Water Science and Engineering xxxx, xxx(xxx): xxx

HOSTED BY

Available online at www.sciencedirect.com

Water Science and Engineering

journal homepage: wse.hhu.edu.cn

Optimising a novel biofilm-based process using *Neurospora discreta* for enhanced treatment of lignin-rich wastewater

Himani Taneja [a], Shamas Tabraiz [b], Asma Ahmed [c,*]

[a] *Department of Vector Biology, Liverpool School of Tropical Medicine, Liverpool L3 5QA, United Kingdom*
[b] *Department of Civil & Environmental Engineering, Imperial College London, London SW7 2AZ, United Kingdom*
[c] *Department of Chemical and Environmental Engineering, University of Nottingham, Nottingham NG7 2RD, United Kingdom*

Received 19 September 2024; accepted 27 January 2025
Available online ■ ■ ■

Abstract

Paper and pulp mills generate substantial volumes of wastewater containing lignin-derived compounds that are challenging to degrade using conventional wastewater treatment methods. This study presents a novel biofilm-based process for enhanced lignin removal in wastewater using the fungus *Neurospora discreta*, which effectively degrades lignin and forms robust biofilms at the air−liquid interface under specific conditions. The process was optimised using the Taguchi design of experiments approach, and three factors including pH, copper sulphate concentration, and trace element concentration were evaluated at three levels. Experimental data were analysed against three responses: lignin degradation efficiency and the activities of two ligninolytic enzymes (polyphenol oxidase and versatile peroxidase). The results indicated that wastewater pH was the most significant parameter affecting lignin degradation efficiency and enzyme activities. Over 70% lignin degradation was achieved at pH levels of 5 and 6 with copper sulphate concentrations above 4 mg/L, while degradation efficiency drastically dropped to 45% at a pH value of 7. Reversed-phase high-performance liquid chromatography analysis demonstrated the effects of the three factors on the polar and non-polar components of lignin in wastewater, revealing a clear decrease in all peak areas after treatment. Additionally, significant relationships were observed between biofilm properties (including porosity, water retention value, polysaccharide content, and protein content) and lignin removal efficiency. This study also reported for the first time the presence of versatile peroxidase, a ligninolytic enzyme, in *Neurospora* sp.

Keywords: Wastewater treatment; Fungal biofilms; *Neurospora discreta*; Lignin degradation; Taguchi design of experiments; Versatile peroxidase; Polyphenol oxidase

1. Introduction

Lignin is a complex and large biopolymer found in plants, playing a crucial role in providing strength and protection to plant structures (Rajesh et al., 2019). It is the second most abundant biopolymer on Earth, following cellulose. The resistance of lignin to degradation is attributed to its highly branched structure, which contains β-O-4 ether bonds, 5-5′ carbon−carbon bonds, and β-5 bonds that are notoriously difficult to break (Sun et al., 1999). In paper production, lignin constitutes a key component of lignocellulosic biomass, which is removed during the pulping process, resulting in the generation of large volumes of lignin-rich wastewater. If discharged untreated, this wastewater poses significant risks to human health, aquatic ecosystems, and plant life due to its high concentrations of lignin and phenolic compounds (Hutchins, 1979; Oikari and Nakari, 1982). The environmental impact of effluent from pulp and paper mills is severe, characterized by dark colouration, turbidity, high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), and low biodegradability. These issues primarily arise from the presence of lignin and its derivatives (Christov and van Driessel, 2003; Srivastava et al., 2005).

This work was supported by the Leverhulme Trust Research Project Grant (Grant No. RPG-2020-021).
* Corresponding author.
E-mail address: asma.ahmed@nottingham.ac.uk (Asma Ahmed).
Peer review under responsibility of Hohai University.

https://doi.org/10.1016/j.wse.2025.02.001

The activated sludge process is widely employed for the biological treatment of pulp mill effluents. However, it encounters challenges, including poor settling and low control over the microbial population within the sludge (Thompson et al., 2001). Moreover, the low tolerance of sludge microorganisms to high concentrations of lignin further restricts its effectiveness (Radjenović et al., 2015). To address these challenges, this study introduces a novel biofilm-based approach using *Neurospora discreta,* a pink ascomycete known for its capacity to degrade lignin in agricultural residues (Pamidipati and Ahmed, 2017). Remarkably, this fungus naturally forms robust biofilms at the air−liquid interface (Ahmed et al., 2020), a characteristic that has recently been exploited to remove nitrogen and phosphorous from lignin-based wastewater in a single-step process (Tabraiz et al., 2022). Biofilms, which consist of aggregates of microorganisms embedded in a three-dimensional matrix of extracellular polymeric substances (EPS) (Flemming and Wingender, 2010), provide stability and protection. This structure enables higher removal efficiencies, improved operational stability, and increased tolerance to toxic substances compared to planktonic (free-floating) microorganisms. Moreover, the ability of *N. discreta* to form robust biofilms at the air−liquid interface eliminates the need for additional support materials or specialised surfaces for biofilm growth (Tabraiz et al., 2022). This characteristic also facilitates direct access to both oxygen and nutrients within wastewater, potentially enhancing degradation efficiency.

This study harnessed the unique properties of *N. discreta* to develop and optimise an innovative biofilm-based process for lignin-rich wastewater treatment. The biofilm structure enhances tolerance to lignin, extends retention times, and leads to greater removal efficiencies (Mishra et al., 2022). Additionally, this approach simplifies downstream processing, as the biofilms can be easily harvested to remove microbial cells from treated wastewater (Ahmed et al., 2020). Another remarkable feature of this process is that the biofilms form at the air−liquid interface without requiring external structures or carriers for support (Zheng et al., 2023).

To optimise this biofilm-based process, the Taguchi design of experiments (DoE) method was employed to investigate relative interactions among key parameters and their effects on lignin degradation, enzyme activities, and biofilm properties. The Taguchi DoE method is a statistical approach designed for processes with multiple independent parameters, referred to as factors, across various levels of interest (Prasad et al., 2005). While the Taguchi DoE method has been used to enhance ligninolytic enzyme activity in biochemical processes (Nandal et al., 2013; Parveen et al., 2022), its application in optimising the treatment of lignin-rich wastewater remains largely unexplored.

This study investigated the impacts of initial culture pH, $CuSO_4$ as an enzyme inducer, and trace elements in wastewater media at three different levels on lignin removal and enzyme activities during the treatment of lignin-rich wastewater using *N. discreta* biofilms. Additionally, this study evaluated the effects of these key parameters on biofilm properties, including microstructure and composition, and discussed the relationship between biofilm properties and lignin removal efficiencies. The novelty of this study includes the following two aspects:

(1) This study introduces an innovative biofilm-based process for treating lignin-rich wastewater, utilising fungal biofilms that form naturally at the air−liquid interface without the need for external carriers or supports. By employing a systematic DoE approach, this study advances the effectiveness of lignin degradation while deepening our understanding of the factors influencing biofilm-based processes, which have not been extensively explored in the treatment of lignin-rich effluents. Moreover, this study provides key insights into the relationships between biofilm properties and lignin removal for the first time.

(2) By addressing lignin concentrations significantly higher than those typically reported, this study more accurately reflects the actual lignin concentrations found in effluents from the paper and pulp industry. This study therefore advances current knowledge and offers valuable insights into the real-world application of lignin degradation, with potential to transform and improve wastewater treatment practices in industries facing substantial environmental challenges due to lignin-rich effluents.

