been demonstrated (Saratale et al., 2011; Dhaouefi et al., 2018). Moreover, withdrawal of heavy metals by some chemicals used as mordents, eventually increases wastewater toxicity (Dhaouefi et al., 2018).

Since many decades ago physicochemical and biological technologies are being developed for textile wastewater treatment. Physicochemical ones have the disadvantage of being expensive; they have high chemical and energy requirements, and produce secondary byproducts, which can endanger their full-scale implementation (Dhaouefi et al., 2018). In some cases, these methods such as adsorption, chemical precipitation or electrochemical precipitation are not able to remove compounds like dyes or their metabolites from the wastewater, only transferring the pollutant from one phase to another, (Hayat et al., 2015; Singh and Singh, 2017), thus failing to solve the problem. Otherwise, biological methods are economic, simple, and environmentally friendly alternatives. Although significant toxicity reduction is observed with these treatments, pollutants mineralization is rarely achieved. As well, microorganism's growth and catalytic activity is often inhibited by the presence of toxic substances, making difficult the extrapolation to-large-scale processes. This leads to the need of modify the natural effluent conditions to carry out research, as for example the use of simulated or diluted effluents, the addition of nutritional supplements and/or previous acclimatization. However, despite the related problems, the treatment of textile wastewater is required not only to do no harm the environment, but also to recover the water from the wastewater and recycle it for irrigation or reuse indoors the factory (Yaseen and Scholz, 2019).

REGARDING BIOLOGICAL TREATMENTS

When we talk about biological treatment, we refer to strategies that use the abilities of microorganisms, plants or enzymes to remove (either by degradation or sorption) a contaminant from a matrix such as soil, sediment, air, or water (Singh and Singh, 2017), and therefore minimize the negative impact in the environment by reducing its toxicity. Among them, treatments that use microorganisms (bacteria, algae, and fungi) are the most widespread.

There is a wide range of references in which microorganisms in pure cultures are used; an *Aeromonas hydrophila* was able to reduce 72% of dyes presents in a simulated wastewater (Thanavel et al., 2019). In other work, a *Trichoderma tomentosum* reached a decolorization of 94.9% in real diluted wastewater and simulated effluent (He et al., 2018). However, nutritional supplements (such as the addition of some carbon or nitrogen sources) and/or previous acclimatization are frequently required in this kind of methodologies to improve results. Even more, achieving the sterile conditions necessary to maintain pure cultures would not be feasible due to the high costs that this would imply (Paździor et al., 2019).

In recent years, the use of mixed microbial cultures (combining several bacterial and/or fungal strains), consortia or microbial communities has gained relevance. Microbial communities have the ability to utilize many different carbon sources by expressing a range of metabolic capabilities

(Eiler, 2006; Shade et al., 2012). As a result of the species diversity and the cooperation between them, the effluent molecules can be attacked at several sites by different strains and the decomposition products generated by the metabolic activity of one strain, may be used as a substrate by the other one (Eiler, 2006; Dafale et al., 2008; Holkar et al., 2016). This gives them a central role in a wide variety of biotechnological applications, like textile wastewater treatment. In contrast, pure cultures are associated to specific compounds, being less versatile in terms of their biodegradation ability (Jadhav et al., 2010; Paździor et al., 2019).

Despite the great advances in the efficiency of the developed biological treatments, there are still some limitations, since most of the researches (80%) are carried out with simulated or synthetic effluents and by the addition of nutritional sources (**Table 1**). For example, microbial community found by Chen et al. (2019) could reach a decolorization efficiency between 71.3 and 96.3% in a synthetic effluent (600 mg L^{-1} dye concentration), but just after the addition of 3.00 g L^{-1} of beef extract, 5.00 g L^{-1} of glucose, and 2.00 g L^{-1} peptone. Other works used even 10 g L^{-1} of glucose (Waghmode et al., 2019). Although good results are achieved in terms of reducing the chemical oxygen demand (COD) and decolorization, usually these values are for dye concentration that does not exceed the 200 mg L^{-1}, and as was shown earlier dye concentration in real textile wastewater can reach until 800 mg L^{-1}. In addition, it should be noted that reactor volumes in many cases are small (Valli Nachiyar et al., 2016; Abd El-Rahim et al., 2017; Hossen et al., 2019; Zahran et al., 2019). As an example, Hossen et al. (2019) studied the decolorization efficiency of different strains in simulated effluents, some of which reached efficiencies of even 90%. Since these results are promising, they were achieved using a batch reactor of 50 mL, a volume that is so far from the real one. This deviation from real situation of textile wastewater makes its subsequent application difficult. Although it is true that keeping the composition of the untreated synthetic wastewater constant is necessary to make valid comparisons of treatment system evaluations (O'Neill et al., 1999), after several decades and thousands of studies that validate the efficiency of biological treatments, it is necessary to leave behind the experimental comfort of simulated effluents to advance one more step in the development of strategies based in real effluents to begin their application.

There are a few cases where real textile wastewater is used (**Table 1**). Samuchiwal et al. (2021) used a microbial consortium to degrade real wastewater, but a significant decreases of color of 70–73% was only achieved by the addition of yeast extract as external input and Pre-Treatment Range (PTR) effluent (with starch) as a carbon source. In the case reported by Ceretta et al. (2018), undiluted real textile wastewater was used, without the addition of an extra nutritional source. The percentage of decolorization achieved was approximately 77% of an effluent with 200 mg L^{-1} dye concentration (Ceretta et al., 2018). Nonetheless, the volume of the treated effluent is still small (120 mL) and there are no larger-scale studies about it.

Paździor and Bilińska (2020) analyzed an example of industrial textile wastewater treatment plant large-scale for the treatment of real undiluted textile wastewater. Despite the overall COD removal efficiency exceeded 93% and varied in a narrow range, the authors concluded that wastewater is only partially biodegradable. Also, the treatment plant consists in a 10 step system which raises installation costs (Paździor and Bilińska, 2020). Therefore, analyzing these reports, first we can conclude that the combination of more than one treatment methodology it would be necessary to achieve a better performance, by combining the degradation efficiency of more than one method. Second, that all the reviewed works have strengths but also weaknesses, either due to the scale in which they are carried out, the need for external inputs, pre-treatments or the use of simulated or diluted effluents. Clearly, textile effluents represent a challenge that has not yet been overcome.

IMPORTANCE OF TOXICITY ASSESSMENT

The removal efficiency of the pollutants in a wastewater depends on the treatment technology, the retention time and on the effluent composition. However, the decrease of the parameters could not be directly related to toxicity reduction as the byproducts could be more toxic than the parental ones. For these reasons, it is important to measure toxicity reduction of the treated wastewater by direct toxicity assays. These can warn the adverse effects of wastewater containing a mixture of pollutants. Nevertheless, a key issue represents which assay will be used. There are many classes of toxicity assays depending on the way by how the effluent will be discharged to the environment and use for example bacteria, microalgae, invertebrates, plants and fishes as sensor organisms. Many studies not only analyze organism growth rate but also mutagenic capacity of pollutants by genotoxicity tests performed on different cell lines. Also, it is important to note that most of the studies used synthetic or simulated wastewater, which may not be reflecting what could happen in a real situation with a real wastewater.

Table 2 shows examples of different toxicity assays performed. Phytotoxicity is a very fast an economic method to test toxicity. It measures seed germination, root elongation, seedling development as main parameters and is the most widespread assay (Valli Nachiyar et al., 2016; Ceretta et al., 2018; Chen et al., 2018; He et al., 2018; Waghmode et al., 2019). For other toxicity tests, as genotoxicity and zootoxicity, there are fewer studies available in literature (Paździor et al., 2017; Dhaouefi et al., 2018; Przystaś et al., 2018; Oliveira et al., 2020). The comparison of the results of phytotoxicity assay with other toxicity test, such as zootoxicity for the same treated wastewater showed differences. For example, Oliveira et al. (2020) showed no reduction of the phytotoxicity, while for zootoxicity a maximum reduction of 47% was obtained. The same was observed for the study of Przystaś et al. (2018), but with opposite results. In addition to these observations, phytotoxicity analysis of wastewater treated only by biological methods, most of the times showed about 50% or less in toxicity reduction, while when biological treatments are combined with other treatments, the toxicity reduction increase (Dhaouefi et al., 2018; Waghmode et al., 2019).

TABLE 1 | Comparison among different biological treatments of textile wastewater.

Effluent type	Dye concentration (mg L^{-1})	Organism	Biological treatment efficiency	References
Synthetic	32.5	Bacterial consortium	90–73.1% COD removal	Oliveira et al., 2020
Synthetic	200	Microbial consortium	31–70% COD removal	Waghmode et al., 2019
Synthetic	600	Bacterial consortium	71.3–96.3% decolorization	Chen et al., 2019
Synthetic	100	*Aeromonas hydrophila*	72% decolorization	Thanavel et al., 2019
Synthetic	200	*Alcaligenes faecalis, B. cereus, Bacillus* ssp.	90% decolorization	Hossen et al., 2019
Real	200	Microbial consortium	77.6% decolorization	Ceretta et al., 2018
Real	n.d.	Activated sludge	10–72% decolorization	Paździor and Bilińska, 2020
Real	n.d.	Bacterial consortium	70–73% decolorization	Samuchiwal et al., 2021
Synthetic and diluted real	100	*Trichoderma tomentosum*	94.9% decolorization	He et al., 2018
Synthetic	100	Bacterial consortium	97% decolorization	Chen et al., 2018
Synthetic and real	100	Microbial consortium	78% COD removal	Kurade et al., 2017

TABLE 2 | Comparison of toxicity analysis in different researches carried out with biological treatment in textile wastewater.