2. Methods

2.1. Fungus maintenance and inoculation

N. discreta cells (Pamidipati and Ahmed, 2017) were maintained on potato dextrose agar (PDA) plates, which were stored at 4°C for spore collection. To prepare the spores for inoculation, Vogel's minimal media, containing no sucrose, were used to flood the plates. Fungal filaments were gently scraped off to collect the spores suspended in the media (Vogel, 1964). The spore suspension was then filtered through sterile muslin cloth and used for inoculation. A haemocytometer was used to determine the spore count, and the spore suspension was adjusted to 1×10^7 spores per millilitre. Subsequently, the reactors were inoculated using 1 mL of this spore suspension.

2.2. Wastewater composition and fermentation setup

Lignin-rich wastewater was prepared by adding 17.5 g/L of water-soluble Kraft lignin (Sigma−Aldrich, 4710003) to Vogel's minimal media containing 2.5 g/L of sucrose. The wastewater was designed to replicate lignin concentrations typically found in effluents from paper and pulp industry bleaching plants while providing essential nutrients required for fungal growth.

Experiments were conducted in 250-mL Erlenmeyer flasks each containing 120 mL of the prepared media, which was sealed with foam bungs. The pH value was adjusted, and other variables, including the concentrations of $CuSO_4$ and trace elements, were modified according to the experimental design outlined in Section 2.4. Each of the nine experimental conditions was established in triplicate.

Please cite this article as: Taneja, H et al., Optimising a novel biofilm-based process using *Neurospora discreta* for enhanced treatment of lignin-rich wastewater, Water Science and Engineering, https://doi.org/10.1016/j.wse.2025.02.001

Negative controls were set up with an uninoculated reactor containing 17.5 g/L of Kraft lignin (Sigma−Aldrich) in Vogel's media with 2.5 g/L of sucrose. The flasks were autoclaved at 121°C for 15 min. Each flask was inoculated under sterile conditions with 1×10^7 spores per millilitre after the media were cooled to room temperature. This process was conducted over a period of 14 d, with samples collected on days 3, 7, 10, and 13 after inoculation.

2.3. Design of experiments (DoE)

In this study, DoE focused on three key factors: culture pH, $CuSO_4$ as an inducer of ligninolytic enzymes (Moshtaghioun et al., 2017), and trace elements. The experiment setup utilised an L9 (3^4) orthogonal array following the Taguchi DoE method, with three levels for each of the three factors. This design was implemented and analysed using MINITAB statistical software. The results from the nine experiments were analysed to identify the most significant factor contributing to enhanced lignin degradation. To ensure the activity of ligninolytic enzymes and maintain lignin solubility in wastewater, three pH levels of 5, 6, and 7 were chosen for the experiments. $CuSO_4$ was tested at three concentrations: 1 mg/L, 4 mg/L, and 8 mg/L, serving as a ligninolytic enzyme inducer. Three different concentrations of trace element solutions (0.05 mL/L, 0.20 mL/L, and 0.30 mL/L in volume-to-volume percentage) were added to the culture media. These solutions contained citric acid, zinc, $CuSO_4$, boric acid, ammonium iron(II) sulphate, sodium molybdate, and manganese sulphate.

2.4. Reversed-phase high-performance liquid chromatography (RP-HPLC)

A Thermo Scientific™ LTQ XL™ RP-HPLC equipped with an ultraviolet (UV) detector was utilised to analyse lignin in the wastewater before and after treatment using *N. discreta* biofilms. This analysis employed a C18 column (with a length of 250 mm, an inner diameter of 4.6 mm, and a particle size of 5 μm) coupled with a guard column (apHera™; with a particle size of 5 μm, a length of 1 cm, and an inner diameter of 4.6 mm). Compounds were detected at a wavelength of 254 nm using a photodiode array detector. A two-solvent gradient elution process was implemented, consisting of HPLC-grade water (solvent A) and 100% acetonitrile (solvent B). The concentration gradient for solvent B was established as follows: 0% at 0 min, 3% at 5 min, 6% at 10 min, 40% at 15 min, and 80% at 20−25 min. The sample injection volume was set at 25 μL, and a flow rate of 1 mL/min was maintained, following a modified version of a previously developed method (Pamidipati and Ahmed, 2017). Data integration was performed using the Xcalibur FreeStyle application. Prior to analysis, samples collected on the last day of fermentation (day 14) were precipitated to remove spent media. The precipitated lignin was redissolved in water at a pH value of 10.5 for RP-HPLC analysis. Column washes and media blanks were run at regular intervals to ensure no carryover of compounds. Media blanks showed no visible peaks.

Kraft lignin (Sigma−Aldrich) solutions at varying concentrations were used to generate a standard curve. The percentage of lignin degradation or removal was calculated based on the total peak areas of polar and non-polar peaks using the following equation:

$$E = \frac{C_0 - C_f}{C_0} \times 100\% \quad (1)$$

where E is the lignin removal percentage, C_0 is the initial lignin concentration, and C_f is the final lignin concentration.

2.5. Activities of ligninolytic enzymes

Polyphenol oxidase (PPO) activity was determined using pyrocatechol as a substrate, with increases in absorbance measured for the formation of p-quinone, which absorbs light at 420 nm as described in Section 2.7 (Bending and Read, 1997). One unit of enzyme activity was defined as an increase in absorbance of 0.001 min^{-1} and is expressed in mmol/(L·min) (Ascacio-Valdés et al., 2014).

Versatile peroxidase (VP) activity was determined by oxidising reactive black dye (RB5) in a 50-mmol/L sodium tartrate buffer at a pH value of 3 (Ravichandran et al., 2019). Decreases in absorbance were recorded at 598 nm at an extinction coefficient (ε_{598}) of 24 000 L/(mol·cm). One unit of enzyme activity is defined as the amount of enzyme that transforms 1 mol of substrate consumed.

Laccase activity was spectrophotometrically quantified by measuring the oxidation kinetics of the substrate 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Bourbonnais et al., 1998). In this assay, 100 μL of centrifuged samples from each reactor were mixed with 100 μL of the 0.1-mol/L citrate buffer at a pH value of 5 in a microwell plate. The mixture was allowed to equilibrate at room temperature for 30 min. Subsequently, 100 μL of 0.3-mol/L ABTS was added to the mixture, and spectral scans at 420 nm commenced immediately. Increases in the concentration of blue-green cation radicals resulting from ABTS oxidation correlated with enzyme activity, using an extinction coefficient (ε_{420}) of 36 000 L/(mol·cm). Enzyme activity was defined as the amount of product formed per unit volume per unit time under the assay conditions (Saito et al., 2003).

Appropriate controls were established for each assay to ensure no interference from non-enzymatic reactions or other background reactions.

2.6. Biofilm composition

To determine the biochemical composition of biofilms, extracellular polymeric substances (EPS) were extracted from weighed biofilms by suspending them in a 10-mL solution of sodium chloride (8.5%) and formaldehyde (0.22%). The suspended biofilms were incubated for at 4°C for 3 h and then centrifuged at 10 000*g* (with *g* denoting the gravitational force) for 10 min. The supernatant was filtered through a 0.45-μm filter and retained for further analysis.

Please cite this article as: Taneja, H et al., Optimising a novel biofilm-based process using *Neurospora discreta* for enhanced treatment of lignin-rich wastewater, Water Science and Engineering, https://doi.org/10.1016/j.wse.2025.02.001

Polysaccharides in EPS were quantified with the dinitrosalicylic acid (DNS) reagent method (Gonçalves et al., 2010). EPS was hydrolysed in glass test tubes by mixing 1 mL of extracted EPS with 0.25 mL of 98% sulphuric acid and heating the mixture in a water bath at 95°C for 1 h. After cooling to room temperature, the solution was neutralised by adding 10-mol/L sodium hydroxide. The reaction mixture was prepared in 96-well plates by adding 25 μL of the DNS solution to 25 μL of the EPS solution. The plates, with the lid on, were heated in an oven at 100°C for 10 min, immediately followed by placing them on ice and adding 250 μL of ice-cold water. Absorbance was measured at 540 nm using a MULTISCAN GO spectrophotometer, and polysaccharide concentrations were calculated using a glucose standard curve.

Protein content in the biofilm EPS samples was estimated using a colorimetric protein kit (77371, Sigma Aldrich, UK), following the manufacturer's protocol.

2.7. Biofilm microstructure

Harvested biofilms were placed on absorbent paper for approximately 1 h to drain excess water. For microscopy, a section of the biofilm was washed with tap water to remove lignin particles and media and then placed on a microscopic slide. The slide was left to dry in an incubator at 25°C for 2 d. The dried biofilms were subsequently imaged using a scanning electron microscope (SEM) at 1 500 and 2 500 magnifications. The obtained images were analysed using ImageJ software (US National Institute of Health) to measure filament length, filament thickness, and total pore surface area (Huang and Wang, 1995). To determine the areal porosity of each biofilm, bright field SEM images were converted into eight-bit images, followed by the application of a threshold algorithm (Huang and Wang, 1995). The pore area was calculated using methods described previously by Ahmed et al. (2020). Filament dimensions, including length and thickness, were measured manually using the segmented line function (Abramoff et al., 2004).

2.8. Statistical analysis

Enzyme activities and lignin degradation were analysed using two-way analysis of variance (ANOVA) with a significance level of 0.05 (95% confidence interval). Section 3 reports the ANOVA findings for lignin degradation, polyphenol oxidase activity, and versatile peroxidase activity. Factors with *p*-values less than 0.05 were considered significant, indicating a rejection of the null hypothesis. The efficacy and significance of these individual factors were further validated through probability analysis.

3. Results and discussion

3.1. Influence of factors on lignin removal efficiency

Lignin degradation efficiency was calculated from the RP-HPLC chromatograms as described in Section 2.4. The characteristic lignin profile of the standards, as well as that of the untreated wastewater, exhibited a prominent polar peak at a retention time of (2.8 ± 0.1) min, along with smaller and relatively non-polar peaks eluting between (15.8 ± 0.1) min and (16.5 ± 0.1) min. After treatment with *N. discreta* biofilms, the wastewater demonstrated a significant decrease in peak areas across all experimental conditions. Fig. 1 shows a representative set of chromatograms obtained before and after treatment at pH levels of 5, 6, and 7. At a pH value of 5, lignin degradation of the polar peak exhibited efficiencies ranging from 68% to 71% across all concentrations of $CuSO_4$ and trace elements, with the peak at 15.8 min becoming undetectable after treatment (Table 1 and Fig. 1). Degradation of the 16.5-min peak was significantly effective at a pH value of 5, with efficiencies ranging from 67% to 83%. At a pH value of 6, the most effective lignin degradation was observed at a $CuSO_4$ concentration of 4 mg/L and a trace element concentration of 0.30 mL/L.

Table A.1 in Appendix A summarises the assignment of variables, parameter levels, and outputs for the L9 design. The overall effects of the three factors are illustrated in the plot of main effects (Fig. A.1 in Appendix A). ANOVA revealed the relative effects of these factors on lignin degradation. Each factor's contribution was calculated using the ratio of pure sum to total sum of squares (SS) adjusted mean square values (Table A.2 in Appendix A). Among all the factors analysed, pH exhibited the most significant effect on lignin degradation efficiency, contributing 68.93% to the overall variance ($p = 0.003$). Trace elements ranked second, albeit with a substantially lower contribution compared to pH. Higher lignin degradation observed at lower pH can be attributed to two factors: enhanced fungal growth at lower pH (Rousk et al., 2009; Taboada-Puig et al., 2011) and increased activity of ligninolytic enzymes, such as peroxidases, at low pH conditions. Previous studies have demonstrated that fungal peroxidases exhibit increased activity on aromatic compounds at pH levels as low as 3 (Heinzkill et al., 1998; Singh et al., 2021). For instance, the optimum pH for ligninolytic enzyme activity in *Phanerochaete chrysosporium* was found to be 4.5−5.5, with a decrease in lignin degradation levels reported at pH values above 5.5 (Garg and Modi, 1999).

At pH levels of 6 and 7, increasing the concentration of trace elements resulted in enhanced lignin degradation efficiencies. However, this effect was not significant at a pH value of 5. At the highest level of trace elements (0.3 mL/L), increasing $CuSO_4$ concentrations led to improved lignin degradation levels.

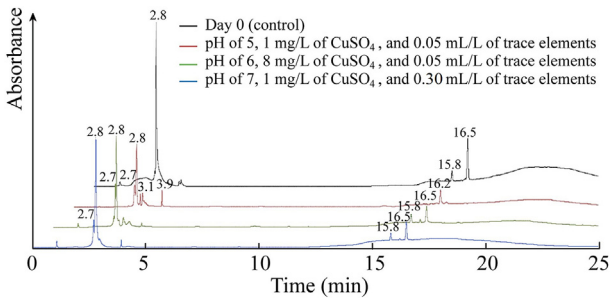


Fig. 1. RP-HPLC profiles for untreated wastewater samples from day 0 and treated wastewater samples under three representative conditions (adapted from Taneja (2023)).

Please cite this article as: Taneja, H et al., Optimising a novel biofilm-based process using *Neurospora discreta* for enhanced treatment of lignin-rich wastewater, Water Science and Engineering, https://doi.org/10.1016/j.wse.2025.02.001

Table 1
Degradation of relatively polar and non-polar components of lignin with varying pH and concentrations of $CuSO_4$ and trace elements after 14 d.

pH	$CuSO_4$ concentration (mg/L)	Trace element concentration (mL/L)	Lignin degradation efficiency (%)		
			Polar peaks	Non-polar peaks	Total
5	1	0.05	66.00 ± 3.0	82.44 ± 0.76	69.00 ± 3.76
	4	0.20	71.77 ± 2.3	67.00 ± 0.47	72.00 ± 2.77
	8	0.30	69.00 ± 1.2	79.85 ± 0.20	77.00 ± 1.40
6	1	0.20	64.88 ± 4.0	64.74 ± 0.56	65.00 ± 4.56
	4	0.30	73.03 ± 2.4	88.55 ± 0.42	74.00 ± 2.82
	8	0.05	52.71 ± 2.3	69.67 ± 0.76	54.00 ± 3.06
7	1	0.30	47.06 ± 6.2	64.74 ± 0.00	50.00 ± 6.20
	4	0.05	46.52 ± 4.2	58.89 ± 0.81	48.00 ± 5.01
	8	0.20	46.80 ± 3.3	52.34 ± 0.56	48.00 ± 3.86

Although the lignin degradation efficiencies reported in this study are comparable to those in the literature, it is important to highlight that this study was conducted at significantly higher lignin concentrations, demonstrating a more efficient process. For instance, most studies reported initial lignin concentrations ranging from 0.05 g/L to 0.10 g/L (Vashi et al., 2018), whereas this study was conducted at a lignin concentration of 17.5 g/L to better reflect the conditions found in effluents from pulping and bleaching processes. This finding underscores the ability of *N. discreta* biofilms to effectively tolerate and treat high levels of lignin in wastewater.