Toxicity assay	Effluent type	Treatment	Toxicity reduction	References
Zootoxicity test (*Daphnia magna*) phytotoxicity test (*Lactuca sativa*)	Synthetic	Bacterial consortium	10–47% (*Daphnia magna*) 0% (*Lactuca sativa*)	Oliveira et al., 2020
Phytotoxicity *Sorghum vulgare* and *Phaseolus mungo*	Synthetic	Sequential photocatalytic and biological treatment	100% (*Phaseolus mungo*) and 90% (*Sorghum vulgare*)	Waghmode et al., 2019
Phytotoxicity *Glycine max* and *Adenanthera microsperma*	Synthetic	*Trichoderma tomentosum*	Not significant	He et al., 2018
Genotoxicity test and Phytotoxicity test (*Raphanus sativus*)	Synthetic	Anoxic-aerobic photobioreactor	Non-genotoxic and enhanced *R. sativus* seedlings	Dhaouefi et al., 2018
Phytotoxicity (*Lactuca sativa*)	Real	Bacterial consortium	50%	Ceretta et al., 2018
Zootoxicity (*Daphnia magna*) and phytotoxicity (*Lemna sp.*)	Synthetic	Pure cultures of fungi *(P. ostreatus, Gloeophyllum odoratum* and *Polyporus picipes)*	Significant reduction for phytotoxicity and a slight decrease for zootoxicity. Variable result depending on the fungus and the support used.	Przystaś et al., 2018
Phytotoxicity Black beans and Rice	Synthetic	Bacterial consortium	50–56%	Chen et al., 2018
Microtox toxicity test (*Vibrio fischeri*)	Real	Microbial consortium	96–98%	Paździor et al., 2017
Phytotoxicity (*Vigna radiata* and *Triticum aestivum*)	Synthetic and real diluted	Bacterial consortium	30% (*Vigna radiata*) and 45% (*Triticum aestivum*)	Valli Nachiyar et al., 2016

Yu et al. (2019) studied the correlation between the toxicity reduction of 12 wastewater treatment plants, from an industrial park, with the treatment process. They used a group of toxicity assays involving microorganism, phytoplankton, zooplankton, plant, and human cell lines. All the influents of wastewater treatment plants induced high toxicities. Seven from all the studied wastewater treatment plants showed a significant toxicity reduction after the treatment. However, the effluents of five of them induced higher toxicity in one or more toxic endpoints compared to the influents. Also, among all toxic endpoints evaluated low correlation coefficients were obtained, indicating that set of toxicity assays was necessary to completely characterize the toxicity and risk of wastewater in industrial parks.

Finally, a more careful analysis has to be done about the toxicity of the wastewater and that it is not enough to choose just a toxicity assay. It is important to select the test taking into account the final objectives of the treatment. For example, if the aim is to reuse the water for irrigation purpose, it will be convenient to use phytotoxicity assays, if the aspiration is the final discharge into water bodies, it may be more appropriate zootoxicity test with aquatic organisms.

AN INTERDISCIPLINARY APPROACH FOR A GENUINE TEXTILE WASTEWATER TREATMENT

Currently, focus is not on technologies that degrade and decrease color, rather on those that can produce reusable water, recuperate salt and/or dyes, fully mineralize the target contaminant and mainly reduce toxicity (Holkar et al., 2016). As we have seen so far, there are a huge diversity treatment types with their strengths and weaknesses, and when they are used separately the mentioned aims can rarely be achieved. Last years, researchers have been working in the development of sustainable water treatment strategies that efficiently combined physical, chemical and biological processes for the treatment of different wastewaters. The design of this integrated treatment processes is also gaining importance in the field of textile wastewater (Waghmode et al., 2019; Bhanot et al., 2020; Paździor and Bilińska, 2020). While in the last decades the number of investigations carried out on the subject has remained constant, in the last 5 years literature in which two or more treatment techniques are combined have quadrupled.

The effects of different combinations like physicochemical pre-treatment followed by a biological treatment or vice versa had been largely studied. For example, sequential photocatalytic and biological treatment with an artificial microbial consortium reach 100% degradation rate in a simulated textile wastewater within 4 h, also decreasing COD and phytotoxicity (Waghmode et al., 2019). In other study, a bacterial consortium isolated from a dyeing factory showed an efficacy of 77.6 ± 3.0% in decolorizing wastewater (Ceretta et al., 2018). When it was coupled with photocatalysis using ZnO/Polypyrrole during only 60 min, the total decolorization efficiency increased to 95.7% and 99.8% TOC degradation (Ceretta et al., 2020). Besides the good efficiency reached, the scaling-up of these processes is still pending. Paździor et al. (2017), investigate different combinations of chemical and biological methods, resulting the best one this combining biodegradation followed by the ozonation with an activated sludge, in a diluted real wastewater, producing the highest toxicity reduction (98%). Similar results were obtained by Aravind et al. (2016), who combined biodegradation followed by photo-assisted electro-oxidation and reaches a decolorization of 98%.

As we saw in this quick analysis, there are many technology designs that combine biological and physicochemical treatments. Researches carried out have shown that integrated treatments significantly improve the removal of organic matter, achieve higher decolorization percentages, reduce toxicity and even reach mineralization of contaminants (Oller et al., 2011; Hayat et al., 2015; Aravind et al., 2016; Paździor et al., 2017, 2019). The efficiency of studies that apply biological pre-treatments followed by physicochemical treatments has been compared with those that did it by the other way. Although there is no consensus in which is the best one, due to the characteristics of the textile effluents, greater difficulties have been observed in the effluent treatment when the physicochemical pre-treatments are employed (Paździor et al., 2019). Furthermore, in this way pre-treatment times are much longer, increasing costs due to the energy and chemical requirements (Qian et al., 2013; Aravind et al., 2016), added to the fact that degradation products can be toxic. Using biodegradation as pre-treatment has the advantage not only of being cheaper, but also due to the enzymatic microbial machine that has the versatility to accommodate any xenobiotic substances which may be present and degrade it (Saratale et al., 2011; Singh and Singh, 2017). Thus, the biological treatment contribute to the overall organic and color removal, and the physicochemical ones cooperate in completing the degradation of pollutants in a shorter time and also contributing to the sterilization of the biotreated wastewater for its subsequent discharge (Paździor et al., 2019).

The case of study of Paździor and Bilińska (2020) is important since analyzed a large-scale industrial textile wastewater treatment plant. In this plant biological, chemical and physical methods (such as filtration, ozonation, biological treatment with activated sludge) are combined. While improving of BOD_5/COD ratio and COD reductions, the authors concluded that after a ten steps system wastewater is only partly biodegradable (Paździor and Bilińska, 2020). These examples bring out the complexity of textile wastewater.

CONCLUSION

The first recorded review on the treatment of textile wastewater, which compiles different methodologies and their efficiency in the removal of dyes, dates from 1971 (Porter, 1971). But after five decades of research and with thousands of accumulated works, according to the United Nations 80% of industrial wastewaters are still being discharged into the environment without treatment (United Nations World Water Assessment Programme, 2017).

To date, some certainties have been reached but there are still a few challenges to overcome. The first ones confirm that progress has been made in the field of textile wastewater treatment:

(i) the use of consortia or microbial communities makes the biodegradation of complex compounds possible;
(ii) the use of combined toxicity assays is necessary to determine the efficiency of the applied treatments;
(iii) the development of integrated treatments by combining two or more methodologies allows to achieve higher percentages of degradation.

Among the challenges that are pending to be solved: the study of treatment methodologies using real effluents, the scaling up of the processes with the corresponding cost analysis, the development of economically attractive alternatives to achieve adoption by the industrial sector, and finally reducing water consumption by reusing it.

It is evident that the combination of physical, chemical and biological methods must be used to carry out a successful treatment, not only in terms of reducing dye concentration and COD, but also for reducing pH, BOD, toxicity. All this objectives will be reached only if the work is carried out in an interdisciplinary way, where scientific evidence, engineering dimensions, but also economic and political ones are taken into account. The implementation of this type of alternatives will only be possible with the support of public policies that value and accompany by supporting, the implementation of effluent treatment strategies for the environment protection.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

The authors would like to thank National University of Mar del Plata and the National Scientific and Technical Research Council (CONICET) for supporting. Thank you very much Natalia Teryda for reading the manuscript and her helpful suggestions. Also, the authors wish to acknowledge Gabriela Fioramonti from Gama S. A. (Mar del Plata, Argentina) for years of providing wastewater samples and dyes.

REFERENCES

Abd El-Rahim, W. M., Moawad, H., Abdel Azeiz, A. Z., and Sadowsky, M. J. (2017). Optimization of conditions for decolorization of azo-based textile dyes by multiple fungal species. *J. Biotechnol.* 260, 11–17. doi: 10.1016/j.jbiotec.2017.08.022

Aravind, P., Subramanyan, V., Ferro, S., and Gopalakrishnan, R. (2016). Eco-friendly and facile integrated biological-cum-photo assisted electrooxidation process for degradation of textile wastewater. *Water Res.* 93, 230–241. doi: 10.1016/j.watres.2016.02.041

Bhanot, P., Celin, S. M., Sreekrishnan, T. R., Kalsi, A., Sahai, S. K., and Sharma, P. (2020). Application of integrated treatment strategies for explosive industry wastewater—a critical review. *J. Water Process Eng.* 35:101232. doi: 10.1016/j.jwpe.2020.101232

Ceretta, M. B., Durruty, I., Orozco, A. M. F., González, J. F., and Wolski, E. A. (2018). Biodegradation of textile wastewater: enhancement of biodegradability via the addition of co-substrates followed by phytotoxicity analysis of the effluent. *Water Sci. Technol.* 2017, 516–526. doi: 10.2166/wst.2018.179

Ceretta, M. B., Vieira, Y., Wolski, E. A., Foletto, E. L., and Silvestri, S. (2020). Biological degradation coupled to photocatalysis by ZnO/polypyrrole composite for the treatment of real textile wastewater. *J. Water Process Eng.* 35:101230. doi: 10.1016/j.jwpe.2020.101230

Chen, Y., Feng, L., Li, H., Wang, Y., Chen, G., and Zhang, Q. (2018). Biodegradation and detoxification of direct black G textile dye by a newly isolated thermophilic microflora. *Bioresour. Technol.* 250, 650–657. doi: 10.1016/j.biortech.2017.11.092

Chen, Y., Zhang, L., Feng, L., Chen, G., Wang, Y., Zhai, Z., et al. (2019). Exploration of the key functional strains from an azo dye degradation microbial community by DGGE and high-throughput sequencing technology. *Environ. Sci. Pollut. Res.* 26, 24658–24671. doi: 10.1007/s11356-019-05781-z

Dafale, N., Rao, N. N., Meshram, S. U., and Wate, S. R. (2008). Decolorization of azo dyes and simulated dye bath wastewater using acclimatized microbial consortium – biostimulation and halo tolerance. *Bioresour. Technol.* 99, 2552–2558. doi: 10.1016/j.biortech.2007.04.044

Dhaouefi, Z., Toledo-Cervantes, A., García, D., Bedoui, A., Ghedira, K., Chekir-Ghedira, L., et al. (2018). Assessing textile wastewater treatment in an anoxic-aerobic photobioreactor and the potential of the treated water for irrigation. *Algal Res.* 29, 170–178. doi: 10.1016/j.algal.2017.11.032

Eiler, A. (2006). Evidence for the ubiquity of mixotrophic bacteria in the upper ocean: implications and consequences. *Appl. Environ. Microbiol.* 72, 7431–7437. doi: 10.1128/AEM.01559-06

Ghaly, A. E., Ananthashankar, R., Alhattab, M., Ramakrishnan, V. V., Ghaly, A., and Ramakrishnan, V. V. (2014). Production, characterization and treatment of textile effluents: a critical review. *J. Chem. Eng. Process Technol.* 5:182. doi: 10.4172/2157-7048.1000182

Gonçalves, P., Bertholdo, R., Dias, J. A., Maestrelli, S. C., and Giraldi, T. R. (2017). Evaluation of the photocatalytic potential of TiO2 and ZnO obtained by different wet chemical methods. *Mater. Res.* 20, 181–189. doi: 10.1590/1980-5373-MR-2016-0936

Hayat, H., Mahmood, Q., Pervez, A., Bhatti, Z. A., and Baig, S. A. (2015). Comparative decolorization of dyes in textile wastewater using biological and chemical treatment. *Sep. Purif. Technol.* 154, 149–153. doi: 10.1016/j.seppur.2015.09.025

He, X., Song, C., Li, Y., Wang, N., Xu, L., Han, X., et al. (2018). Efficient degradation of Azo dyes by a newly isolated fungus *Trichoderma tomentosum* under non-sterile conditions. *Ecotoxicol. Environ. Saf.* 150, 232–239. doi: 10.1016/j.ecoenv.2017.12.043

Holkar, C. R., Jadhav, A. J., Pinjari, D. V., Mahamuni, N. M., and Pandit, A. B. (2016). A critical review on textile wastewater treatments: possible approaches. *J. Environ. Manage.* 182, 351–366. doi: 10.1016/j.jenvman.2016.07.090

Hossen, M. Z., Hussain, M. E., Hakim, A., Islam, K., Uddin, M. N., and Azad, A. K. (2019). Biodegradation of reactive textile dye Novacron Super Black G by free cells of newly isolated *Alcaligenes faecalis* AZ26 and *Bacillus* spp obtained from textile effluents. *Heliyon* 5:e02068. doi: 10.1016/j.heliyon.2019.e02068

Işik, M., and Sponza, D. T. (2004). Monitoring of toxicity and intermediates of C.I. Direct Black 38 azo dye through decolorization in an anaerobic/aerobic sequential reactor system. *J. Hazard. Mater.* 114, 29–39. doi: 10.1016/j.jhazmat.2004.06.011

Jadhav, J. P., Kalyani, D. C., Telke, A. A., Phugare, S. S., and Govindwar, S. P. (2010). Evaluation of the efficacy of a bacterial consortium for the removal of color, reduction of heavy metals, and toxicity from textile dye effluent. *Bioresour. Technol.* 101, 165–173. doi: 10.1016/j.biortech.2009.08.027

Kurade, M. B., Waghmode, T. R., Patil, S. M., Jeon, B.-H., and Govindwar, S. P. (2017). Monitoring the gradual biodegradation of dyes in a simulated textile effluent and development of a novel triple layered fixed bed reactor using a bacterium-yeast consortium. *Chem. Eng. J.* 307, 1026–1036. doi: 10.1016/j.cej.2016.09.028

Oliveira, J. M. S., de Lima E Silva, M. R., Issa, C. G., Corbi, J. J., Damianovic, M. H. R. Z., and Foresti, E. (2020). Intermittent aeration strategy for azo dye biodegradation: a suitable alternative to conventional biological treatments? *J. Hazard. Mater.* 385:121558. doi: 10.1016/j.jhazmat.2019.121558

Oller, I., Malato, S., and Sánchez-Pérez, J. A. (2011). Combination of advanced oxidation processes and biological treatments for wastewater decontamination-a review. *Sci. Total Environ.* 409, 4141–4166. doi: 10.1016/j.scitotenv.2010.08.061

O'Neill, C., Hawkes, F. R., Hawkes, D. L., Lourenço, N. D., Pinheiro, H. M., and Delée, W. (1999). Colour in textile effluents - sources, measurement, discharge consents and simulation: a review. *J. Chem. Technol. Biotechnol.* 74, 1009–1018. doi: 10.1002/(SICI)1097-4660(199911)74:11<1009::AID-JCTB153<3.0.CO;2-N

Paździor, K., and Bilińska, L. (2020). Microscopic analysis of activated sludge in industrial textile wastewater treatment plant. *Autex Res. J.* 1–7. doi: 10.2478/aut-2020-0050. [Epub ahead of print].

Paździor, K., Bilińska, L., and Ledakowicz, S. (2019). A review of the existing and emerging technologies in the combination of AOPs and biological processes in industrial textile wastewater treatment. *Chem. Eng. J.* 376:120597. doi: 10.1016/j.cej.2018.12.057

Paździor, K., Wrębiak, J., Klepacz-Smółka, A., Gmurek, M., Bilińska, L., Kos, L., et al. (2017). Influence of ozonation and biodegradation on toxicity of industrial textile wastewater. *J. Environ. Manage.* 195, 166–173. doi: 10.1016/j.jenvman.2016.06.055

Porter, J. (1971). *State of the Art of Textile Waste Treatment*. Washington, DC: Environmental Protection Agency, Water Quality Office.