3.2. Influence of factors on ligninolytic enzyme activities

Under nutrient-limiting conditions, cultures transition to a secondary metabolic state during which ligninolytic enzymes are produced (Baldrian, 2006). Enzyme activity for PPO and VP was observed under all conditions, as detailed in the following sections. Enhanced enzyme activity in acidic cultures was noted from day 3 onwards, coinciding with the formation of fully formed biofilms on the wastewater surface. However, laccase activity was not detected in any of the cultures. The absence of laccase activity can be attributed to the presence of lignin degradation products in the wastewater, which are known inhibitors of laccase, as demonstrated in previous studies (Pamidipati and Ahmed, 2020).

3.2.1. Polyphenol oxidase activity

The highest PPO activity was obtained at the lowest pH (5) and the lowest $CuSO_4$ concentration (1 mg/L) (Fig. 2(a) and Table A.3 in Appendix A). The mechanism and activity of fungal PPOs are highly dependent on copper, which is essential for their activity. Studies have shown that the removal of copper from enzymes results in inactive forms, which can be reactivated by adding excess copper (Yoruk and Marshall, 2003). The effect of varying copper concentrations on enzyme activity depends on the type of fungus (Viswanath et al., 2014). For instance, in *Trametes trogii*, the addition of 1 000 μmol/L of $CuSO_4$ strongly induced the production of laccase, whereas in *Pleurotus ostreatus,* PPO was induced at much lower levels (150 μmol/L) of $CuSO_4$ (Levin et al., 2002; Palmieri et al., 1993). However, higher levels of copper have been reported to inhibit fungal growth and PPO activity in *T. trogii* (Levin et al., 2002). In this study, increasing $CuSO_4$ concentration resulted in lower PPO activity in *N. discreta* biofilms, indicating an inhibitory effect of copper ions on PPO at high concentrations. This observation explains the effect of $CuSO_4$ on lignin degradation efficiency, as discussed in the previous section.

A significant decrease in PPO activity was observed with increasing pH (Fig. 2(a)). The growth of PPO is associated with an optimum pH in the acidic range, consistent with the preference of most fungi for acidic conditions (Palmieri et al., 1993). This explains the enhanced PPO activity observed in acidic cultures. Trace element concentration did not significantly affect PPO activity. Consequently, the interaction between pH and trace element concentration (Fig. 2(b)) was prominently influenced by pH. As shown in Fig. 2(c), the highest PPO activity was observed at the lowest levels of both $CuSO_4$ and trace elements. These observations suggest several influences on PPO activity: (1) pH dominance, overshadowing any potential effects from other factors; (2) copper sensitivity, indicating an inhibitory effect of $CuSO_4$ at higher concentrations; and (3) minimal influence of trace element concentration, likely due to sufficient quantities already present in the medium.

ANOVA for PPO activity presented in Table A.3 indicated that initial pH and the addition of $CuSO_4$ significantly influenced lignin degradation ($p = 0.048$), while trace element concentration showed no significant effect ($p > 0.05$). The analysis also revealed the contributions of various factors to PPO activity: pH accounted for the highest contribution at 58.23%, followed by $CuSO_4$ concentration at 19.14%, and trace element concentration at 8.57%, which was the lowest. This ranking underscores that pH was the most critical factor for PPO activity, with $CuSO_4$ concentration being the second most important.

3.2.2. Versatile peroxidase activity

VP (E.C.1.11.1.16) has been primarily reported in genera such as *Pleurotus* sp., *Bjerkendera* sp., and *Physisporinus* sp. (Knop et al., 2015). This study represents the first report of VP production in *Neurospora* sp., which have commonly been associated with laccase and PPO production (Luke and Burton, 2001). Additionally, there is limited evidence of VP production from biofilm structures in the literature (Sridhar, 2016).

In this study, VP activity was significantly affected by pH (Fig. 2(d) through (f) and Table A.3 in Appendix A). The results revealed complex interactions among pH, $CuSO_4$ concentration, and trace element concentration. At a pH value of 5, increasing $CuSO_4$ and trace element concentrations led to enhanced VP activity, contrasting with the observed pattern for PPO activity. This resulted in relatively consistent overall lignin degradation across all $CuSO_4$ levels due to the opposing effects of VP and PPO. Conversely, in higher pH conditions, increasing $CuSO_4$ concentrations caused a decrease in VP activity. However, at a pH value of 7, VP activity remained low regardless of $CuSO_4$ concentrations. These findings highlight the intricate balance between different enzymatic activities in lignin degradation and underscore the importance of pH in regulating VP activity.

ANOVA for VP activity (Table A.3 in Appendix A) indicated that pH was the most significant factor for VP activity,

Please cite this article as: Taneja, H et al., Optimising a novel biofilm-based process using *Neurospora discreta* for enhanced treatment of lignin-rich wastewater, Water Science and Engineering, https://doi.org/10.1016/j.wse.2025.02.001

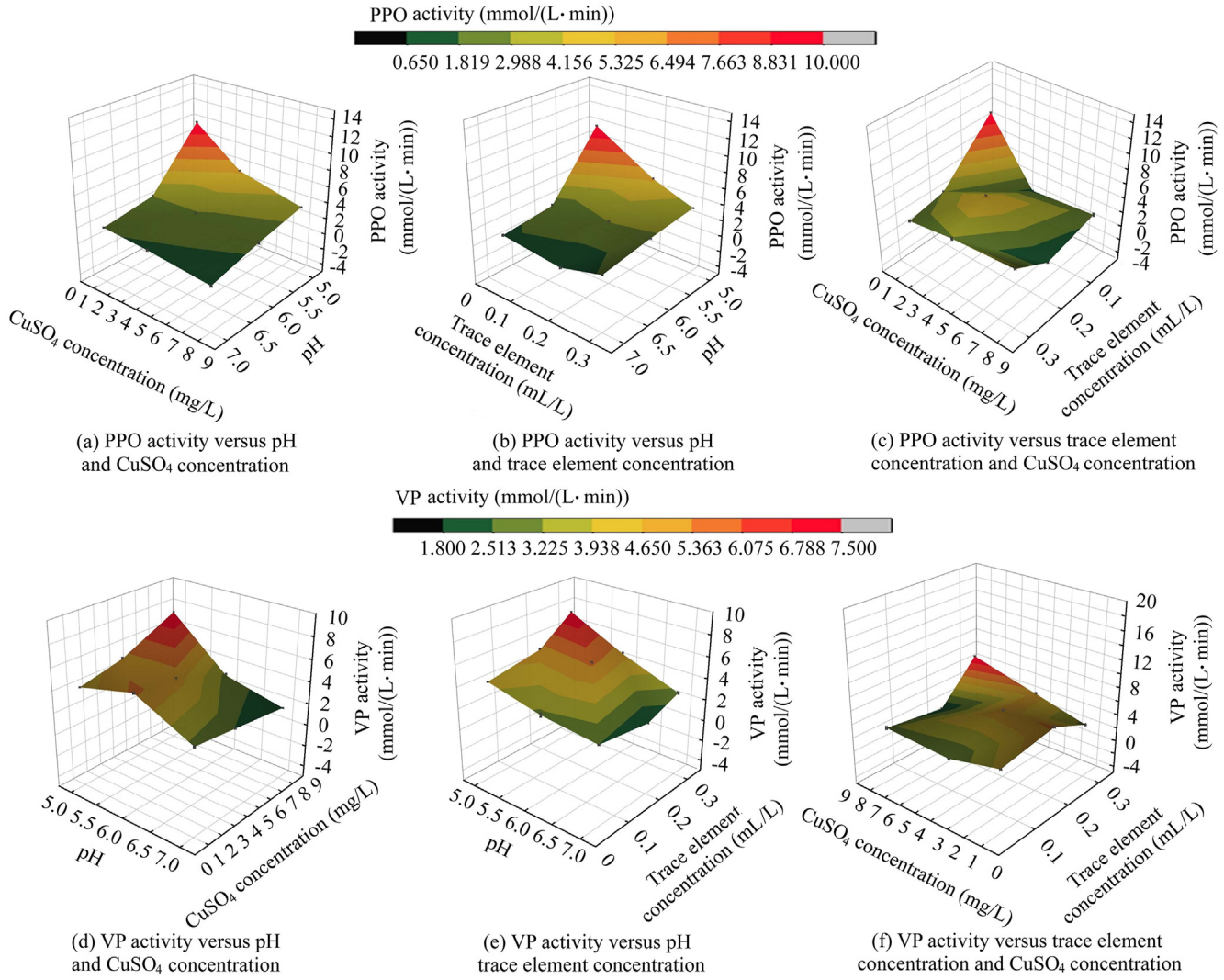


Fig. 2. Interaction plots showing enzyme activity of PPO and VP in response to changes in pH, $CuSO_4$ concentration, and trace element concentration.

contributing 59.2% to the variance ($p = 0.012$). Similar to PPO activity, pH ranked as the most influential factor among the three factors. However, unlike PPO, variations in $CuSO_4$ concentration did not significantly affect VP activity ($p > 0.05$).

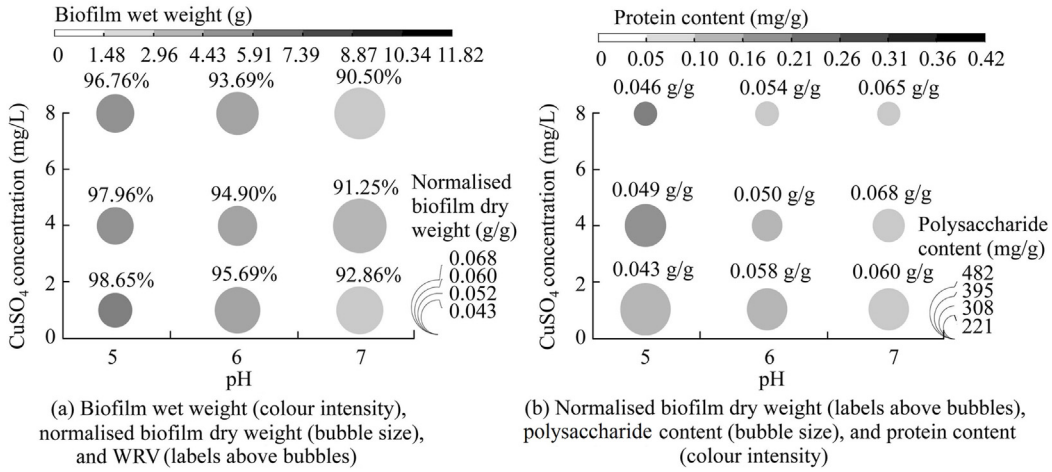
3.3. Influence of factors on biofilm composition and water retention value

Biofilms were observed at the air−liquid interface of all reactors approximately 24 h after inoculation, with their thickness increasing daily as new layers formed on the surface (Fig. A.2 in Appendix A). A strong positive correlation between lignin removal efficiency and the water retention value (WRV) of biofilms was observed with a two-tailed significance test, yielding a Pearson's correlation coefficient (R) of 0.854 and an associated p-value of 0.003. This indicates that the properties of biofilms were closely related to their ability to degrade lignin. A higher WRV suggests a greater water holding capacity, which can facilitate mass transfer and thereby influence lignin degradation efficiencies.

Fig. 3(a) shows the wet and dry weights of biofilms and WRV as influenced by pH and $CuSO_4$ levels. Compared to $CuSO_4$ concentration, pH had a more significant effect on biofilm thickness, which was notably higher at a pH value of 5 across all conditions. The biofilm wet weight and WRV decreased with increasing pH across all $CuSO_4$ concentration levels. However, the normalised dry weight increased with rising pH. This can be attributed to the enhanced solubility of lignin at high pH levels, leading to greater availability of carbon sources for cell growth.

Chemical analysis of EPS extracted from the biofilms revealed higher polysaccharide and protein concentrations in biofilms grown under more acidic conditions (Fig. 3(b)). Given that polysaccharides are hydrophilic, biofilms in acidic conditions absorb more water (Ahmed et al., 2020), resulting in improved lignin degradation. Copper is known to influence biofilm formation in both bacterial (Vargas-Straube et al., 2020) and fungal (Gomes et al., 2020) biofilms, with increased copper levels inhibiting EPS production. In this study, increasing $CuSO_4$ concentrations resulted in decreased polysaccharide and protein contents, particularly at lower pH levels (Fig. 3(b)), indicating that copper ions affected biofilm growth and EPS composition.

Please cite this article as: Taneja, H et al., Optimising a novel biofilm-based process using *Neurospora discreta* for enhanced treatment of lignin-rich wastewater, Water Science and Engineering, https://doi.org/10.1016/j.wse.2025.02.001



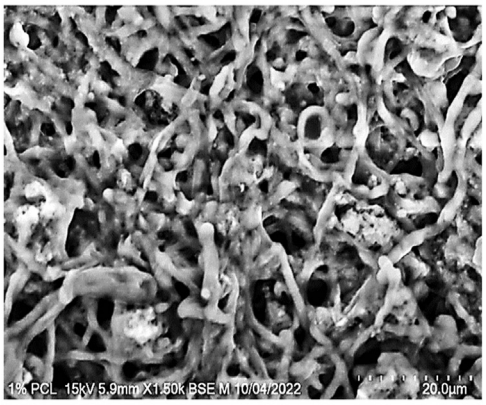
(a) Biofilm wet weight (colour intensity), normalised biofilm dry weight (bubble size), and WRV (labels above bubbles)

(b) Normalised biofilm dry weight (labels above bubbles), polysaccharide content (bubble size), and protein content (colour intensity)

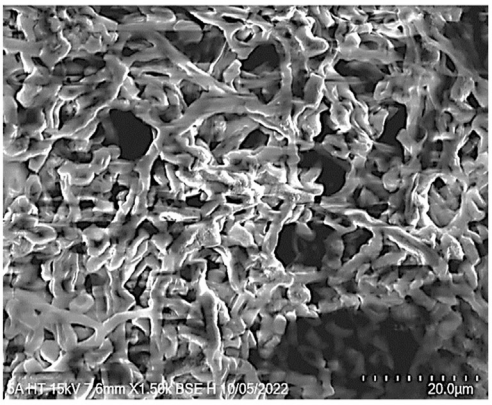
Fig. 3. Bubble plots for comparing biofilm wet weight, normalised biofilm dry weight, and WRV and for comparing normalised biofilm dry weight, polysaccharide content, and protein content.

3.4. Influence of factors on biofilm microstructure

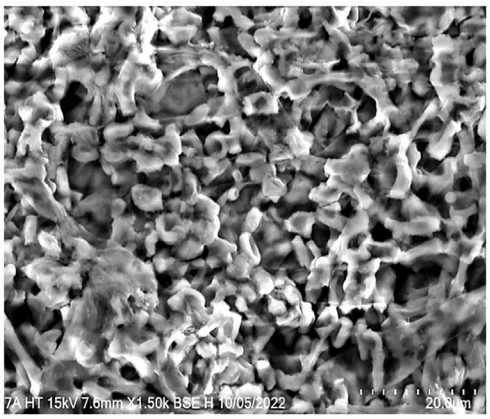
The SEM images (Fig. 4(a) through (c)) of the harvested biofilms were analysed to measure filament length, filament diameter, and pore area using the ImageJ application. This analysis aimed to establish the relationship between the microstructural features of the biofilms and their lignin removal capabilities. Culture pH is known to affect fungal



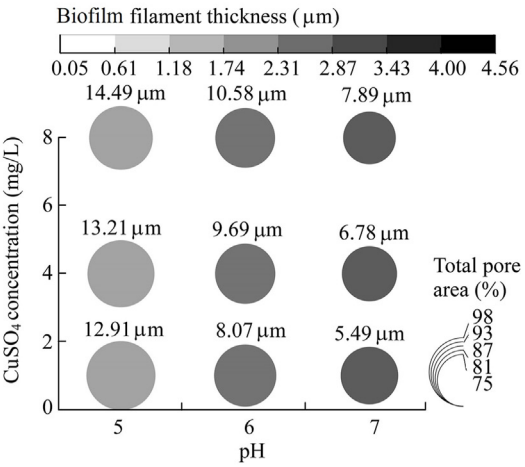
(a) SEM image of biofilms grown at pH of 5



(b) SEM image of biofilms grown at pH of 6



(c) SEM image of biofilms grown at pH of 7



(d) Bubble plot for comparing biofilm filament length (labels above bubbles), thickness (colour intensity), and total pore area (bubble size)

Fig. 4. Representative SEM images of biofilms grown at pH values of 5, 6, and 7 (with SEM accelerating voltage of 15 kV, 1 500 magnifications, and backscattered electron mode) and bubble plot for comparing biofilm filament length, thickness, and total pore area (adapted from Taneja (2023)).

Please cite this article as: Taneja, H et al., Optimising a novel biofilm-based process using *Neurospora discreta* for enhanced treatment of lignin-rich wastewater, Water Science and Engineering, https://doi.org/10.1016/j.wse.2025.02.001

morphology, with an increase in pH resulting in shorter hyphal length and increased hyphal thickness (Papagianni, 2004). The biofilms grown at a pH of 5 exhibited longer and thinner hyphae compared to those grown under neutral conditions, which displayed a more globular appearance (Fig. 4(a) and (c)). As the solubility of lignin is lower in acidic conditions (Norgren and Edlund, 2014), the lower pH of wastewater leads to reduced availability of lignin for the cells, prompting them to grow longer in search of nutrients, as observed in previous studies (Ahmed et al., 2020). A significant correlation was found between lignin removal efficiency and the total pore area of biofilms using a two-tailed significance test, yielding a Pearson's correlation coefficient (R) of 0.780 7 and a p-value of 0.013.

Additionally, the total pore area decreased with increasing pH (Fig. 4(d)). These results are consistent with previous studies, which reported an inverse correlation between filament diameter and total pore area (Ahmed et al., 2020). The pore area observed in biofilms under neutral pH conditions was significantly lower, resulting in thinner and less water-absorbing biofilms. Higher biofilm porosity is desirable as it allows for greater wastewater flow through the channels in biofilms, thereby exposing a greater surface area of filaments.

3.5. Correlations between lignin removal efficiency, enzyme activities, and biofilm properties

This study conducted an in-depth analysis to identify correlations between lignin removal efficiency, enzyme activities, and biofilm properties, revealing several significant relationships (Fig. 5). Lignin removal efficiency exhibited a strong positive correlation with VP activity ($R = 0.86$ and $p < 0.05$). In contrast, no significant correlation was observed between lignin removal efficiency and PPO activity. Additionally, a positive correlation was found between lignin removal efficiency and total protein content in the spent media.

This study identified strong correlations between lignin removal efficiency and various biofilm properties, consistent with previous findings (Tabraiz et al., 2022). Specially, lignin removal efficiency showed positive correlations with WRV ($R = 0.85$ and $p < 0.05$), total pore area ($R = 0.78$ and $p < 0.05$), and filament length ($R = 0.71$ and $p < 0.05$). In contrast, a negative correlation was observed with filament diameter ($R = -0.72$ and $p < 0.05$).

These findings underscore the intricate relationship between biofilm characteristics and lignin removal efficiency, highlighting the crucial role of the extracellular matrix in pollutant degradation and its significant influence on water adsorption, as noted by Yadav et al. (2023). This comprehensive analysis provides valuable insights into the factors affecting lignin removal efficiency and may inform future strategies for optimizing biofilm-based pollutant degradation processes.

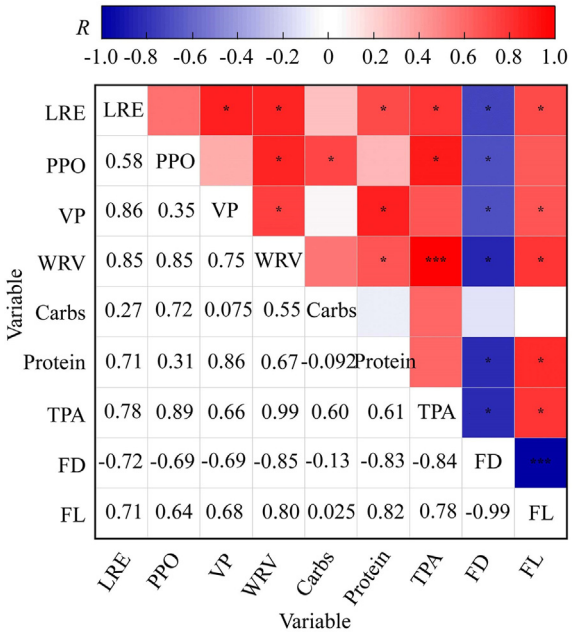


Fig. 5. Pearson's correlation plot showing correlations between lignin removal efficiency (LRE), polyphenol oxidase activity (PPO), versatile peroxidase activity (VP), water retention value (WRV), polysaccharide content (Carbs), protein content (Protein), total pore area (TPA), filament diameter (FD), and filament length (FL) (asterisks in upper triangle signify variables exhibit significant correlations, with "*", "**", and "***" denoting $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; red and blue colours denote positive and negative correlations, respectively; and Pearson's correlation coefficient (R) values are presented in lower triangle).

4. Conclusions

This study utilized the Taguchi DoE approach to identify key process parameters and their optimal values for lignin removal in high-strength wastewater using *N. discreta* biofilms. The findings reveal complex interactions between environmental factors, enzyme activities, and biofilm properties, all of which contribute to lignin removal.

Wastewater pH emerged as the most significant factor affecting the lignin removal efficiency of *N. discreta* biofilms. At a pH value of 5, other factors had minimal influence on overall lignin removal. Interestingly, at this pH, increasing $CuSO_4$ concentration enhanced VP activity while decreasing PPO activity, resulting in relatively consistent overall lignin degradation across various $CuSO_4$ levels. This highlights the intricate balance between different enzymatic activities in the lignin degradation process.

This study also revealed significant positive correlations between lignin removal efficiency and various biofilm properties, including WRV ($R = 0.85$ and $p < 0.05$), total pore area ($R = 0.78$ and $p < 0.05$), filament length ($R = 0.71$ and $p < 0.05$). A negative correlation was observed between lignin removal efficiency and filament diameter ($R = -0.72$ and $p < 0.05$). Additionally, the extracellular matrix composition,

Please cite this article as: Taneja, H et al., Optimising a novel biofilm-based process using *Neurospora discreta* for enhanced treatment of lignin-rich wastewater, Water Science and Engineering, https://doi.org/10.1016/j.wse.2025.02.001

particularly protein content, showed a positive correlation with lignin removal efficiency.

This research represents the first report of VP production in *Neurospora* sp., expanding our understanding of lignin-degrading capabilities across fungal species and growth forms. The biofilm-based process discussed in this study demonstrated up to 88% lignin removal from wastewater containing relatively high lignin concentrations (17.5 g/L), significantly higher than those typically reported in the literature (0.05−0.10 g/L). Given the ability of fungal biofilms to tolerate and treat high concentrations of lignin, the proposed process has the added advantage of footprint reduction and the potential for modular scalability, making it a sustainable and cost-effective alternative to large-scale wastewater treatment.

The novel biofilm-based process for the treatment of high-strength lignin-containing wastewater can potentially reduce the need for diluting effluents from paper and pulp industries. The insights gained into the relationships between process parameters, enzyme activities, and biofilm properties provide a foundation for optimizing biofilm-based wastewater treatment systems.

Declaration of competing interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wse.2025.02.001.

References

Abramoff, M.D., Magalhães, P.J., Ram, S.J., 2004. Image processing with ImageJ. Biophot. Int. 11(7), 36.

Ahmed, A., Narayanan, R.A., Veni, A.R., 2020. Influence of carbon source complexity on porosity, water retention and extracellular matrix composition of *Neurospora discreta* biofilms. J. Appl. Microbiol. 128(4), 1099−1108. https://doi.org/10.1111/jam.14539.

Ascacio-Valdés, J.A., Buenrostro, J.J., De la Cruz, R., Sepúlveda, L., Aguilera, A.F., Prado, A., Contreras, J.C., Rodríguez, R., Aguilar, C.N., 2014. Fungal biodegradation of pomegranate ellagitannins. J. Basic Microbiol. 54(1), 28−34. https://doi.org/10.1002/jobm.201200278.

Baldrian, P., 2006. Fungal laccases − Occurrence and properties. FEMS Microbiol. Rev. 30(2), 215−242. https://doi.org/10.1111/j.1574-4976.2005.00010.x.

Bending, G.D., Read, D.J., 1997. Lignin and soluble phenolic degradation by ectomycorrhizal and ericoid mycorrhizal fungi. Mycol. Res. 101(11), 1348−1354. https://doi.org/10.1017/S0953756297004140.

Bourbonnais, R., Leech, D., Paice, M.G., 1998. Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. Biochim. Biophys. Acta Gen. Subj. 1379(2), 381−390. https://doi.org/10.1016/S0304-4165(97)00117-7.

Christov, L., van Driessel, B., 2003. Waste water bioremediation in the pulp and paper industry. Indian J. Biotechnol. 2(3), 444−450.

Flemming, H.C., Wingender, J., 2010. The biofilm matrix. Nat. Rev. Microbiol. 8(9), 623−633. https://doi.org/10.1038/nrmicro2415.

Garg, S.K., Modi, D.R., 1999. Decolorization of pulp-paper mill effluents by white-rot fungi. Crit. Rev. Biotechnol. 19(2), 85−112. https://doi.org/10.1080/0738-859991229206.

Gomes, I.B., Simões, M., Simões, L.C., 2020. Copper surfaces in biofilm control. Nanomaterials 10(12), 2491. https://doi.org/10.3390/nano10122491.

Gonçalves, C., Rodriguez-Jasso, R.M., Gomes, N., Teixeira, J.A., Belo, I., 2010. Adaptation of dinitrosalicylic acid method to microtiter plates. Anal. Methods 2, 2046−2048. https://doi.org/10.1039/C0AY00525H.

Heinzkill, M., Bech, L., Halkier, T., Schneider, P., Anke, T., 1998. Characterization of laccases and peroxidases from wood-rotting fungi (family *Coprinaceae*). Appl. Environ. Microbiol. 64, 1601−1606. https://doi.org/10.1128/aem.64.5.1601-1606.1998.

Huang, L., Wang, M.J., 1995. Image thresholding by minimizing the measures of fuzziness. Pattern Recogn. 28(1), 41−51. https://doi.org/10.1016/0031-3203(94)E0043-K.

Hutchins, F.F., 1979. Toxicity of Pulp and Paper Mill Effluent: A Literature Review. U. S. Environmental Protection Agency, Washington DC.

Knop, D., Yarden, O., Hadar, Y., 2015. Ligninolytic peroxidases in the genus *Pleurotus*: Divergence in activities, expression, and potential applications. Appl. Microbiol. Biotechnol. 99(3), 1025−1038. https://doi.org/10.1007/s00253-014-6256-8.

Levin, L., Forchiassin, F., Ramos, A.M., 2002. Copper induction of lignin-modifying enzymes in the white-rot fungus *Trametes trogii*. Mycologia 94(3), 377−383. https://doi.org/10.1080/15572536.2003.11833202.

Luke, A.K., Burton, S.G., 2001. A novel application for *Neurospora crassa*: Progress from batch culture to a membrane bioreactor for the bioremediation of phenols. Enzym. Microb. Technol. 29(6−7), 348−356. https://doi.org/10.1016/S0141-0229(01)00390-8.

Mishra, S., Huang, Y., Li, J., Wu, X., Zhou, Z., Lei, Q., Bhatt, P., Chen, S., 2022. Biofilm-mediated bioremediation is a powerful tool for the removal of environmental pollutants. Chemosphere 294, 133609. https://doi.org/10.1016/j.chemosphere.2022.133609.

Moshtaghioun, S.M., Dadkhah, M., Bahremandjo, K., Haghbeen, K., Aminzadeh, S., Legge, R.L., 2017. Optimization of simultaneous production of tyrosinase and laccase by *Neurospora crassa*. Biocatal. Biotransform. 35(1), 1−10. https://doi.org/10.1080/10242422.2016.1266617.

Nandal, P., Ravella, S.R., Kuhad, R.C., 2013. Laccase production by *Coriolopsis caperata* RCK2011: Optimization under solid state fermentation by Taguchi DOE methodology. Sci. Rep. 3(1), 1386. https://doi.org/10.1038/srep01386.

Norgren, M., Edlund, H., 2014. Lignin: Recent advances and emerging applications. Curr. Opin. Colloid Interface Sci. 19(5), 409−416. https://doi.org/10.1016/j.cocis.2014.08.004.

Oikari, A.O., Nakari, T., 1982. Kraft pulp mill effluent components cause liver dysfunction in trout. Bull. Environ. Contam. Toxicol. 28, 266−270. https://doi.org/10.1007/BF01608505.

Palmieri, G., Giardina, P., Marzullo, L., Desiderio, B., Nitti, G., Cannio, R., Sannia, G., 1993. Stability and activity of a phenol oxidase from the ligninolytic fungus *Pleurotus ostreatus*. Appl. Microbiol. Biotechnol. 39, 632−636. https://doi.org/10.1007/BF00205066.

Pamidipati, S., Ahmed, A., 2017. Degradation of lignin in agricultural residues by locally isolated fungus *Neurospora discreta*. Appl. Biochem. Biotechnol. 181, 1561−1572. https://doi.org/10.1007/s12010-016-2302-6.

Pamidipati, S., Ahmed, A., 2020. A first report on competitive inhibition of laccase enzyme by lignin degradation intermediates. Folia Microbiol. 65, 431−437. https://doi.org/10.1007/s12223-019-00765-5.

Papagianni, M., 2004. Fungal morphology and metabolite production in submerged mycelial processes. Biotechnol. Adv. 22(3), 189−259. https://doi.org/10.1016/j.biotechadv.2003.09.005.

Parveen, S., Ali, M.I., Aslam, M., Ali, I., Jamal, A., Al-Ansari, M.M., Al-Humaid, L., Urynowicz, M., Huang, Z., 2022. Optimizing biocatalytic potential of *Dipodascus australiensis* M-2 for degrading lignin under laboratory conditions. Microbiol. Res. 265, 127179. https://doi.org/10.1016/j.micres.2022.127179.

Prasad, K.K., Mohan, S.V., Rao, R.S., Pati, B.R., Sarma, P.N., 2005. Laccase production by *Pleurotus ostreatus* 1804: Optimization of submerged culture conditions by Taguchi DOE methodology. Biochem. Eng. J. 24(1), 17−26. https://doi.org/10.1016/j.bej.2005.01.019.

Radjenović, J., Matošić, M., Mijatović, I., Petrović, M., Barceló, D., 2015. Membrane bioreactor (MBR) as an advanced wastewater treatment

Please cite this article as: Taneja, H et al., Optimising a novel biofilm-based process using *Neurospora discreta* for enhanced treatment of lignin-rich wastewater, Water Science and Engineering, https://doi.org/10.1016/j.wse.2025.02.001

technology. In: Barceló, D., Petrovic, M. (Eds.), Emerging Contaminants from Industrial and Municipal Waste. Springer, Berlin, Heidelberg, pp. 37−101. https://doi.org/10.1007/978-3-540-79210-9_2.

Rajesh, J., Kavitha, S., Yukesh Kannah, R., Poornima Devi, T., Gunasekaran, M., Kim, S., Kumar, G., 2019. A review on biopolymer production via lignin valorization. Bioresour. Technol. 290, 121790. https://doi.org/10.1016/j.biortech.2019.121790.

Ravichandran, A., Rao, R.G., Thammaiah, V., Gopinath, S.M., Sridhar, M., 2019. A novel versatile peroxidase from *Lentinus squarrosulus* towards enhanced delignification and *in vitro* digestibility of crop residues. Bioresources 14(3), 5132−5149. https://doi.org/10.15376/biores.14.3.5132-5149.

Rousk, J., Brookes, P.C., Bååth, E., 2009. Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. Appl. Environ. Microbiol. 75(6), 1589−1596. https://doi.org/10.1128/AEM.02775-08.

Saito, T., Hong, P., Kato, K., Okazaki, M., Inagaki, H., Maeda, S., Yokogawa, Y., 2003. Purification and characterization of an extracellular laccase of a fungus (family *Chaetomiaceae*) isolated from soil. Enzym. Microb. Technol. 33(4), 520−526. https://doi.org/10.1016/S0141-0229(03)00158-3.

Singh, A.K., Bilal, M., Iqbal, H.M.N., Raj, A., 2021. Lignin peroxidase in focus for catalytic elimination of contaminants — A critical review on recent progress and perspectives. Int. J. Biol. Macromol. 177, 58−82. https://doi.org/10.1016/j.ijbiomac.2021.02.032.

Sridhar, M., 2016. Versatile peroxidases: Super peroxidases with potential biotechnological applications − A mini review. J. Dairy Vet. Ani. Res. 4(2), 277−280. https://doi.org/10.15406/jdvar.2016.04.00116.

Srivastava, V.C., Mall, I.D., Mishra, I.M., 2005. Treatment of pulp and paper mill wastewaters with poly aluminium chloride and bagasse fly ash. Colloids Surf. A Physicochem. Eng. Asp. 260(1), 17−28. https://doi.org/10.1016/j.colsurfa.2005.02.027.

Sun, R., Tomkinson, J., Bolton, J., 1999. Separation and characterization of lignins from the black liquor of oil palm trunk fiber pulping. Separ. Sci. Technol. 34(15), 3045−3058. https://doi.org/10.1081/SS-100100821.

Taboada-Puig, R., Lú-Chau, T., Moreira, M.T., Feijoo, G., Martínez, M.J., Lema, J.M., 2011. A new strain of *Bjerkandera* sp. production, purification and characterization of versatile peroxidase. World J. Microbiol. Biotechnol. 27(1), 115−122. https://doi.org/10.1007/s11274-010-0435-2.

Tabraiz, S., Aiswarya, N.M., Taneja, H., Narayanan, R.A., Ahmed, A., 2022. Biofilm-based simultaneous nitrification, denitrification, and phosphorous uptake in wastewater by *Neurospora discreta*. J. Environ. Manag. 324, 116363. https://doi.org/10.1016/j.jenvman.2022.116363.

Taneja, H., 2023. The Development and Optimisation of a Lab-Scale Process for Biological Treatment of Lignin-Rich Wastewater Using Biofilms Formed by *Neurospora discreta*. PhD Dissertation. Canterbury Christ Church University, Christ Church. https://repository.canterbury.ac.uk/item/978y9/the-development-and-optimisation-of-a-lab-scale-process-for-biological-treatment-of-lignin-rich-wastewater-using-biofilms-formed-by-nuerospora-discreta.

Thompson, G., Swain, J., Kay, M., Forster, C.F., 2001. The treatment of pulp and paper mill effluent: A review. Bioresour. Technol. 77, 275−286. https://doi.org/10.1016/S0960-8524(00)00060-2.

Vargas-Straube, M.J., Beard, S., Norambuena, R., Paradela, A., Vera, M., Jerez, C.A., 2020. High copper concentration reduces biofilm formation in *Acidithiobacillus ferrooxidans* by decreasing production of extracellular polymeric substances and its adherence to elemental sulfur. J. Proteonomics 225, 103874. https://doi.org/10.1016/j.jprot.2020.103874.

Vashi, H., Iorhemen, O.T., Tay, J.H., 2018. Degradation of industrial tannin and lignin from pulp mill effluent by aerobic granular sludge technology. J. Water Proc. Eng. 26, 38−45. https://doi.org/10.1016/j.jwpe.2018.09.002.

Viswanath, B., Rajesh, B., Janardhan, A., Kumar, A.P., Narasimha, G., 2014. Fungal laccases and their applications in bioremediation. Enzym. Res. 163242. https://doi.org/10.1155/2014/163242, 2014.

Vogel, H.J., 1964. Distribution of lysine pathways among fungi: Evolutionary implications. Am. Nat. 98(903), 435−446. https://doi.org/10.1086/282338.

Yadav, S., Tripathi, S., Purchase, D., Chandra, R., 2023. Development of a biofilm-forming bacterial consortium and quorum sensing molecules for the degradation of lignin-containing organic pollutants. Environ. Res. 226, 115618. https://doi.org/10.1016/j.envres.2023.115618.

Yoruk, R., Marshall, M.R., 2003. Physicochemical properties and function of plant polyphenol oxidase polyphenol oxidase: A review. J. Food Biochem. 27, 361−422. https://doi.org/10.1111/j.1745-4514.2003.tb00289.x.

Zheng, Y., Song, H., Chen, Q., Hou, Y., Zhang, X., Han, S., 2023. Comparing biofilm reactors inoculated with *Shewanella* for decolorization of Reactive Black 5 using different carrier materials. Biotechnol. J. 19(1), 2300299. https://doi.org/10.1002/biot.202300299.

Please cite this article as: Taneja, H et al., Optimising a novel biofilm-based process using *Neurospora discreta* for enhanced treatment of lignin-rich wastewater, Water Science and Engineering, https://doi.org/10.1016/j.wse.2025.02.001