Przystaś, W., Zabłocka-Godlewska, E., and Grabińska-Sota, E. (2018). Efficiency of decolorization of different dyes using fungal biomass immobilized on different solid supports. *Braz. J. Microbiol.* 49, 285–295. doi: 10.1016/j.bjm.2017.06.010

Qian, F. Y., Sun, X. B., and di Liu, Y. (2013). Effect of ozone on removal of dissolved organic matter and its biodegradability and adsorbability in biotreated textile effluents. *Ozone Sci. Eng.* 35, 7–15. doi: 10.1080/01919512.2013.720211

Rather, L. J., Akhter, S., and Hassan, Q. P. (2018). "Bioremediation: green and sustainable technology for textile effluent treatment," in *Sustainable Innovations in Textile Chemistry and Dyes. Textile Science and Clothing Technology*, ed. S. S. Muthu (Singapore: Springer), 75–91. doi: 10.1007/978-981-10-8600-7_4

Samuchiwal, S., Gola, D., and Malik, A. (2021). Decolourization of textile effluent using native microbial consortium enriched from textile industry effluent. *J. Hazard. Mater.* 402:123835. doi: 10.1016/j.jhazmat.2020.123835

Saratale, R. G., Saratale, G. D., Chang, J. S., and Govindwar, S. P. (2011). Bacterial decolorization and degradation of azo dyes: a review. *J. Taiwan Inst. Chem. Eng.* 42, 138–157. doi: 10.1016/j.jtice.2010.06.006

Shade, A., Peter, H., Allison, S. D., Baho, D. L., Berga, M., Bürgmann, H., et al. (2012). Fundamentals of microbial community resistance and resilience. *Front. Microbiol.* 3:417. doi: 10.3389/fmicb.2012.00417

Singh, P. K., and Singh, R. L. (2017). Bio-removal of azo dyes: a review. *Int. J. Appl. Sci. Biotechnol.* 5, 108–126. doi: 10.3126/ijasbt.v5i2.16881

Thanavel, M., Kadam, S. K., Biradar, S. P., Govindwar, S. P., Jeon, B.-H., and Sadasivam, S. K. (2019). Combined biological and advanced oxidation process for decolorization of textile dyes. *SN Appl. Sci.* 1:97. doi: 10.1007/s42452-018-0111-y

United Nations World Water Assessment Programme (2017). *The United Nations World Water Development Report 2017. Wastewater: The Untapped Resource*, ed. UNESCO, Paris. Available online at: https://unesdoc.unesco.org/ark:/48223/pf0000247647 (accessed October 2, 2019).

Valli Nachiyar, C., Ananth, P. B., Sunkar, S., Karunya, A., and Jabasingh, S. A. (2016). Decolorization and biodegradation of textile azo dye Acid Black 24 by developed bacterial consortium CN-1A. *Ind. J. Biotechnol.* 15, 412–419.

Vikrant, K., Giri, B. S., Raza, N., Roy, K., Kim, K.-H., Rai, B. N., et al. (2018). Recent advancements in bioremediation of dye: current status and challenges. *Bioresour. Technol.* 253, 355–367. doi: 10.1016/j.biortech.2018.01.029

Waghmode, T. R., Kurade, M. B., Sapkal, R. T., Bhosale, C. H., Jeon, B. H., and Govindwar, S. P. (2019). Sequential photocatalysis and biological treatment for the enhanced degradation of the persistent azo dye methyl red. *J. Hazard. Mater.* 371, 115–122. doi: 10.1016/j.jhazmat.2019.03.004

Yaseen, D. A., and Scholz, M. (2019). Textile dye wastewater characteristics and constituents of synthetic effluents: a critical review. *Int. J. Environ. Sci. Technol.* 16, 1193–1226. doi: 10.1007/s13762-018-2130-z

Yu, Y., Wu, B., Jiang, L., Zhang, X. X., Ren, H. Q., and Li, M. (2019). Comparative analysis of toxicity reduction of wastewater in twelve industrial park wastewater treatment plants based on battery of toxicity assays. *Sci. Rep.* 9:3751. doi: 10.1038/s41598-019-40154-z

Zahran, S. A., Ali-Tammam, M., Hashem, A. M., Aziz, R. K., and Ali, A. E. (2019). Azoreductase activity of dye-decolorizing bacteria isolated from the human gut microbiota. *Sci. Rep.* 9:5508. doi: 10.1038/s41598-019-41894-8

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 12 May 2021
doi: 10.3389/fmicb.2021.633004

Biochemical Characterization of a Novel Bacterial Laccase and Improvement of Its Efficiency by Directed Evolution on Dye Degradation

Shuang Dai[1], Qian Yao[1], Gen Yu[1], Shan Liu[2], Jeonyun Yun[2], Xiong Xiao[2], Zujun Deng[1] and He Li[1]**

[1] Guangdong Key Laboratory of Pharmaceutical Bioactive Substances, College of Life Science and Biopharmaceuticals, Guangdong Pharmaceutical University, Guangzhou, China, [2] Guangzhou Base Clean Cosmetics Manufacturer Co., Ltd., Guangzhou, China

OPEN ACCESS

Edited by:
Kunal R. Jain,
Sardar Patel University, India

Reviewed by:
Wong Yee Shian,
Universiti Malaysia Perlis, Malaysia
Peter-Leon Hagedoorn,
Delft University of Technology, Netherlands

***Correspondence:**
Zujun Deng
dengzujun66@163.com
He Li
lihe32@163.com

Specialty section:
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

Received: 24 November 2020
Accepted: 07 April 2021
Published: 12 May 2021

Citation:
Dai S, Yao Q, Yu G, Liu S, Yun J, Xiao X, Deng Z and Li H (2021) Biochemical Characterization of a Novel Bacterial Laccase and Improvement of Its Efficiency by Directed Evolution on Dye Degradation. Front. Microbiol. 12:633004. doi: 10.3389/fmicb.2021.633004

Laccase is a copper-containing polyphenol oxidase with a wide range of substrates, possessing a good application prospect in wastewater treatment and dye degradation. The purpose of this research is to study the degradation of various industrial dyes by recombinant laccase rlac1338 and the mutant enzyme lac2-9 with the highest enzyme activity after modification by error-prone PCR. Four enzyme activities improved mutant enzymes were obtained through preliminary screening and rescreening, of which lac2-9 has the highest enzyme activity. There are four mutation sites, including V281A, V281A, P309L, S318G, and D232V. The results showed that the expression of the optimized mutant enzyme also increased by 22 ± 2% compared to the unoptimized enzyme and the optimal reaction temperature of the mutant enzyme lac2-9 was 5°C higher than that of the rlac1338, and the optimal pH increased by 0.5 units. The thermal stability and pH stability of mutant enzyme lac2-9 were also improved. With ABTS as the substrate, the k_{cat}/K_m of rlac1338 and mutant strain lac2-9 are the largest than other substrates, 0.1638 and 0.618 $s^{-1}M^{-1}$, respectively, indicating that ABTS is the most suitable substrate for the recombinant enzyme and mutant enzyme. In addition, the K_m of the mutant strain lac2-9 (76 μM) was significantly lower, but the k_{cat}/K_m (0.618 $s^{-1}M^{-1}$) was significantly higher, and the specific enzyme activity (79.8 U/mg) increased by 3.5 times compared with the recombinant laccase (22.8 U/mg). The dye degradation results showed that the use of rlac1338 and lac2-9 alone had no degradation effect on the industrial dyes [indigo, amaranth, bromophenol blue, acid violet 7, Congo red, coomassie brilliant blue (G250)], however, adding small molecular mediators Ca^{2+} and ABTS at the same time can significantly improve the degradation ability. Compared to the rlac1338, the degradation rates with the simultaneous addition of Ca^{2+} and ABTS of mutant enzyme lac2-9 for acid violet 7, bromophenol blue and coomassie brilliant blue

significantly improved by 8.3; 3.4 and 3.4 times. Therefore, the results indicated that the error-prone PCR was a feasible method to improve the degradation activity of laccase for environmental pollutants, which provided a basis for the application of laccase on dye degradation and other environmental pollutants.

Keywords: laccase, dye degradation, directed evolution, enzymatic characteristics, expression

INTRODUCTION

Laccase, first discovered in the sap of the Japanese lacquer tree *Rhus vernicefera* (Wang and Chen, 2003) is one of the earliest enzymes studied. It belongs to the copper oxidase family. Laccase can use O_2 to oxidize various aromatics and non-aromatic compounds through free radical catalytic reactions. Laccase has a wide range of sources, mainly from plants, fungi, and bacteria. With the gradual discovery of the function of laccase in dye degradation, the research on laccase has received extensive attention.

At present, there are more than 10,000 industrial dyes, according to the composition, it can be divided into azo, indigo, anthraquinone and triphenylmethane dyes. The mechanism of laccase on dyes from different sources is very different (Zhang et al., 2017). Laccase can directly oxidize anthraquinone dyes (Li, 2013) and azo dyes (Si et al., 2011); for some recalcitrant dyes, laccase often requires the participation of small molecular mediators to achieve efficient decolorization. For example, Susana et al. (2007) used the laccase mediator system to degrade reactive black and sky blue, the results showed that the laccase mediator system had a degradation rate of 90% for reactive black within 30 min, and the degradation rate for sky blue was also up to 30%, and the laccase system alone has almost no degradation effect on these two dyes.

At present, enzyme modification methods are mainly grouped into two categories, namely rational design methods and non-rational design methods (Mills et al., 1967). Among them, non-rational design does not need to understand the relationship between protein structure and function. By simulating the process of natural evolution in the laboratory, after random mutation, recombination and selection, the long natural evolution process can be simulated in a short time. The error-prone PCR (error-prone PCR) selected in this experiment is a non-rational design method, which can carry out directed evolution and selection of the coding genes of enzyme molecules, thereby improving the stability of the enzyme and the specificity of the substrate. It is an important research tool to the current protein engineering. The *in vitro* molecular directed evolution technology based on error-prone PCR can produce large phenotypic differences through minimal sequence changes, and target strains can be screened out, which simplifies the comparative analysis of sequences to a great extent. However, because error-prone PCR can only mutate a small sequence in the original protein, it is generally suitable for smaller gene fragments (<2,000 bp) (Wang et al., 2017).

In this study, a laccase gene *lac1338* from the marine microbial metagenome was synthesized by a codon optimization for *E. coli* expression. Then the laccase rlac1338 with high expression and thermal stability was obtained through prokaryotic expression. The laccase can degrade various dyes, but due to its low enzymatic activity than comparable bacterial or fungal laccases, the dye degradation rates are also low, which limits its application to a certain extent. Therefore, we modified the *lac1338* gene by an error-prone PCR directed evolution strategy, and used enzyme activity as a screening indicator to obtain mutant strains with increased laccase activity. By comparing the optimum temperature and temperature stability, optimum pH and pH stability, enzymatic kinetics, and dye degradation rate between the recombinase and the mutant enzyme, we have determined that error-prone PCR modification of laccase is a feasible method. Further explore its application prospects in industrial dye processing, and provide a certain foundation for its suitability for industrial applications.

MATERIALS AND METHODS

Strains and Plasmids

The recombinant plasmid pUC118-lac1338 (GenBank, accession number HM623889) was constructed and stored in our laboratory. pET-32a (+) and *Escherichia coli* BL2l (DE3) were purchased from Novagen.

Main Reagents

A gel recovery kit, a plasmid extraction kit, and a PCR product recovery kit (all from Omega), a protein purification kit (Novagen, Germany), and a Diversify® PCR random mutagenesis kit (Clontech). A 170 kD prestained protein ladder was purchased from Guangzhou Saizhe Biotechnology Co., Ltd. Other reagents included ABTS (Amresco), IPTG and ampicillin (both TaKaRa). Other conventional reagents were all analytically pure.

Recombinant Plasmid Construction and Protein Expression

According to the sequencing results, primer pairs were designed on Premier 5:

lac1338-F: 5′-CCGGAATTCATGCGCAAAAGTCCCGGAGT CACTTTTTCA-3′ (the underlined part is the *Bam*H I restriction site);
lac1338-R: 5′-AGCAAGCTTTCAGTCGGGCATGTTGGGGA TTTCAGG-3′ (the underlined part is the *Hind* III restriction site).

Diversify® PCR random mutagenesis kit was used for PCR amplification. *Bam*H I and *Hind* III restriction sites were added at both ends of the sequence, ligated to the *Bam*H I and *Hind*

III sites of the expression vector pET-32a(+), the recombinant plasmid was obtained and verified by double enzyme digestion. The recombinant plasmid pET-32a-lac1338 was transformed into *E. coli* BL21(DE3) and placed under shaking culture at 37°C and 200 rpm/min until the OD_{600} reached about 0.8. After that, (Isopropyl-β-D-thiogalactopyranoside), and 0.5 mmol/L Cu^{2+} (final concentrations) were added, followed by incubation at 30°C and 200 rpm/min under shaking for 16 h to induce protein expression, centrifuge the bacteria to collect the precipitate and ultrasonically break it. Referred to the instruction of His·Bind® Purification Kit (Novagen) for protein purification and SDS-PAGE analysis. With bovine serum albumin (BSA) as the standard, the purified protein concentration was determined using the bicinchoninic acid (BCA) method (Brown et al., 1989).

Directed Evolution of lac1338

With lac1338 as the template, error-prone PCR was performed according to the instruction of the Diversify® PCR random mutagenesis kit. A mutation rate of 2.3 bp/kb was selected, and the reaction system was: 10 × TITANIUM Taq Buffer 5 μl; 50 × Diversify dNTP Mix 1 μl; Template DNA (∼1 ng/μl) 1 μl; Mut-F (250 ng/μl) 1 μl; Mut-R (250 ng/μl) 1 μl; TITANIUM Taq Polym 1 μl; MnSO4 1 μl; dGTP (2 mM) 1 μl; PCR Grade Water 39 μl. The reaction conditions were: 94°C 30 s; 94°C 30 s, 68°C 90 s, 25 cycles; 72°C 5 min. The error-prone PCR product was cloned and constructed into *E. coli* BL21 (DE3) and the mutants with the highest enzyme activity were selected for experimental research through preliminary screening and re-screening of highly active laccase mutants. Among them, the initial screening was through the ABTS color reaction, and the enzyme activity was judged according to the color depth; the second screening was to identify whether the size of the mutant laccase was consistent with the size of the recombinant laccase by SDS-PAGE gel electrophoresis, and then selected by the enzyme activity determination method the mutant enzyme with the highest enzyme activity (Feng and Li, 2015).

Activity Determination and Enzymatic Properties of Recombinant Laccase rlac1338 and Mutant Strain lac2-9

Enzyme Activity Determination

With ABTS as the substrate, the reaction system of 3 mL citric acid-sodium citrate buffer solution contains 5 mmol/L ABTS, 6 mmol/L Cu^{2+} and a certain concentration of the enzyme solution. After uniform mixing, the sample system reacted in a water bath at 55°C for 3 min, followed by measurement of OD_{405}, measured three times in parallel and repeated the experiment three times. A blank control was prepared without adding the enzyme solution. Under these conditions, the amount of enzyme required to catalyze the oxidation of 1 μmol ABTS per minute was defined as 1 enzyme activity unit (U).

Determination of Optimal Reaction Conditions and Stability Analysis

The optimal reaction temperature was determined by measuring the enzyme activity from 30 to 70°C (5°C interval). The optimal reaction pH of the enzyme was identified within pH 4–10 (Britton-Robinson buffer solution). To determine the thermostability of the enzyme, its activity under the optimum pH was measured from 35 to 80°C (5°C interval) for 2 h. The enzyme activity of the enzyme solution stored at 4°C was considered as 100%. To evaluate the pH stability of the enzyme, the activity at the optimum temperature was measured after 4 h of incubation at pH of 4–8. The activity of the untreated enzyme solution was taken as 100%.

Determination of Kinetic Parameters of Enzymatic Reaction of Recombinant Laccase rlac1338 and Mutant Strain lac2-9

The Michaelis constant K_m and the maximum rate V_{max} were determined:

Different common substrates of laccase were selected (2,6-DMP, ABTS, Guaiacol, Catecho, 1-naphthol) to react. The OD_{405} was determined to calculate the initial speed of the enzyme reaction and plot a Lineweaver-Burk double reciprocal graph.

Comparison of Dye Degradation by rlac1338 and lac2-9

The effects of rlac1338 and mutant enzyme lac2-9 on the degradation of various industrial dyes (amaranth, isatin, bromophenol blue, acid violet 7, crystal violet, orange red G, Congo red, rhodamine B, methylene blue and coomassie brilliant blue G250) were investigated. Inoculated 0.2 mL of recombinant laccase rlac1338 and mutant enzyme lac2-9, respectively, in 50 mL of liquid medium, and kept them at the optimum temperature (55, 60°C) and the suitable pH (6, 6.5), shook overnight at 220 rpm. After 16 h, added the above industrial dyes to make the final concentration of the dye 100 mg/L. After decolorizing at 37°C and 220 rpm for 24 h, the absorbance of each dye solution was determined at the maximum wavelength of the spectrophotometer. Then the effects of Ca^{2+} and ABTS on the degradation rate were simultaneously investigated.

$$\text{Degradation rate (I) was calculated as } I = (A_0 - A_1)/A_0 \times 100\%,$$

where A_0: light absorption of the blank control; A_1: light absorption of a sample dye.

Simulation of the Three-Dimensional Structure of the Mutant Strain

The amino acid sequence of the target protein was inputted into PHYRE2[1] to predict structure. The PDB file was opened with Pymol, and the mutation site was marked. According to the predicted structure information, the site mutation effect was analyzed together with a comparison of enzymatic properties between the mutant enzyme and the wild-type enzyme.

[1]http://www.sbg.bio.ic.ac.uk/

RESULTS

Recombinant Plasmid Construction and Protein Expression

The 1,338 bp *lac1338* gene was amplified by PCR (**Supplementary Figure 1**) and double enzyme digestion resulted in two bands of ∼1,338 and 5,900 bp (**Supplementary Figure 2**), respectively, which were the target gene and the empty linear plasmid pET-32a(+), which proved that the target gene was successfully ligated to the expression vector. SDS-PAGE showed that the rlac1338 was a single band with a molecular weight of ∼68 kD (18 kD was the fusion protein tag on the expression vector) (**Supplementary Figure 3**), which was the same as the theoretically predicted protein molecular weight. The purified protein concentration measured by the BCA method was 0.25 g/L.

Directed Evolution of rlac1338

Error-Prone PCR to Generate Mutant Laccase Gene and Construction of Mutant Library

Using mutant primers Mut-F and Mut-R, a mutant gene fragment with *Hind* III and *Bam*HI restriction sites was amplified, the size was about 1.3 kbp, and the target strip was recovered by 1% agarose gel electrophoresis band. Randomly picked clones and extracted plasmids for double enzyme digestion, and subjected the digested products to gel electrophoresis analysis. According to statistics, the number of clones in the library can reach 10^8, which meet the requirements of library screening.

Primary Screening of Highly Active Laccase Mutants

After being cultured, the positive transformants were developed with ABTS, and the enzyme activity was judged by the intensity of the color, that is, the darker the color, the higher the enzyme activity (**Supplementary Figure 4**). The results showed that about 40% of the mutants had no color change or lighter color than the wild-type laccase, about 40% of the activity was basically unchanged, and only about 20% of the mutants had an increase in activity. It can be seen that the mutations affected the laccase enzyme active.

Rescreening of Highly Active Laccase Mutants

The mutants with higher activity obtained in the preliminary screening were identified by SDS-PAGE electrophoresis, and the results showed that the size of the laccase protein produced by the mutants was similar to that of the rlac1338 (**Figure 1**).

Mutant Gene Sequence Sequencing

The strains with higher enzyme activity than the wild type were obtained by functional screening (Xia et al., 2014; Lv et al., 2015), which were named lac1-16, lac1-19, lac2-1, and lac2-9 after sequencing (**Table 1**). Obviously, the enzyme activity of the mutant strain lac2-9 was significantly improved.

Properties of Enzymes

The optimal reaction temperature of the mutant enzyme was 5°C higher than that of rlac1338 (60 vs. 55°C) (Figure 2). The thermal stability was also improved, and the relative enzyme activity was still over 50% after being kept at 55°C for 4 h (**Figure 2**). The results of pH and pH stability were shown in **Figure 3**. With ABTS as the reaction substrate, the mutant strain had a pH increase of 0.5 unit compared with the recombinant laccase, but its stability was not significantly different.

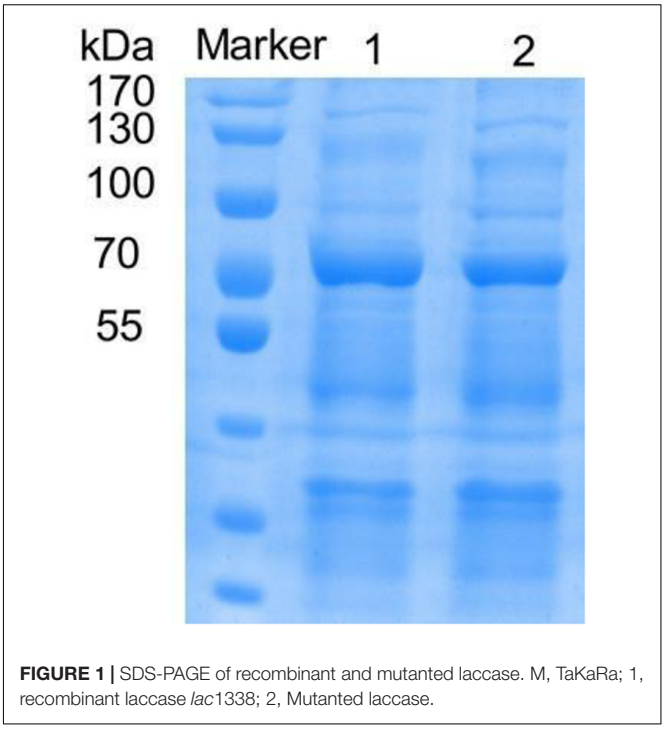


FIGURE 1 | SDS-PAGE of recombinant and mutanted laccase. M, TaKaRa; 1, recombinant laccase *lac*1338; 2, Mutanted laccase.

TABLE 1 | Nucleotide substitution and amino acid changes of the mutant enzymes.

Mutant	Mutant base	Mutant amino acid	Specific enzyme activity (U/mg)
rlac1338	–	–	22.8 ± 1.5
1-16	T234C/T258C/ A943G	S314G	45.6 ± 2.2*
1-19	T496C/T975C	M317T	34.25 ± 1.9*
2-1	T72A/ A104G	V15D/I26V	34.25 ± 1.4*
2-9	T243C/T267C/ T552A		
	T842C/C926T/ A952G/T698A	V281A/P309L/ S318G/D232V	79.8 ± 2.7*

**Indicates that the data is significant, that is $p < 0.05$.*

Kinetic Properties of Recombinant Laccase rlac1338 and Mutant lac2-9

The Michaelis constant K_m and specific enzyme activity of rlac1338 and mutant lac2-9 for non-phenolic or phenolic substrates were measured at the optimal reaction pH and temperature (**Table 2**). With ABTS as the substrate, the k_{cat}/K_m of rlac1338 and mutant strain lac2-9 are the largest than other substrates, 0.1638 and 0.618 $s^{-1}M^{-1}$, respectively, indicating

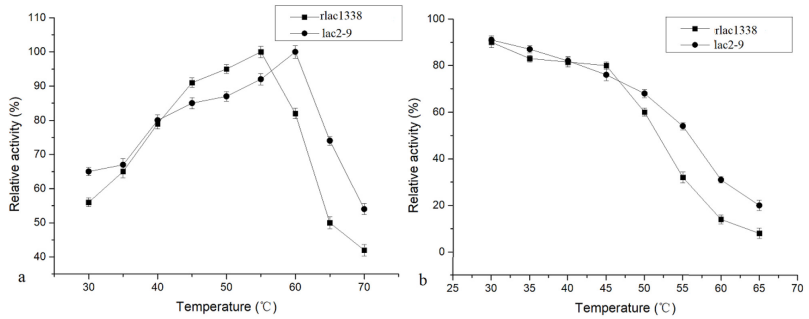


FIGURE 2 | Comparison of optimal temperature and thermal stability between mutant strain lac2-9 and recombinant rlac1338.

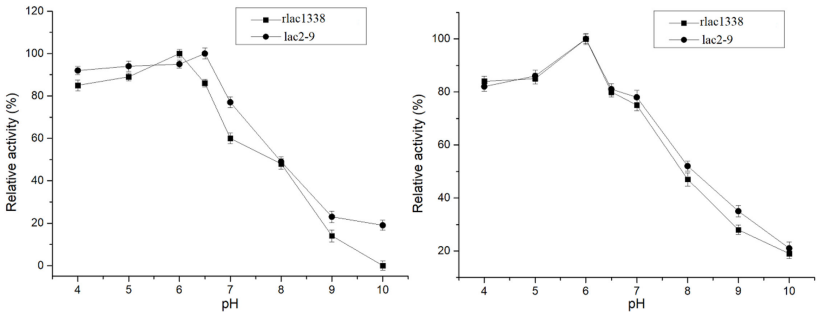


FIGURE 3 | Comparison of optimal pH and pH stability of mutant strain lac2-9 and recombinant rlac1338.

TABLE 2 | Comparison of kinetic parameters and enzyme activity between mutant enzyme lac2-9 and rlac1338.

Enzyme	Substrate	K_m(μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)	Specific enzyme activity (U/mg)
rlac1338	2,6-DMP	654 ± 2.1	9.43 ± 0.8	1.44×10^{-2}	1.12 ± 0.02
	ABTS	210 ± 3.3	34.39 ± 1.1	0.16	22.80 ± 1.5
	Guaiacol	4, 900 ± 1.8	1.34 ± 0.04	2.7×10^{-4}	0.64 ± 0.04
	Catecho	507 ± 2.5	13.99 ± 1.0	2.76×10^{-2}	10.60 ± 1.6
	1-naphthol	5, 600 ± 2.4	3.56 ± 0.4	6.4×10^{-4}	0.13 ± 0.01
lac2-9	2,6-DMP	560 ± 3.7	11.23 ± 1.2	0.02	2.67 ± 0.05
	ABTS	76 ± 1.8	46.94 ± 2.5	0.62	79.80 ± 3.6
	Guaiacol	4, 300 ± 1.6	1.57 ± 0.3	3.7×10^{-4}	1.37 ± 0.05
	Catecho	570 ± 2.9	30.76 ± 1.6	5.4×10^{-2}	18.90 ± 1.3
	1-naphthol	6, 040 ± 3.1	3.89 ± 0.7	6.4×10^{-4}	8.9×10^{-2}

The reaction system of 3 mL citric acid-sodium citrate buffer solution includes 5 mmol/L substrate, 6 mmol/L Cu^{2+}, 55°C, 3 min.

that ABTS is the most suitable substrate for the recombinant enzyme and mutant enzyme. In addition, the K_m of the mutant strain lac2-9 (76 μM) was significantly lower, but the k_{cat}/K_m (0.618 $s^{-1}M^{-1}$) was significantly higher, and the specific enzyme activity (79.8 U/mg) increased by 3.5 times compared with the recombinant laccase (22.8 U/mg), the specific enzyme activity to other substrates also improved to different degrees compared with the recombinant laccase.

Comparison of Dye Degradation by lac2-9 and rlac1338

Compared with the recombinant laccase and rlac1338, the degradation rates with the simultaneous addition of Ca^{2+} and ABTS of mutant strain lac2-9 over acid violet 7, bromophenol blue, coomassie brilliant blue and amaranth increased from 10.9, 20, 25, and 13.7 to 90.5, 67.8, 85, and 14.5%, respectively (**Figure 4**). Hence, it is feasible to obtain functionally enhanced mutant enzymes through error-prone PCR.

Homologous Three-Dimensional Structure Model of Mutant Strain

The amino acid sequences of the mutant enzyme and recombinant laccase were submitted to the Phyre2 protein online analysis server (see text footnote 1). Using homology modeling, its three-dimensional structure is similar to Crystal structure of Lac15 from a marine microbial metagenome

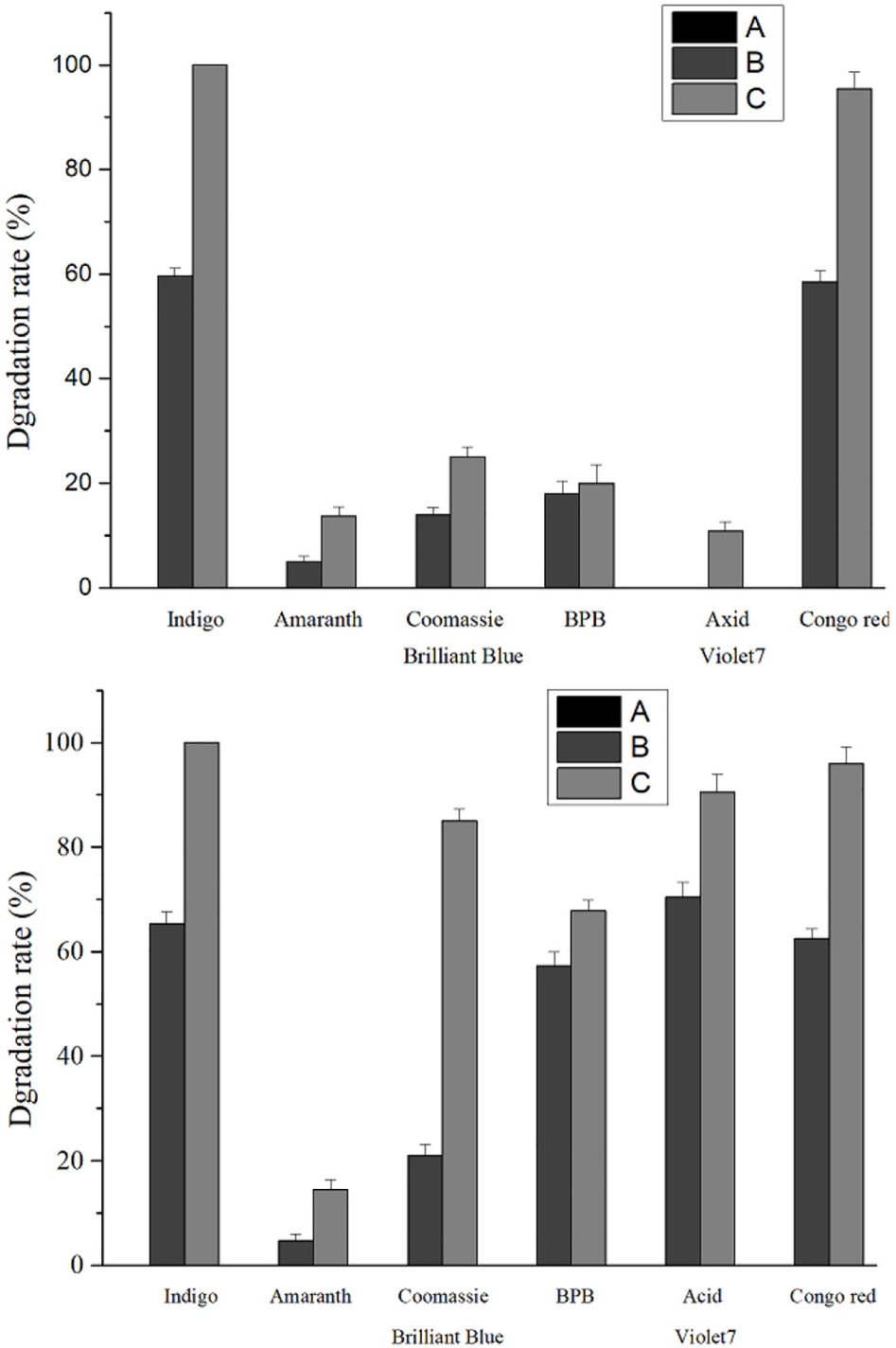


FIGURE 4 | Comparison of dye degradation rate between mutant enzyme lac2-9 and rlac1338 in the presence of small molecule mediator ABTS and Ca^{2+}. A, no ABTS and Ca^{2+}; B, 100 mM Ca^{2+}; C, 100 mM Ca^{2+} and 20 μM ABTS. Measurement conditions: 37°C, decolorization for 24 h.

(4f7k.1.A). Its three-dimensional structure was simulated, and copper in the structure atoms and mutant amino acids was marked using Pymol. Results showed that the enzyme existed in the form of a single subunit protein (**Figure 5**). The entire monomer molecule was composed of three cupredoxin-like domains (Domains 1, 2, 3), which were divided into 3 regions accordingly. Each domain has a β-barrel shape (the β-ropes are arranged into β-hinges to form the so-called Greek pattern). The amino acid sequences of the mutant enzyme and the recombinant laccase lac1338 were compared. The

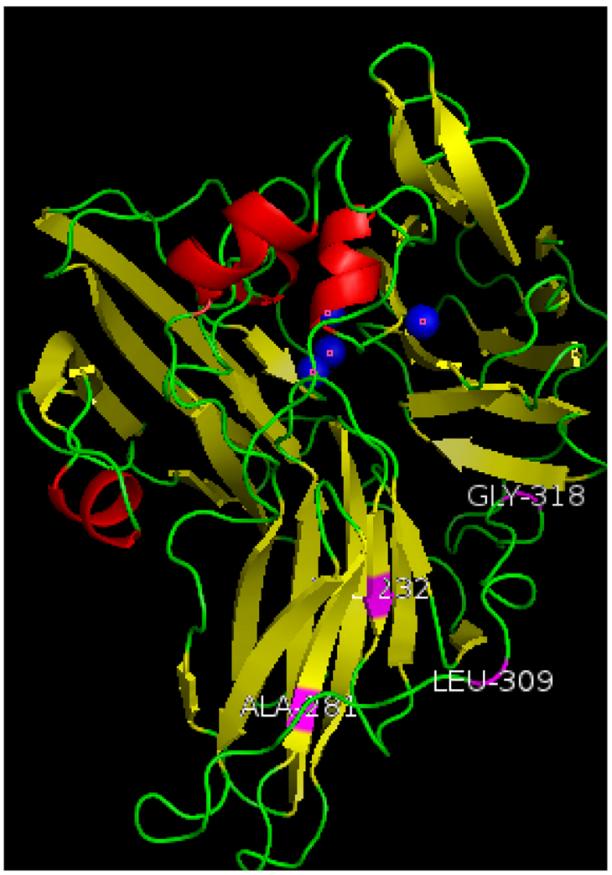


FIGURE 5 | Three-dimensional structure simulation diagram of mutant enzyme lac2-9. α-Helixes (red), β-sheets (yellow), loops (green) and copper atoms (blue) are depicted. The mutation sites of the mutant enzyme (shown in purple in the **Figure 5**) V281A, P309L, S318G, and D232V are far away from the active center of laccase, and are located on the surface or ring region of the laccase protein. Among them, 309 and 318 are on the loop of the spherical structure, and 281 and 232 are on the α-spiral.

mutation sites (shown in purple in the figure) V281A, P309L, S318G, and D232V are far from the active center of the laccase, and are located on the surface or loop region of the laccase protein.

DISCUSSION

The gene encoding codon optimized *lac1338* was synthesized. The biggest advantage of this method is that the protein can be expressed at a high level. The optimum reaction temperature of most fungal laccases discovered so far is between 25 and 50°C. Zhao (2012) screened a white rot fungus with high laccase production, the optimum temperature of the laccase produced by the bacteria is 30°C. However, bacterial laccase generally has a relatively high optimal reaction temperature. Li et al. (2020) selected a strain of laccase from *Bacillus thuringiensis* that produces a high-temperature laccase, and its optimal temperature is 75°C. The optimum temperature of the laccase synthesized in this experiment is 55°C, and the optimum temperature of lac2-9 is 60°C. Although it is lower than some bacterial laccases, it is also higher than most fungal laccases. This may be an advantage for the application of this laccase in industrial production in high temperature environment.

At present, directed evolution methods mainly include error-prone PCR, DNA shuffling, random *in vitro* recombination, and staggered extension (Wang et al., 2017). Directed evolution technology belongs to the category of non-rational design. It does not need to know the three-dimensional structure information and mechanism of protein in advance. Instead, it creates a process similar to natural evolution *in vitro*, causes a large number of mutations in genes, and then selects the required ones through specific screening methods. The nature or function of the target gene. In particular, it has certain advantages in modifying the enzymatic properties of enzymes, such as improving the thermal stability of the enzyme, expanding the range of substrates, making the optimal reaction pH more acidic or alkaline, and improving enzyme activity. Directed evolution is usually divided into three steps: the first step is to generate diverse genes through random mutation or *in vitro* recombination; the second step is to construct a mutation library after the mutated gene is introduced into an appropriate vector; the third step is to select by appropriate screening methods Mutants of desired properties. The whole process can be cycled many times until the enzyme with the desired properties is obtained. The error-prone PCR technology selected in this study is a relatively simple and fast random mutation strategy that only needs to change a single condition to produce greater mutation benefits. After the mutation library is constructed, the choice of an efficient and sensitive screening method is the success of directed evolution to transform the protein molecule. According to laboratory conditions, by simulating the activity screening method of 96-well plates, the apparent enzyme activity of the mutants is preliminarily determined based on the color reaction of the substrate, that is, the faster the color reaction speed and the darker the color, the higher the corresponding mutant laccase enzyme activity; the detection of enzyme activity in the re-screening process avoids misjudgment and improves the accuracy and authenticity of the screening results. Explore the amino acids that affect enzyme activity and stability through the three-dimensional structure, and the results show that in the mutation sites of the mutant enzyme lac2-9, positions 281 and 309 were replaced by non-polar amino acids with polar amino acids, and position 318 was mutated from the neutral amino acid serine to the non-polar amino acid glycine. These positions may be involved in maintaining the spatial conformation required for laccase catalytic oxidation. The mutation at position 232 from the polar positively charged aspartic acid to the non-polar amino acid valine improves the surface hydrophobic effect on the mutant enzyme molecule, making it more suitable for binding to the substrate at high temperatures (Autore et al., 2009), and affects the combination of T2/T3 copper trinuclear active center and hydroxide radical, so that the optimal pH changes (Madzak et al., 2006). Therefore, the mutation at this site improves the optimum temperature and stability of the mutant enzyme.

Studies show that the mutant enzyme has improved dye degradation types and degradation rates compared to the recombinant laccase rlac1338, indicating that it directed evolution is feasible for obtaining a mutant enzyme with more industrial application value. Miele et al. (2010) improved the enzyme activity of *Pleurotus ostreatus* laccase POXA1b by directed evolution, which increased the degradation rates of acid yellow 49, acid red 266 and direct yellow 106. By increasing the enzymatic activity of the laccase CotA of *Bacillus licheniformis*, Koschorreck et al. (2009) enhanced the degradation effects of the laccase on the dyes such as alizarin red S, brilliant blue R, and isatin. But laccase can only degrade phenolic dyes, but cannot directly oxidize non-phenolic dyes. Studies have found that after adding some small molecule compounds called laccase mediators, it can promote the catalytic effect of laccase on non-phenolic substrates, and can significantly improve the catalytic efficiency of phenolic substrates, thereby further expanding laccase the scope of the substrate. These mediators themselves are also laccase substrates, acting as electron transfer intermediates, allowing electrons to be transferred between the enzyme and the substrate. At present, the most commonly used synthetic mediator is 2,2-azide-bis (3-ethylbenzothiazole-6-sulfonic acid) (ABTS). Laccases from various sources can quickly oxidize ABTS to $ABTS^{+}$ intermediate body, and then oxidize the substrate. ABTS can enhance the dye degradation efficiency of laccase, and mediate the oxidation between laccase and non-enzymatic substrate dyes, so that laccase can degrade the dyes of non-laccase substrates (Singh and Kumar, 2010). For example, azo dyes are not the substrates of laccase, and cannot be directly degraded by most laccases (Camarero et al., 2005). Nevertheless, rlac1338 can completely degrade the azo dye rhodamine under the synergistic effect of ABTS and Ca^{2+}. Laccase lac1338 has a pI of 5.05, and its optimum pH is 6.0, so the laccase protein has a positive charge. Ca^{2+} is a positive ion, which binds to the dye anion, which reduces the charge repulsion between the laccase protein and the dye ion, and improves the rate at which the enzyme adsorbs the dye molecules (Sheng, 2019). In the next work, we will combine the three-dimensional structure of the laccase protein and its bioinformatics to further explore the reasons why the mutant base changes its properties.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SD: conceptualization, original draft preparation, and editing. QY: manuscript reviewing. GY, SL, JY, and XX: supervision. HL and ZD: project administration and supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the Chinese National Natural Science Foundation (No. 31971384), Guangzhou Science and Technology Plan (No. 201802030009), Innovation and Strengthening School Project from Guangdong Pharmaceutical University (Nos. 2016KTSCX067 and 2016SFKC_28), and Science and Technology Plan of Guangdong Province (Nos. 2017A010105011 and 2014A020208134).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.633004/full#supplementary-material

REFERENCES

Autore, F., Vecchio, C. D., Fraternali, F., Giardina, P., Sannia, G., and Faraco, V. (2009). Molecular determinants of peculiar properies of a pleurotus ostreatus laccase: analysis by site-directed mutagenesis. *Enzyme. Microb. Tech.* 45, 507–513. doi: 10.1016/j.enzmictec.2009.08.004

Brown, R. E., Jarvis, K. L., and Hyland, K. J. (1989). Protein measurement using bicinchoninic acid: elimination of interfering substances. *Anal. Biochem.* 180, 136–139. doi: 10.1016/0003-2697(89)90101-2

Camarero, S., Ibarra, D., Martinez, M. J., and Martinez, A. T. (2005). Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. *Appl. Environ. Microb.* 71, 1775–1784. doi: 10.1128/aem.71.4.1775-1784.2005

Feng, J., and Li, H. (2015). Screening, cloning, expression and enzymatic properties of a novel phospholipase A1 gene from the mangrove soil metagenomic library. *Microbiol. China.* 3, 489–496.

Koschorreck, K., Schmid, R. D., and Urlacher, V. B. (2009). Improving the functional expression of a Bacillus licheniformislaccase by random and site-directed mutagensis. *BMC. Biotechnol.* 9:12. doi: 10.1186/1472-6750-9-12

Li, M. (2013). *Study On the Degradation of Dyes with Different Structures by White-rot Fungi and Their Laccase*. Wuhan: Huazhong University of Science and Technology.

Li, X. Y., Wang, J. D., Huang, B. S., Liao, S. H., and Chen, G. Y. (2020). Study on the screening, identification and enzymatic properties of *Bacillus thuringiensis* strain Lac-72 produced by thermostable Laccase. *J. Shandong. Agr. U. (Nat. Sci. Ed.)* 51, 797–803.

Lv, J. T., Wei, L., Cao, X. L., Yang, J. Y., Guo, X. J., Li, L. B., et al. (2015). Screening, identification and bacteriostat qualitative analysis of antagonistic strain against colletotrichum gloeosporioides. *J. HN. Agric. Sci.* 44, 89–93. doi: 10.15933/j.cnki.1004-3268.2015.07.020

Madzak, C., Mimmi, M. C., Caminade, E., Brault, A., Baumberger, S., Briozzo, P., et al. (2006). Shifting the optimal pH of activity for a laccase from the fungus Trametes versicolor by structure-based mutagenesis. *Protein. Eng. Des. Sel.* 19, 77–84. doi: 10.1093/protein/gzj004

Miele, A., Giardina, P., Sannia, G., and Faraco, V. (2010). Random mutants of a *Pleurotus ostreatus* laccase as new biocatalysts for industrial effluents bioremediation. *J. Appl. Microbiol.* 108, 998–1006. doi: 10.1111/j.1365-2672.2009.04505.x

Mills, D. R., Peterson, R. L., and Spiegelam, S. (1967). An extracellular dawinian experiment with a self-duplieating nucleic acid molecule. *Pans.* 58, 217–224. doi: 10.1073/pnas.58.1.217

Sheng, N. (2019). *Analysis of the Influence of Calcium and Magnesium Ions on the Properties of Liquid Dyes*. Tianjin: Polytech.

Si, J., Cui, B. K., He, S., and Dai, Y. C. (2011). Optimization of conditions for laccase production by perenniporia subacida and its application in dye decolorization. *J.Appl. Environ. Biol.* 17, 736–741.

Singh, A. D., and Kumar, S. R. (2010). Ligninolytic fungal laccases and their biotechnological applications. *Appl. Biochem. Biotech.* 160, 1760–1788. doi: 10.1007/s12010-009-8676-y

Susana, C., David, I., Angel, T. M., Javier, R., Ana, G., Jose, C., et al. (2007). Paper pulp delignification using laccase and natural mediators. *Enzyme. Microb. Tech* 40, 1264–1271. doi: 10.1016/j.enzmictec.2006.09.016

Wang, G. D., and Chen, X. Y. (2003). The properties, functions, catalytic mechanism and applicability of laccase. *Bull. Bot.* 4, 469–475.

Wang, S. M., Gu, Z. B., Zhu, Y. X., Leng, J., Feng, L., Li, Q., et al. (2017). Review on directed evolution of protein. *Feed. China* 14, 15–52.

Xia, Y. L., Cheng, L. H., and Li, H. (2014). Preliminary screening and identification of anti-tumor active strains in marine sediments of the South China Sea. *J. GD. Coll. Pharm.* 1, 76–80.

Zhang, Z. X., Liu, H. Y., Xing, H., and Ma, Y. (2017). Research progress on substrate species degraded by laccase. *Biol. Bull.* 33, 97–102.

Zhao, M. L. (2012). *Studies on the Production of Laccase From White rot Fungus and its Degradation Application in Printing Wastewater*. Nanchang: Nanchang University.

Conflict of Interest: SL, JY, and XX were employed by company Guangzhou Base Clean Cosmetics Manufacturer Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership