Section of Natural and Applied Sciences

THE DEVELOPMENT AND OPTIMISATION OF A LAB-SCALE PROCESS FOR BIOLOGICAL TREATMENT OF LIGNIN-RICH WASTEWATER USING BIOFILMS FORMED BY *NEUROSPORA DISCRETA*

By

Himani Taneja

Canterbury Christ Church University

Thesis submitted

for the Degree of Doctor of Philosophy

2023

Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of Canterbury Christ Church University or any other University or institute of learning.

Himani Taneja

November 2023

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr Asma Ahmed, for providing me with this unique opportunity. I extend my sincere gratitude for your invaluable guidance and encouragement throughout my doctoral years. Your expertise and insightful feedback have been instrumental in shaping my academic journey. Thanks to your belief in me, I have gained the confidence to pursue independent research. I am grateful to Dr Naeem Syed, my chair of studies, for approving my work during review meetings.

I express my sincere gratitude to my esteemed university, Canterbury Christ Church, for the enriching academic environment and to The Leverhulme Trust for funding my research. A big thanks to Dave Belsom, Krupa Bhatt, David Lisgarten, Hannah Shilling, Ali Ariss, and Ryan Steers for their technical support and friendly advice in the labs. Thank you, Shamas, for conducting two enzyme assays. I am deeply grateful to my friends Yashila Dewan and Giselle Dabbs and my colleagues for consistently reminding me of my abilities during those moments of self-doubt.

I would also like to extend my gratitude to my family for being my rock and standing by me through thick and thin. I am deeply grateful to my beloved husband, Himanshu, whose unwavering support and understanding made this journey possible. Your constant encouragement has helped me believe in myself and overcome any obstacles. To my precious daughter, Sanya, your patience, and love have fuelled my determination and made this achievement as much yours as it is mine.

Finally, I want to express my heartfelt appreciation to my parents for constantly reminding me that I can do anything I want. My father, who affectionately called me “Dr Sahib” even when I was a child, has been my anchor, providing the strength and motivation to navigate the challenges of pursuing a PhD. Papa - I finally did it, and this one is for you.

"Better once than never, for never too late."

— William Shakespeare, The Taming of the Shrew

Table of Contents

Acknowledgements iii
Abbreviations and Symbols ix
List of Figures xi
List of Tables xvi
Abstract 1
1. Introduction 3
1.1 Background and Overview 3
1.2 Lignin Structure 4
1.3 Recalcitrant Nature of Lignin 7
1.4 Pulp and Paper-Making Process 7
1.5 Characteristics of Pulp and Paper Mill Wastewaters 9
1.6 Structural Changes in Lignin During Kraft Process 11
1.7 Effect of Lignin on the Environment 12
1.8 Existing Wastewater Treatment Methods 12
1.8.1 Physical Processes 13
1.8.2 Chemical Process 13
1.8.3 Biological Treatment 14
1.9 Challenges in the Existing Biological Treatment Systems 25
1.10 Fungal Biofilms in Wastewater Treatment 28
1.11 Gaps in Existing Research 29
1.12 Research Questions and Aim 31
1.13 Rationale 32
1.14 Objectives 33
1.15 Thesis Organisation 36
2. Materials and Methods 37
2.1 Background 37
2.2 Fungal Strain Maintenance 37
2.3 Minimal Media and Wastewater Composition 38
2.4 Wastewater Media 39
2.5 Inoculation of Reactors 39
2.6 Sample Preparation for Lignin Estimation 41
2.7 pH Estimation 42
2.8 Lignin Estimation 42

2.8.1 Ultraviolet-visible Spectrometry 42
2.8.2 Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) 43
2.9 Chemical Oxygen Demand 46
2.10 Biofilm Growth and Harvest 46
2.11 Water Retention Value 46
2.12 Extracellular Polymeric Substances 48
2.13 Activity of Lignin Degrading Enzymes 48
2.13.1 Laccase Activity 48
2.13.2 Polyphenol Oxidase Activity 49
2.13.3 Versatile Peroxidase Activity 49
2.14 Microscopy 50
2.14.1 Scanning Electron Microscopy 50
2.14.2 Environment Scanning Electron Microscopy 50
2.14.3 Image Analysis 51
3. Optimisation of Process Conditions for Treatment of Lignin Wastewater Using Novel Biofilm Formed by *Neurospora discreta* 52
3.1 Introduction 52
3.2 Materials and Methods 54
3.2.1 Fungus Maintenance and Inoculation 54
3.2.2 Wastewater Composition and Fermentation Setup 55
3.2.3 Design of Experiments 55
3.2.4 Reversed-Phase High-Performance Liquid Chromatography 56
3.2.5 Activities of Lignin-Degrading Enzymes 57
3.2.6 Biofilm Composition 58
3.2.7 Biofilm Microstructure 58
3.2.8 Statistical Analysis 59
3.3 Results and Discussion 59
3.3.1 Effect of Process Parameters on Lignin Degradation Efficiency 59
3.3.2 Activities of Ligninolytic Enzymes 64
3.3.3 Biofilm Growth, Water Retention Value and Composition 68
3.3.4 Biofilm Microstructure Using Scanning Electron Microscopy 70
3.4 Conclusion 72
4. Addition of Lignin-Degradation Intermediates for Enhanced Lignin and COD Removal from Industrial Wastewater in a Biofilm-Based Process 73
4.1 Introduction 73

4.2 Materials and Methods 76
4.2.1 Wastewater Composition 76
4.2.2 Inoculum 77
4.2.3 Fermentation Setup 77
4.2.4 Feeding 78
4.2.5 Sample Preparation for UV-Based Lignin Estimation 78
4.2.6 Lignin Estimation Using Reverse Phase Liquid Chromatography 79
4.2.7 Identification of Lignin Degradation Compounds 80
4.2.8 Chemical Oxygen Demand 80
4.2.9 Water Retention Value and Dry Weight 80
4.2.10 Enzymes Activity 81
4.2.11 Extracellular Polymeric Substances Extraction 81
4.2.12 Polysaccharides and Protein Estimation in ECM 82
4.3 Results and Discussion 82
4.3.1 Lignin Estimation 82
4.3.2 Chemical Oxygen Demand Removal Efficiency 89
4.3.3 Activity of Lignin-Degrading Enzymes 90
4.3.4 Identification of Compounds Fed for Enhanced Degradation 92
4.3.5 Biofilm Microstructure and Composition 99
4.3.6 Correlations Between Process Efficiencies and Biofilm Properties 102
4.4 Conclusion 104
5. Evaluation of a Repeated-Batch Process for Treating Lignin-Rich Industrial Effluent Using Biofilms Formed by *Neurospora discreta* 106
5.1 Introduction 106
5. 2 Materials and Method 110
5.2.1 Fermentation Setup 110
5.2.2 Wastewater Media Composition 110
5.2.3 Inoculum 111
5.2.4 Lignin Estimation 112
5.2.5 Chemical Oxygen Demand 112
5.2.6 Enzyme activity 113
5.2.7 Biofilm Characterisation 113
5.2.8 Microscopy 113
5.3 Results and Discussion 114
5.3.1 Biofilm Growth 114

5.3.2 Lignin Estimation 115
5.3.3 Chemical Oxygen Demand Removal Efficiency 123
5.3.4 Activity of Lignin-Degrading Enzymes 124
5.3.5 Biofilm Characterisation 129
5.3.6 Correlations Between Process Efficiencies and Biofilm Properties 132
5.4 Conclusion 132
6. Evaluation of a Scale-Up Process for the Treatment of Lignin-Rich Wastewater by *Neurospora discreta* Biofilms in a Repeated-Batch System 135
6.1 Introduction 135
6.2 Materials and Methods 137
6.2.1 Fermentation Setup in Tray Reactors 137
6.2.2 Wastewater Media 138
6.2.3 Inoculation 139
6.2.4 Lignin Estimation 139
6.2.5 Chemical Oxygen Demand 140
6.2.6 Enzyme Activity 140
6.2.7 Biofilm Characterisation 141
6.3 Results and Discussion 141
6.3.1 Biofilm Growth 141
6.3.2 Lignin Estimation 142
6.3.4 Chemical Oxygen Demand Removal Efficiency 150
6.3.5 Activity of Lignin-Degrading Enzymes 152
6.3.6 Biofilm Composition - Extracellular Matrix Components 156
6.3.7 Correlations Between Process Efficiencies and Biofilm Properties 161
6.4 Discussion 161
6.5 Conclusion 163
7. Conclusion and Future Work 165
7.1 Thesis Highlights 165
7.2 Future Work 167
7.3 Concluding Remarks and Practical Consideration 169
8 Publications and Other Research Activities Arising from This Thesis 170
8.1 Publications 170
8.2 Manuscripts in Preparation 170
8.3 Presentations 170
9 References 171

Abbreviations and Symbols

COD	Chemical Oxygen Demand
BOD	Biological Oxygen Demand
VPO	Versatile Peroxidase
PPO	Polyphenol Oxidase
HPLC	High-Performance Liquid Chromatography
RP-HPLC	Reverse Phase High-Performance Liquid Chromatography
MS	Mass Spectrometry
UV	Ultraviolet
DOE	Design of Experiments
ESI	Electron Spray Ionization
PDA	Potato Dextrose Agar
RT	Retention Time
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline- 6-sulfonate
R	Pearson coefficient
ε	extinction coefficient
µm	microns
µM	Micromolar
µmoles	Micromoles
U	Unit of specific enzyme activity (µmoles-1min-1)
rpm	Rotations per minute
mg	Milligram
mL	Milliliter

µL	micro-liter
L	Litre
g	gram
mg	milligram
°C	degree Celsius
hrs	hours
mg L^{-1}	milligrams per litre

List of Figures

Figure

Page

Figure 1.1 Structure of lignin (a) Schematic representation of components of lignocellulosic biomass used for paper making (b) Monomeric unit of lignin precursors with H, G, and S (c) Structure of lignin macromolecule. 6

Figure 1.2 Schematic representation of pulp and paper making process 8

Figure 1.3 A schematic representation showing characteristics of wastewater generated during the pulp and paper-making process. 9

Figure 2.1 *Neurospora discreta* growing on PDA plates. 38

Figure 2.2 Fermentation set up for wastewater treatment in a stationary incubator 40

Figure 2.3 Standard curve for Kraft lignin 43

Figure 2.4 Chromatogram profile of lignin standard curve (a) Kraft lignin standard (b) Lignin precipitated from wastewater from paper and pulp mill 44

Figure 2.5 Biofilm microstructure (a) SEM (2D) image of biofilm (b) 3D surface plot of the SEM image 59

Figure 2.6 SEM image converted into 8-bit with thresholding algorithm applied in red..... 50

Figure 3.1 Lignin estimation (a) Reversed-phase high-performance liquid chromatography profiles for untreated wastewater samples compared with treated samples (b) Degradation of relatively polar and non-polar components of lignin 59

Figure 3.2 Main effects plot showing the effects of pH, $CuSO_4$ and Trace Elements on lignin degradation efficiency. 60

Figure 3.3 Interaction plots for lignin degradation efficiency (%) as an effect of changes in three variables: pH, copper sulphate and trace elements. 62

Figure 3.4 Interaction plots showing enzyme activity of polyphenol oxidase as an effect of changes in three variables: pH, copper sulphate and trace elements 64

Figure 3.5 Interaction plot showing enzyme activity of versatile peroxidase as an effect of changes in three variables: pH, copper sulphate and trace elements: 66

Figure 3.6 Bubble plot comparing biofilm properties: (a) wet weight, dry biomass and water retention value (b) biofilm normalised dry weight, polysaccharides and proteins. 68

Figure 3.7 Scanning electron microscopic images and biofilm microstructure 69

Figure 4.1 Lignin estimation (a) Comparison of lignin degradation efficiencies in unfed and culture-fed spent media on day 19 of incubation. (b) Residual lignin concentration in a culture fed with day 10 spent media and unfed samples on days 6, 8, 11, 16, and 19 post-inoculation.. 81

Figure 4.2 Chromatograms profile of untreated wastewater sample showing (a) Complete profile (b) Enlarged polar peak area 83

Figure 4.3 Chromatograms overlay of fungal treated unfed and fed cultures compared to untreated samples (a) Complete profile (b) Enlarged polar peak area. 84

Figure 4.4 Chromatogram overlay of wastewater media samples comparing lignin content in untreated and fungal treated treated cultures (a) Complete chromatogram profile (b) Enlarged polar peak area. 85

Figure 4.5 Chromatograms overlay of wastewater media comparing day-wise residual lignin content in untreated samples with fungal treated cultures fed and unfed. 86

Figure 4.6 Comparison of COD removal efficiencies in unfed and spent-media-fed cultures on day 19 of incubation. 87

Figure 4.7 Enzyme activity in cultures fed with lignin degradation intermediates in comparison with unfed culture (a) Polyphenol oxidase (b) Versatile peroxidase. 89

Figure 4.8 Chromatogram overlay of fed spent wastewater media comparing showing the skewed chromatogram profile. 92

Figure 4.9 Chromatograms of spent wastewater media comparing new lignin degradation intermediates formed on day 10 with (a) Untreated samples (b) Standard mix..93

Figure 4.10 Structure of (a) Sinapic acid (b) Resveratrol (c) trans-Cinnamic acid.....94

Figure 4.11a MS spectra of spent media from day 10 of fungal treatment using targeted the method. 95

Figure 4.11b Mass spectra of standard Nordihydroguaiaretic acid 96

Figure 4.12 Scanning electron microscope images showing biofilm structure of biofilms grown in cultures: (a) Unfed (b) Fed with spent media from day 10. 97

Figure 4.13 Biofilm properties comparing (a) wet weight, water retention value and dry biomass, (b) Polysaccharides per unit biomass and protein per unit biomass....99

Figure 4.14 Pearson's correlation plot showing the correlations between lignin removal efficiency……...100

Figure 5.1 Lignin removal efficiency for Cycles 1, 2 and 3 wastewater selectively supplemented with sucrose (Set A and B).. 116

Figure 5.2 Chromatogram overlay of Cycle 1 spent media comparing untreated and treated samples (a) Complete profile (b) Enlarged polar peak 119

Figure 5.3 Chromatogram overlay of Cycle 2 Set A spent media comparing untreated and treated samples (a) Complete profile (b) Enlarged polar peak 120

Figure 5.4 Chromatogram overlay of Cycle 2 Set B spent media comparing untreated and treated samples (a) Complete profile (b) Enlarged peak area 121

Figure 5.5 Chromatogram overlap of Cycle 3 Set A and B spent wastewater media comparing lignin content in untreated with fungal treated samples from day 12. 122

Figure 5.6 COD Removal efficiency (%) for Cycles 1, 2 and 3 wastewater selectively supplemented with sucrose (Set A and B).. 124

Figure 5.7 Laccase Activity (UL^{-1}) for cultures for Cycle 2 and Cycle 3 of repeated batch wastewater treatment 126

Figure 5.8 Polyphenol oxidase enzyme activity (UL^{-1}) in three cycles of wastewater treatment 128

Figure 5.9 Versatile peroxidase enzyme activity (UL^{-1}) in three cycles of wastewater treatment 129

Figure 5.10 Properties of biofilms showing dry biomass, water retention value, polysaccharides per unit biomass and proteins per unit biomass in the EPS extracted from biofilms. .....**Error! Bookmark not defined.**

Figure 5.11 Scanning electron microscopy images of biofilms in native condition showing the microstructure……127

Figure 5.12 Pearson's correlation plot. The plot shows a correlation between lignin removal efficiency (%), COD removal efficiency (%) and biofilm properties. 132

Figure 6.1 Fermentation setup for repeated batch treatment of wastewater for upscaled wastewater treatment process 138

Figure 6.2 Image of reactor set-up showing biofilms at the beginning of Cycle 3 of wastewater treatment. 142

Figure 6.3 Lignin removal efficiency (%) for biofilm treatment for comparing the treatment for Cycle 2 and Cycle 3 144

Figure 6.4 Chromatogram overlay of spent wastewater media samples from Cycle 1 Set B of wastewater treatment experiment comparing lignin content in untreated and treated samples. 146

Figure 6.5 Chromatogram overlay of spent wastewater media samples from Cycle 2 of wastewater treatment experiment comparing lignin content in untreated and treated samples showing (a) Complete profile (b) Enlarged peak area 147

Figure 6.6 Chromatogram overlay of spent wastewater media samples from Cycle 2 of wastewater treatment experiment comparing lignin content in untreated and treated samples from tray and flask (a) Set A (b) Set B 148

Figure 6.7 Chromatogram overlay of spent wastewater media samples from Cycle 3 of wastewater treatment experiment comparing lignin content in untreated and treated samples from tray and flask (a) Set A (b) Set B. 149

Figure 6.8 COD removal efficiency in wastewater treatment done by biofilms grown in Vogel's media (Set A) and in wastewater (Set B) in flask and tray reactors ..151

Figure 6.9 Laccase activity in cultures treated with biofilms grown on Vogel's media and industrial wastewater in Cycles 2 and 3 of the repeated batch wastewater treatment process. 152

Figure 6.10 Polyphenol oxidase activity in cultures treated with biofilms grown on Vogel's media (Set A) and on industrial wastewater (Set B) during the three cycles of repeated batch wastewater treatment 154

Figure 6.11 Versatile peroxidase activity in cultures treated with biofilms grown on Vogel's media (Set A) and industrial wastewater (Set B) during the three cycles of repeated batch wastewater treatment. 156

Figure 6.12 Biofilm properties showing dry biomass per unit biofilm weight as dry weight, water retention value, polysaccharides per unit biomass, and protein per unit biomass. 158

Figure 6.13 Scanning electron microscopy image of biofilms 159

Figure 6.14 Biofilm microstructure analysis 160

Figure 6.15 Correlations between process efficiencies and biofilm properties……. 157

List of Tables

Tables Page

Table 1.1 - Summary of previous studies on fungal degradation of lignin……..…18

Table 2.1- Characteristics of wastewater provided by the pulp and paper-making company..39

Table 3.1 Assignment of variables and parameter levels using L9 design…….60

Table 3.2 Two-way Analysis of Variance (ANOVA) for lignin degradation efficiency…….60

Table 3.3 Two-way Analysis of Variance (ANOVA) for polyphenol oxidase activity……...65

Table 3.4 Two-way Analysis of Variance (ANOVA) for versatile peroxidase activity……..66

Table 4.1 Spent media collection in Cycle 1 of fungal wastewater treatment process to be fed in Cycle 2…….....76

Table 4.2 Quantification of biofilm characteristics by analysing SEM images with ImageJ software…….....97

Table 5.1 Details of repeated-batch wastewater treatment showing the experiment cycles, sets, total HRT of the biofilms and wastewater media composition…….107

Table 6.1 Experiment design with concentrations of lignin and sucrose in wastewater media used in Cycle 1, 2 and 3 and in Sets A and B of experiments……...134

Abstract

Lignin is a complex biopolymer found in lignocellulosic materials used as raw material in pulp and paper making. Lignin is processed as a by-product of low value and is discarded in the wastewater. This wastewater is highly polluting due to dissolved lignin degradation products, which give it an intense colour and high chemical oxygen demand (COD), causing harm to aquatic life, plants, and animals. Removal or degradation of lignin has been shown to improve water quality in industrial wastewater, however, the complex structure of lignin makes it difficult to be degraded. Advancements in wastewater treatment methods, such as the conventional physiochemical and thermochemical methods employed, have a detrimental impact on the environment due to the production of hazardous by-products and high energy requirements. Biological treatment of lignin using fungi has the potential to overcome many of these roadblocks and lead to a successful process.

This thesis aims to develop a single-step biological process for treating wastewater from the kraft process used in paper-making. *Neurospora discreta*, an ascomycete fungus, has been reported to degrade lignin effectively in lignocellulosic biomass, as it possesses the ligninolytic enzymatic machinery required for lignin degradation. It also has a unique ability to form robust biofilms at the air and liquid interface. In this research, *N. discreta* was evaluated for its ability to treat lignin-rich wastewater for the first time.

The process optimisation was initially developed on synthetic wastewater using alkali lignin, followed by studies using wastewater provided by a pulp and paper-producing company. Firstly, the Taguchi statistical design of experiments, was used to identify the critical process levers for enhancing lignin degradation. Secondly, the addition of naturally formed lignin degradation intermediates in the fungal-treated wastewater spent media was evaluated as a strategy to increase lignin and COD removal. Finally, the biofilms were tested in a continuous repeated batch process, where actively metabolising mature biofilms were

transferred to fresh wastewater in repeated cycles. The process was then scaled-up eightfold to tray reactors.

This research has developed a fungal biofilm-based sustainable, eco-friendly and scalable alternative for lignin and COD removal in industrial wastewater. The fungal biofilm treatment proved to be efficient in removing 67.8% of standard kraft lignin in synthetic wastewater. The process efficiencies, while treating real wastewater from pulp and paper mill, were improved significantly by using lignin degradation intermediates as additives. The lignin and COD removal efficiencies of 70% were noted in cultures fed with lignin degradation intermediates compared to 57% and 50% respectively, in unfed culture. Enzyme activity for polyphenol oxidase (PPO), versatile peroxidase (VPO) and laccase were also seen where VPO was reported for the first time in a *Neurospora* species. The repeated-batch treatment process was evaluated and resulted in an efficient scalable process.

Keywords: Wastewater Treatment, *Neurospora discreta*, biofilms, lignin-rich wastewater, ligninolytic enzymes, lignin degradation intermediates.

1. Introduction

1.1 Background and Overview

Lignin and lignin-derived compounds are common water pollutants in industrial wastewater discharged by pulp and paper mills (Pradeep *et al.,* 2015). Lignin, a large heterogenous, three-dimensional, complex biopolymer, is a building block for lignocellulosic biomass. In plants, lignin is primarily responsible for giving strength to the plant parts and comprises up to 30% of the plant biomass (Rajesh *et al.,* 2019). It remains an underutilised by-product of the pulp and paper-making process as it is a critical component of the lignocellulosic biomass used as raw material in papermaking (Haq, Mazumder and Kalamdhad, 2020). Depending on the process, a significant volume of wastewater is generated during the papermaking processes and can go up to 150 m^3 per ton of paper produced (Sharma, Iqbal and Chandra, 2022). The wastewater generated is highly polluting as it has a high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) (Thompson *et al.,* 2001). The low biodegradability of the generated wastewater is due to the presence of dissolved lignin, which is discarded as a byproduct (Christov and Van Driessel, 2003; Srivastava, Mall and Mishra, 2005). Discharging untreated wastewater rich in lignin and lignin degradation phenolic compounds is potentially polluting and poses severe risks to human health, aquatic life, and plants. Hence, such compounds are considered priority pollutants (Oikari and Nakari, 1982).

The UK was introduced to the Integrated Pollution Control (IPC) by the Environment Protection Act 1990 (EPA) to conserve the environment by treating pollution at the source (Jordan, 1993). As part of the IPC authorisation, UK-based pulp and paper mills were required to showcase the Best Available Techniques Not Entailing Excessive Cost (BATNEEC) for pollution treatment (Thompson *et al.,* 2001). The European paper and pulp mills are under social and political pressure to close the wastewater circuits (Simstich and Oeller, 2010).

For successful water resource management, efficient use of water is deemed critical, and to aid the process economy, the process water can be reused, saving on operational costs (Habets and Driessen, 2007). However, only a certain level of process optimisation can be achieved to reuse the process water generated from the pulp and paper mill without further treatment, beyond which secondary and tertiary treatments are required for BOD and COD removal (Blanco, Hermosilla and Negro, 2016) .

For wastewater treatment, the pulp and paper mill in the UK applies the primary physio-chemical technique for clarification, followed by the secondary-biological or tertiary processes (Thompson *et al.,* 2001). However, these processes are not efficient for the complete degradation of lignin. Due to our limited understanding of the heterogenous structural features of lignin that make it recalcitrant by nature, lignin degradation has been a persistent area of research for years (Ruiz-Dueñas and Martínez, 2009).

This chapter outlines the characteristics of lignin, its resistant nature, and the properties of wastewater from pulp and paper mills by providing a detailed account of the paper-making process. Further, the existing methods used for transforming and breaking down this biopolymer are discussed, focusing on biological methods, particularly fungi.

1.2 Lignin Structure

Lignocellulosic biomass comprises about 50% cellulose and 30% hemicellulose; the remaining biomass comprises lignin and ash (Figure 1.1 a) (Arora, 2003). Lignin is present between the lamella and plasma membrane and provides strength and structure to the plant by binding the cells together (Christopher, Yao and Ji, 2014). It is a large, heterogeneous, complex biopolymer found in lignocellulosic biomass and is part of the raw material for paper-pulp making (Bruijnincx, Rinaldi and Weckhuysen, 2015). Lignin synthesis in nature is a result of enzymatic dehydrogenative polymerisation of three monolignols, which are three

phenylpropanoid units: sinapyl alcohol (with syringyl precursor unit, S), coniferyl alcohol (with guaiacyl precursor unit, G) and p-coumaryl alcohol (with p-hydroxyphenyl precursor unit, H) linked together by several aryl ethers and carbon-carbon bonds (Figure 1.1 b) (Haq, Mazumder and Kalamdhad, 2020; Pamidipati and Ahmed, 2017). These monolignols produce syringyl, guaiacyl and p-hydroxyphenyl residues in lignin (Calvo-Flores and Dobado, 2010). Different proportions of these monolignols contribute towards cell wall lignification. Therefore, the durability of the biomass species depends on the S and G ratio (Pamidipati and Ahmed, 2017). The lignin components differ from plant to plant (Pérez *et al.*, 2002). Lignin in hardwood is syringyl-guaiacyl type, and softwood is typically guaiacyl with some p-hydroxyphenyl lignin in both (Katiyar, Srivastava and Kushwaha, 2020).

There are various C-C and C-O linkages between the monolignols (Calvo-Flores and Dobado, 2010). The β-aryl ether bonds (β-O-4) are the most abundant linkage, accounting for almost 80% of hardwood lignin and 50% of softwood lignin (Adler, 1977). The recalcitrant nature of the biopolymer is due to other linkages which are most resistant to most of the degradation processes, including β - β, 4-O-5, β-5, 5-5 and β-1 linkages (Patil, 2014). The heterogeneous structure of lignin is due to numerous cross-linkages forming a three-dimensional aromatic polymer (Figure 1.1 c). The linear structure of lignin, with minimum branching, was reported in milled wood (Crestini *et al.*, 2011). In another study, ball-milled wood was subjected to endoglucanase treatment, which resulted in a complex structure of lignin-carbohydrate with β-O-4 structures linearly coupled to the structure (Lawoko, Henriksson and Gellerstedt, 2005). Naturally synthesised lignin differs from industrial lignin, produced as by-products of various industrial processes (Vishtal and Kraslawski, 2011). During industrial processes, lignin undergoes structural changes due to chemicals and impurities in the process involved, resulting in diversity in the macromolecular structure. The

modified form of lignin, hence produced due to the specific treatment process, increases the recalcitrant nature of lignin.

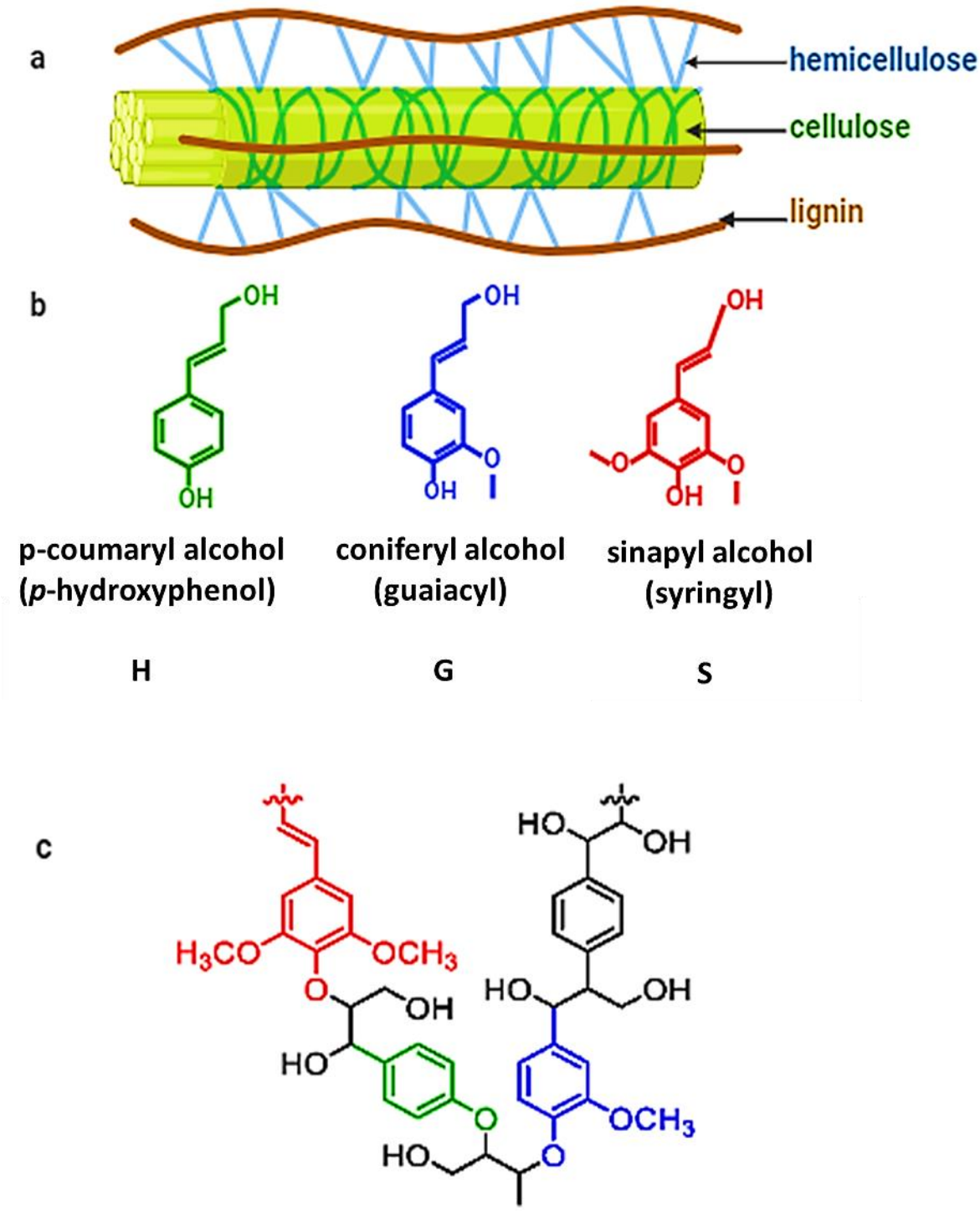


Figure 1.1 - Structure of lignin (a) Schematic representation of components of lignocellulosic biomass used for paper making (b) monomeric unit of lignin precursors with H, G, and S (c) structure of lignin macromolecule (Karunarathna and Smith, 2020; Christopher, Yao and Ji, 2014). Created on

1.3 Recalcitrant Nature of Lignin

Although the three lignin precursors are phenolic compounds, the giant polymer formed by these monolignols is not phenolic (Ruiz-Dueñas and Martínez, 2009; Ralph *et al.,* 2004). During plant lignin biosynthesis, the monolignols are oxidised to form phenoxy radicals (Higuchi, 2012). The dimeric dilignols are formed by resonant forms of phenoxy radicals coupling chemically (Sangha *et al.,* 2014). The dilignols are further oxidised enzymatically to form a lignin polymer (Sakakibara and Sano, 2000). The side chains of the phenylpropanoid precursors and ether linkage of phenolic positions are more predominant in the polymer as the coupling products are highly stable and the radical forms are abundant. The three-dimensional structure of the polymer is the result of the chain branching and coupling process during polymerisation. The recalcitrant nature of lignin is due to its non-phenolic nature, because of which it is not easy to oxidise by low-redox-oxidoreductases such as those found in plants during the polymerisation process (Duval and Lawoko, 2014). The three-dimensional bulky structure of the polymer poses another hindrance to biodegradation as the accessibility to enzymatic action is reduced (Ruiz-Dueñas and Martínez, 2009).

1.4 Pulp and Paper-Making Process

The pulp and paper-making process (Figure 1.2) is highly water-intensive and produces large volumes of wastewater rich in lignin (Toczyłowska-Mamińska, 2017). It is estimated that 70 to 225 m^3 of wastewater is generated for every ton of paper produced, and around 80 to 100 m^3 is sourced from the bleaching process (Singh *et al.,* 2021). The pulp and paper industry alone produces 50 -70 million tonnes of lignin annually, and by 2030, this number is estimated to increase by 225 million tonnes annually (Bajwa *et al.,* 2019). The raw materials used for making paper include wood, agricultural residues, plant material, and recycled paper (Haile *et al.,* 2021). The non-wood raw material usually constitutes bagasse, bamboo, jute, straw, esparto grass and sisal (Ogunsile and Quintana, 2010). The prominent steps in pulp

and papermaking are pulping, bleaching and finally papermaking (Thompson *et al.*, 2001). The wastewater is generated throughout the pulp and papermaking process, from wood pulping to the finished paper.

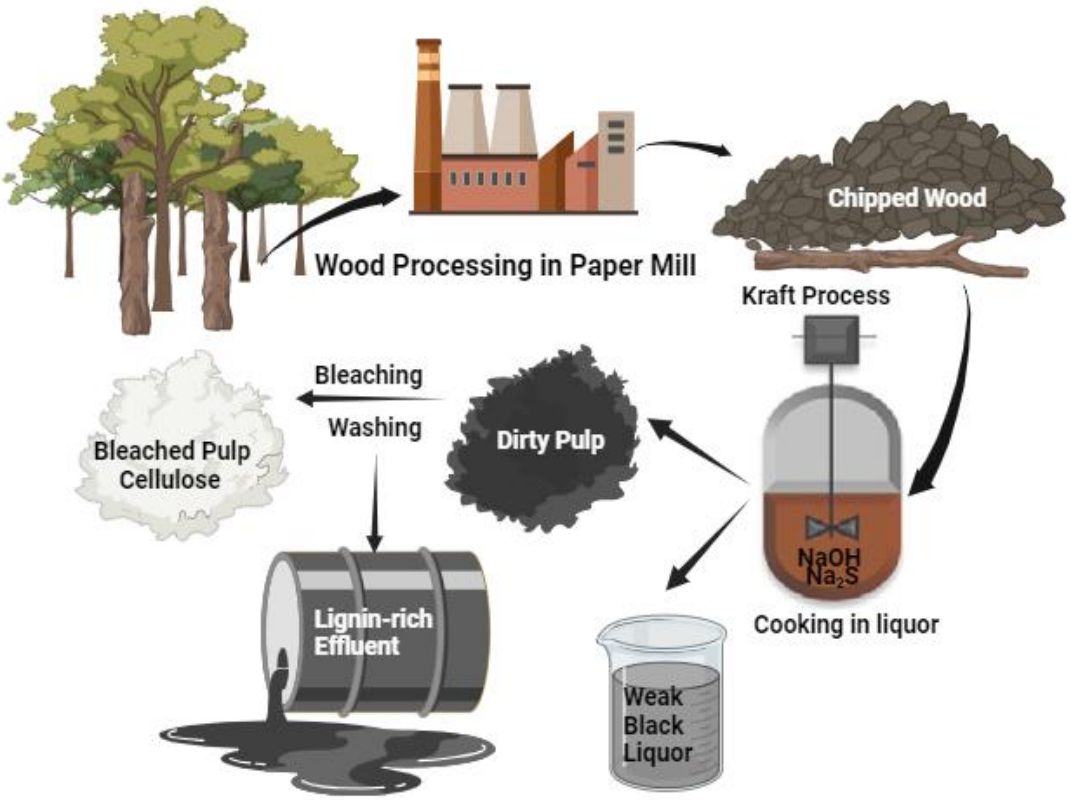


Figure 1.2 - Schematic representation of pulp and paper making process using kraft pulping technique. Created in BioRender.com

Pulping is the process where plant raw material is broken into small chips and can be done by chemical, mechanical, or thermal processes (Bajpai and Bajpai, 2015). The chemical pulping processes used are either the kraft or sulphide process. In the kraft pulping process, the cooking of wood chips is done in white liquor made of sodium sulfide (Na_2S) and sodium hydroxide (NaOH) at 170^{o} C for 1 to 2 hours to remove lignin from the pulp wood (Gierer, 1980; Sainlez and Heyen, 2013). The sulphide pulping process uses only sodium hydroxide. Of the two methods, the kraft process dominates the industry due to the advantage of the strength of the pulp produced, and the spent cooking chemicals can be recovered. To prepare

paper products, lignin is separated from the cellulosic biomass, during which large volumes of wastewater rich in lignin are generated. This viscous aqueous solution is known as black liquor.

The following steps of bleaching and washing also generate large volumes of wastewater rich in lignin. All the lignin cannot be removed during the pulping, neither by the kraft nor the sulfite process. About 10-15% of the lignin remains in the pulp produced as extended pulping may degrade the polysaccharide factions (Kringstad and Lindström, 1984). The dark colour of the pulp is due to the remaining lignin, which further requires a multistep bleaching process. The bleaching step results in wastewater with a high pH of 11 and is rich in lignin and other toxic compounds such as chlorinated and organic halogens, phenols, etc. The exclusion of lignin from the cellulosic material is done during bleaching, which accounts for around 70 kg of total organic compounds, mostly lignin, per ton of pulp produced (Kringstad and Lindström, 1984; Gupta, Liu and Shukla, 2019). The process of delignification during the entire paper-making process, when lignin is removed from the cellulosic material, is the leading cause of wastewater generated is rich in lignin (Toczyłowska-Mamińska, 2017).

1.5 Characteristics of Pulp and Paper Mill Wastewaters

The pulp and paper industry remains one of the most polluting industries, of which the most polluted is the kraft pulping and bleaching wastewater (Almonti, Baiocco and Ucciardello, 2021; Hutchins, 1901). Globally, the pulp and paper industry is estimated to discharge 695.7 million m^3 of wastewater (Haq, Mazumder and Kalamdhad, 2020). The wastewater characteristics significantly differ from the diverse processes implemented, such as washing and bleaching of the pulp, wood and kraft process, and the raw material used (Figure 1.3) (Gupta, Liu and Shukla, 2019).

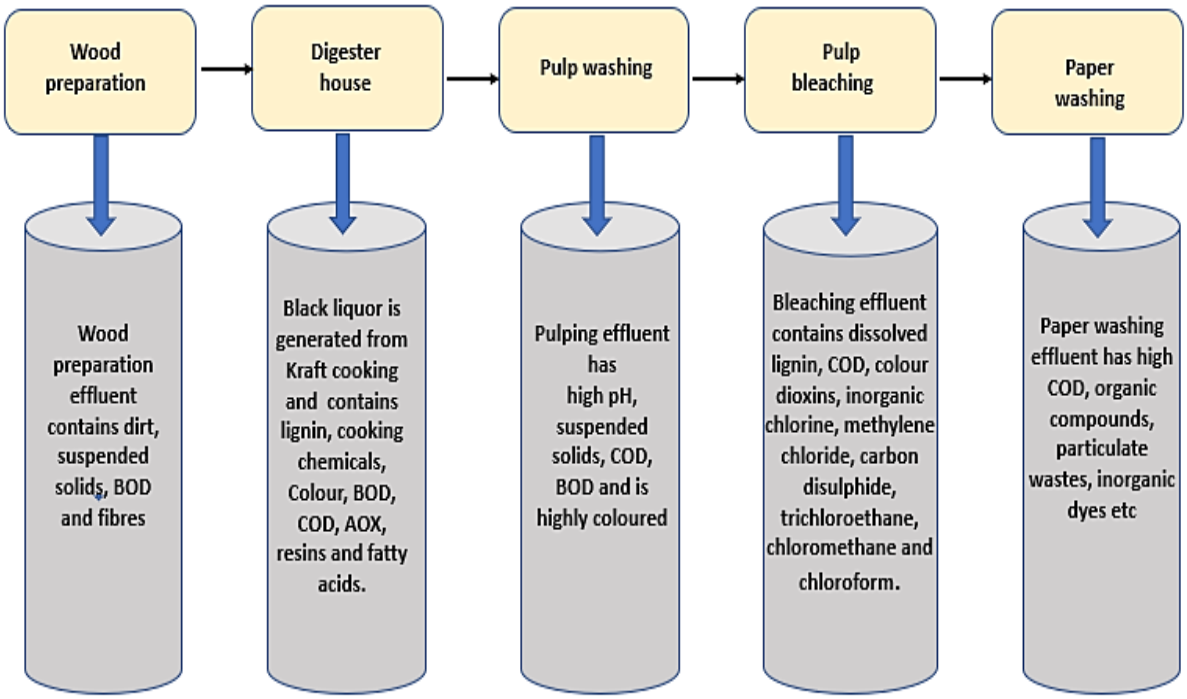


Figure 1.3 - A schematic representation showing characteristics of wastewater generated during the pulp and paper making process.

The wastewater generated contains non-biodegradable and biodegradable fractions, of which the toxic non-biodegradable compounds include chlorinated lignin, unsaturated fatty acids, phenolics, and resin acids (Zhang and Chuang, 1998). The biodegradable organic matter contributes to one-third of the total dissolved solids, including substances derived during pulping and bleaching processes and part of the raw material used contributing to the organic load (Cabrera, 2017). The wastewater produced during the pulp and paper-making process consists of lignin-derived phenolic compounds generated during the processing of wood and other lignocellulosic raw materials (Villegas *et al.*, 2016; Saadia and Ashfaq, 2010; Christov and Van Driessel, 2003). The colour of wastewater is even darker in the process where chemical pulping is employed (Thompson *et al.*, 2001).

During lignin removal treatment, the reaction between the lignin, hydroxide and hydrosulphide anions results in the fragmentation of lignin into smaller molecular weight phenols, thio- and alkali lignin, which are water-soluble (Gellerstedt and Lindfors, 1984). The

wastewater generally exhibits high pollution parameters, such as biochemical oxygen demand (BOD) and chemical oxygen demand (COD) (Upadhyaya and Singh, 1991). The untreated wastewater released from pulp and paper mills can be very polluting, with COD of 11000 mg L^{-1} (Wang, 2006; Thompson *et al.*, 2001). Other characteristics, such as high pH, colour, and total and suspended solids, have been reported in the review (Pokhrel and Viraraghavan, 2004).

1.6 Structural Changes in Lignin During Kraft Process

The kraft-pulping process removes 90% of the lignin, and the bleaching process eliminates the remaining lignin from the pulp via chlorine treatment and alkali extraction methods (Eriksson and Kolar, 1985). Kraft lignin from the pulping process differs from the naturally occurring lignin as it undergoes structural modifications during the kraft treatment. Lignin isolated from black liquor is highly alkaline, and further acid treatment by using carbon dioxide or sulfuric acid brings the pH down to 2-5 (Eriksson and Kolar, 1985; Bhoria *et al.*, 2012). During the alkaline treatment and neutralisation process, several changes occur in the lignin structure due to cross-linking and condensation processes. After the chlorine treatment used for the bleaching process, the chlorolignin formed has low molecular weight and contains conjugated carbonyl groups, phenolic hydroxyl, and carboxyl groups (Eriksson and Kolar, 1985; Bhoria *et al.*, 2012).

At high pH, quinonic intermediates are formed due to α-aryl ether cleavage, alongside sizeable modification occurs at phenolic hydroxyl groups due to β-aryl ether bond cleavage (Chakar and Ragauskas, 2004). As a result of the mesomeric effect and free phenolic hydroxyl groups being produced, the α-aryl ether bonds become more susceptible to cleavage than β-aryl ether bonds (Doherty, Mousavioun and Fellows, 2011). Simple heating of the biomass in water also results in extensive structural changes due to cleavage at the α-aryl

ether bond due to nucleophilic substitution or the formation of quinone methide intermediates (Chakar and Ragauskas, 2004).

1.7 Effect of Lignin on the Environment

The untreated wastewater generated by paper pulp mills is discharged into the receiving waters, either indirectly or directly (Gupta, Liu and Shukla, 2019). It has deleterious effects on the environment and health, as reported by various authors (Chandra *et al.,* 2018; Bajwa *et al.,* 2019). During the existing wastewater treatment process, most harmful pollutants are removed, but some are copious enough to be released into the environment. The strong contamination of European rivers and other industrial regions is majorly due to the presence of polychlorinated biphenyls (PVBs) and polycyclic aromatic hydrocarbons (PAHs) (Singh *et al.,* 2021).

Most of the lignin and its compounds, the primary pollutant in the wastewater, react with other organic compounds and form organic halides (AOX). Of all the harmful effects on the environment, lethal effects on aquatic life, mainly fish, causing respiratory stress, genotoxic effects, and liver damage have been reported commonly (Pokhrel and Viraraghavan, 2004). The impact on human health due to exposure to paper pulp wastewater being discharged into the environment causes nausea, headaches, diarrhoea, and eye infections (Gupta, Liu and Shukla, 2019). The inability to degrade lignin makes it difficult to degrade and accumulate as a pollutant (Pessala *et al.,* 2004).

1.8 Existing Wastewater Treatment Methods

Several advanced and conventional methods are used to treat lignin-rich wastewater, which degrades, remove, or even recovers lignin alongside reducing the COD and colour, which have been reviewed by (Kamali and Khodaparast, 2015; Pokhrel and Viraraghavan, 2004; Thompson *et al.,* 2001). There are physio-chemical and biological methods applied for the treatment of wastewater.

1.8.1 Physical Processes

The wastewater discharged by pulp and paper mills is first subjected to primary treatment processes. The physical methods used for the treatment of lignin-rich wastewaters are precipitation (sedimentation coagulation and flocculation), adsorption on activated carbon and biological sludge, and reverse osmosis and filtration are applied for the wastewater treatment process. Physical methods are employed to remove the suspended solids.

In a manner, immediate treatment aims to secure the secondary treatment equipment system (Möbius, 2006). The physical methods have limitations due to sludge formation and are ineffective in lignin degradation. Even though the sedimentation process is the preferred choice, it has limited application as it can only remove 80% of the suspended solids (Saunamaki, 1997). The immobilisation method attaches the solids to the bubbles floating on the surface. It requires a further skimming downstream process, which can be avoided by using dissolved air floatation, which adds more cost to the process (Thompson *et al.*, 2001). Larger particles are removed by the course screening process, which may harm the equipment used due to the clogging of pipes (Biermann, 1997). Mechanical clarifiers use the settling down property of solids. However, the primary sludge formed is centrally landfilled, which poses a further problem. These methods are not effective enough to remove COD and BOD, which can be released in small amounts only. The physical processes are inefficient in treating lignin, which undergoes spatial rather than chemical change and thus persists in different forms.

1.8.2 Chemical Process

The elimination of suspended solids can be done using the primary treatment processes; however, the pulp and paper mill wastewater is abundant in dissolved organics, which need

further treatment methods (Hagelqvist, 2013). The chemical methods used to degrade lignin and dissolved lignin degradation intermediates are oxidation, reduction, ion exchange and complexometric methods. Advanced oxidation processes (AOP) are known for degrading complex lignin structures, which must be broken down before advancing to the biological processes (Hermosilla *et al.*, 2015). The process depends on the hydroxyl radicle produced, which is selective (Al-Rasheed, 2005). The wet catalytic oxidation using activated charcoal resulted in 86% COD removal (Garg, Mishra and Chand, 2007). Ozone treatment of wastewater resulted in a 50% reduction in lignin (Kishimoto *et al.*, 2010). The catalytic bi oxidation, using Cu/Mn or Cu/Pb, exhibits 84% COD removal (Akolekar *et al.*, 2002). The chemical processes are expensive and add cost to treating pulp and paper mill wastewater (Buyukkamaci and Koken, 2010).

1.8.3 Biological Treatment

Compared to available physiochemical methods, renewed attention has been given to biological treatment since it offers milder operating conditions, requires reduced energy demands, and is environment-friendly (Zhu *et al.*, 2017; Costerton *et al.*, 1995). The biological treatment process is applied as a single-step process that involves the metabolisation of lignin by bacteria, fungi, and enzymes, individually or in combination with primary or tertiary processes (Singhal and Thakur, 2009). Compared to the physio-chemical processes, the biological processes are preferred as they are economical, eco-friendly, and sustainable for BOD, COD and colour removal in wastewater rich in lignin (Ram *et al.*, 2020). Most UK mills use onsite biological treatment processes as the secondary treatment method, of which the activated sludge process is the most common (Thompson *et al.*, 2001). The microbial degradation of lignin is attributed to the production of ligninolytic enzymes that catalyse the lignin degradation process (Bugg *et al.*, 2011).

The microbial breakdown of lignin is a complex process that often occurs by simultaneous utilisation of cellulose and hemicellulose present in biomass. The treatment of technical lignin is often facilitated by the addition of cellulose slurry that encourages the oxidation of lignin by the peroxidase enzymes produced by the microorganisms (Nousiainen *et al.*, 2014). Most of the lignocellulosic biomass treatment processes start with the separation of the valuable carbohydrate components, leaving lignin behind as a by-product. Selective lignin-degrading microbial strains have been identified in nature that can depolymerise lignin selectively in plants leaving behind the cellulose intact (Watanabe *et al.*, 2002).

The conventional biological processes for secondary treatment of pulp and paper mill wastewater are the anaerobic and aerobic biological treatment processes (Habets and Driessen, 2007; Tezel *et al.*, 2001). Aerobic digestion is the process when complex organics are utilised by microorganisms in the presence of trace elements and oxygen to produce carbon dioxide and biomass (Spellman, 2013). During aerobic treatment, the biodegradable matter is oxidised first, followed by the cellular material produced by the microbe (Wang, 2006; Wang, Shammas and Hung, 2007). Aerobic processes have found more application in pulp and papermaking mills, owing to their controlled degree of degradation and promising results (Rittmann and McCarty, 2001). The treatment methods applying the aerobic process available include aerobic membrane reactors, activated sludge reactors, sequencing batch reactors, aerated lagoons, moving bed reactors and aerobic membrane reactors (Patel *et al.*, 2021). Anaerobic biodegradation of lignin is less understood, and most studies focus on treating small molecular weight lignin degradation compounds (Khan and Ahring, 2019).

1.8.3.1 Bacterial Treatment

Lignin-degrading bacteria represent three classes mainly - Actenomycetes, γ-Proteobacteria, and α-Proteobacteria, which have also been found in the digestive systems of wood-infesting insects and termites (Bugg *et al.,* 2011). These organisms are ubiquitous and produce several extracellular peroxidases, which can oxidise the β-aryl ether bonds in model lignin catalytically. Low molecular weight phenolic compounds were identified as lignin degradation intermediates during the lignin treatment with *Rhodococcus* RHA1 and *Pseudomonas putida* (Ahmad *et al.,* 2010). The extracellular peroxidases - lignin peroxidases and manganese peroxidases- produced by the bacterial cells are responsible for the action of the lignin-degrading bacteria (Ram *et al.,* 2020). Treatment of kraft lignin with *Comamonas* sp. resulted in degradation into low molecular compounds, and the results correlated with 32% COD removal efficiency after seven days of incubation (Chen *et al.,* 2012). Several bacterial species have been assessed, but the dominant species are *Micrococcus luteus* and *Bacillus subtilis*, which can potentially reduce COD, BOD and lignin in wastewater. Despite the progress, only one bacterial lignin peroxidase has been successfully cloned, and manganese peroxidase structural information is available. Though few researchers have emphasised the bacterial degradation of lignin owing to their pH tolerance, only a few bacterial strains can degrade lignin (Chen *et al.,* 2012). Furthermore, the bacterial treatment of wastewater suffers from much lower enzyme activity than the more widely studied white-rot fungi. The production of these enzymes at large-scale applications suffers from low expression levels (Chauhan, 2020; Bugg *et al.,* 2011).

1.8.3.2 Fungal Treatment

Microorganisms, such as fungi and bacteria, have been instrumental in the biodegradation of lignin and are known to utilise lignin as a carbon and energy source (Basha, Rajendran and Thangavelu, 2010). Fungi are ubiquitously present in wastewaters high in concentrations of

pollutants, and around 1600-1700 species of fungi are known to degrade lignin (Janusz *et al.,* 2017). The application of fungi to clean up environmental pollution, a process known as mycoremediation, has remained an area of interest as long as they are natural degraders adapted to use lignin for growth. The eukaryotic nature of fungi makes them better suited to handle inhibitory compounds (Schueffler and Anke, 2014). In comparison with bacteria, the fungal mycelial morphology and cell wall structure of fungi make them more tolerant to toxic pollutants (Sankaran *et al.,* 2010). Secretion of regulated ligninolytic enzymes target specific bonds in the lignin polymer, breaking it down, resulting in lignin degradation.

Depending on the mechanism of wood degradation, fungi are classified as soft-rot, brown-rot, and white-rot fungi (El-Bestawy *et al.,* 2008). The most potent and widespread lignin degraders belong to litter-decomposing white-rot fungi, which include few ascomycetes and many species of basidiomycetes (Buswell, Odier and Kirk, 1987). The model organism primarily employed for lignin biodegradation studies is *Phanaerochete chrysosporium*, primarily due to the ability to secrete ligninolytic enzymes, to grow in chemically defined medium and versatility concerning rapid growth in temperatures up to 40^{o} C (Kirk and Farrell, 1987). In the previous studies, supplementing lignin-rich medium with carbohydrates proved to be the key towards initiating the lignin degradation process using *P. chrysosporium* (Ulmer *et al.,* 1983; Reid, 1979) *Neurospora crassa*, an ascomycete, was studied to investigate its ability to biodegrade phenolic compounds and the capacity of its enzymatic system to produce oxidative enzymes (Luke and Burton, 2001). Nitrogen repression has also been reported to trigger lignin degradation in *Trametes versicolor* (Mikiashvili *et al.,* 2005). Several authors have reported lignin degradation by applying white rot fungi by supplementing the growth medium with either carbon or nitrogen (Singh and Chen, 2008). Concomitant degradation of lignin with fungal growth makes this process popular.

Fungal growth is widespread in pulp and paper mill wastewater. It is very popular for treating lignin-rich wastewater owing to the extracellular ligninolytic enzyme system produced, which enables lignin degradation (Yang *et al.,* 2011a). Compared to bacteria, fungi can survive in high concentrations of pollutants. Various filamentous fungi, mainly basidiomycetes and ascomycetes, can degrade lignin to different extents (Pokhrel and Viraraghavan, 2004).

Table 1.1 - Summary of previous studies on fungal degradation of lignin.

Fungus	**Lignin**	**COD**	**Colour**	**Reference**
P.chrysosporium MTCC	71%	56%	86%	(Chopra and Singh, 2012)
Trametes versicolor	-	82%	80%	(Pedroza-Rodríguez and Rodríguez-Vázquez, 2013)
Fusariumsam bucinum	79%	89.4%	78.6%	(Malaviya and Rathore, 2007)
Aspergillus flavus	39-61%	38.2%	31-51%	(Barapatre and Jha, 2016)
Mycena spp. *J24*	32.95%	33.14%	72.11%	(Subowo, 2019)
Pleurotus ostreatus	37.7–46.5%	-	-	(Li *et al.,* 2019)
Pleurotus streatus	77%	60%	80%	(Choudhury *et al.,* 1998)
Nigrospora sp., Curvularia,lunata LDF21	76.1%	80%	82.3%	(Rajwar, Paliwal and Rai, 2017)
Paraconiothyrium variabile	22.99%	-	-	(Gao *et al.,* 2011)
Neurospora sitophila, Chrysonilia sitophila	25%	-	-	(Rodríguez *et al.,* 1997)

The fungal wood decay is classed into three groups - The white-rot fungi, basidiomycetes degrade lignin, cellulose and hemicellulose and selectively delignify wood in some cases. They produce enzymes such as peroxidases, cellulases and laccases that are extracellular and participate in lignin degradation. Brown rot basidiomycetes leave behind lignin after selectively degrading the wood polysaccharides. The brown rotters lack the necessary

oxidative enzymes present in white rot fungi. Similar to brown rot fungi, the soft rot ascomycetes (*Chaetomium globosum, Ustulina deusta*) and deuteromycetes (e.g. *Aureobasidium pullulans, Phialophora* spp. *and Trichoderma* spp.) degrade the polysaccharides more readily but degrade lignin as well (Shary, Ralph and Hammel, 2007; Martínez *et al.,* 2005).

Several authors have reported different fungal species for their varying capabilities in lignin, COD, and colour removal (Zhang *et al.,* 2012; González, Sarria and Sánchez, 2010; Sakurai *et al.,* 2002; Chandra and Singh, 2012). Table 1.1 illustrates lignin, COD, and colour removal efficiencies of different fungal species from the kraft wastewater from pulp and paper making using various treatment strategies. The most studied wood-decaying white-rot fungi, *P. chrysosporium* and *T. versicolor*, are potent lignin degraders and decolourise lignin-rich wastewater (Bajpai and Kondo, 2012). This group of basidiomycetes has a remarkable ability to degrade lignin due to robust oxidative bioremediation capabilities and high environmental tolerance to toxic pollutants. They can withstand a wide pH range (Asgher *et al.,* 2008). *P. chrysosporium,* also used as an organism modelled for lignin degradation, is known to produce extracellular and non-specific lignin-degrading enzymes such as manganese peroxidases (MP), lignin peroxidases (LiP) and laccases (Verma and Madamwar, 2002).

Representatives of the Ascomycota group cause soft wood-rot decay by eroding the secondary cell wall and degrading the acid-insoluble (Klason) lignin in softwood (Worrall, Anagnost and Zabel, 1997) (Janusz *et al.,* 2017). Little is known about the lignin degradation mechanism brought about by ascomycetes (Worrall, Anagnost and Zabel, 1997). Some ascomycetes are shown to degrade radiolabelled lignin but only to 10%, indicating the natural lignin-degrading ability of ascomycetes is limited (Worrall, Anagnost and Zabel, 1997). The other possibility is that ascomycetes attack the phenolic units in lignin, which comprise

roughly 10% of the polymer, and the remaining include nonphenolic units (Hammel *et al.,* 2002). The oxidation of nonphenolic lignin requires strong oxidants such as laccase. However, phenolic units can be easily oxidised by oxidising agents such as phenol oxidase (Shary, Ralph and Hammel, 2007). Laccase production is typically associated with basidiomycetes and has yet to be convincingly shown in soft-rot ascomycetes (Shary, Ralph and Hammel, 2007). Degradation of lignin is mainly in aquatic areas that are moist, supporting the growth of soft rot fungi in waterlogged wood (Katiyar, Srivastava and Kushwaha, 2020). These fungi prefer lignin removal on hardwood over softwood, as lignin degradation in hardwood is easier (Katiyar, Srivastava and Kushwaha, 2020). The lignin degradation process is slow as the enzymes produced by soft-rot fungi may not show activity on the guaiacyl unit but actively degrade the syringyl unit (Rodriguez *et al.,* 1996).

Compared to basidiomycetes fungi, only a few ascomycetes fungi are known to degrade lignin. The species of *Aspergillus niger, Tametes versicolor, Lentinus edodes, Tinctoria borbonica, Coriolus versicolor, Ceriporiopsis* sp. and *Irpex lacteus* have been identified to degrade lignin significantly (Singh, 2018). *Daldinia concentrica* degraded 44% of lignin and 53% of total wood loss (López, Silva and Santos, 2017). The lignin degradation abilities of *Chrysonilia scatophilia* (red bread mould) were also reported with a simultaneous degradation of lignin and loss of carbohydrates, causing 20% weight loss (Madadi and Abbas, 2017). The soft rot-like decay in crop plants by *Botrytis cinerea* was reported (Thurston, 1994).

1.8.3.3 Lignin Degrading Enzymes

Microbial enzymes play an essential role in the degradation of organic waste in the pulp and paper industries (Patel *et al.,* 2021). Fungal degradation of lignin occurs as a part of secondary metabolism, which is triggered by depleting nutrients, slow growth rate or biosynthesis of inducers or supplements in the media (Yang *et al.,* 2011b; Demain, 1998).

Ligninolytic enzymes are known to be triggered as a response to growth-limiting conditions such as carbon, nitrogen or sulfur depletion during the idiophasic metabolism (Govender, Pillay and Odhav, 2010). Extracellular enzymes, which are oxidative and unspecific, catalyse the depolymerisation of lignin first to form unstable compounds, which further undergo oxidative reactions to form low molecular products (Pérez *et al.,* 2002). The white-rot fungi are known to produce more than two types of ligninolytic enzymes- lignin peroxidase (LiP) and manganese peroxidase (MnP) that are reported to catalyse the lignin degradation process (He *et al.,* 2015). *P. chrysosporium* is known to efficiently degrade and mineralise lignin due to its ability to secrete ligninolytic enzymes, such as laccases and peroxidase (Hatakka and Hammel,2011). Each lignin-degrading enzyme is specific to a particular chemical bond, and others vary in potentiality. Filamentous ascomycetes, such as *N. crassa*, are known to produce laccase and polyphenol oxidase, which are two different oxidoreductase enzymes efficient in the degradation of phenols (Froehner and Eriksson, 1974).

1.8.3.3.1 Laccases

Laccases (EC.1.10.3.2) are secreted by most lignin-degrading fungi, such as ascomycetes, basidiomycetes and deuteromycetes, as extracellular enzymes (Baldrian and Šnajdr, 2006). Ascomycetes such as *N. crassa, Magnaporthe grisea, Melanocarpus albomyces*, *Myrothecium verrucaria* 24G-4 and *Chaetomium thermophile* are reported to produce laccase (Dashtban *et al.,* 2010). Laccases are blue multicopper oxidases that reduce molecular oxygen to water while oxidising several substrates such as polyphenols, di-, mono-, diamines, and aminophenols (Froehner and Eriksson, 1974). These enzymes can oxidise phenols and begin a polymerisation process; hence, their role in lignin degradation is arguable (Berka *et al.,* 1997; Gianfreda, Xu and Bollag, 1999). The repolymerisation, however, can be prevented by mediators such as 2,2-azinobis ABTS (3-ethylbenzothiazoline-6-sulphonic acid), which enables laccase to target the non-phenolic units in lignin (Gianfreda, Xu and Bollag, 1999). It

has been reported that changes in pH affect the affinity and specificity of various laccase, and the ideal pH for phenols is between 3-7 for fungal laccase (Gianfreda, Xu and Bollag, 1999). Catalysis of Laccase can be hindered by alkaline pH due to the presence of hydroxyl ions (Berka *et al.,* 1997).

1.8.3.3.2 Polyphenol Oxidase

Polyphenol oxidases (PPO, EC.1.10.3.1), referred to as 'tyrosinases', 'phenol oxidase' and 'polyphenolase', are used interchangeably (Yoruk and Marshall, 2003). Polyphenol oxidases (PPO) are copper-containing monooxygenases and are involved in two types of reactions resulting in the *ortho*-hydroxylation of phenols to catechols and the other being the oxidation of catechols to *ortho*-quinones (Burton, 1994). The enzyme PPO is responsible for lignin degradation and has been reported in white-rot fungi, proving a correlation between the degradation of lignin and the production of polyphenol oxidases (Lerch, 1983). The first extracellular PPO was characterised by *Trichoderma reesei*- filamentous fungi (Selinheimo *et al.,* 2006). The structure of fungal PPO from *N. crassa* was first studied to elucidate the active copper and the enzyme is reported to be growth-associated (Lerch, 1983). The mechanism and action of the fungal PPO are dependent on copper, which is essential for PPO activity as the removal of copper from the enzyme results in inactive forms, which can be restored by adding excess copper (Yoruk and Marshall, 2003). This enzyme is different from laccase due to its ortho-hydroxylation activity. The quinones condense readily to form brown polymers called melanins, which have high molecular weight (Burton, 1994).

1.8.3.3.3 Lignin Peroxidases (LiP)

The peroxidase enzymes such as versatile peroxidases (VPO), lignin peroxidase (LiP), manganese peroxidase (MnP) and the more evolved polyphenol oxidases are majorly produced by ascomycetes and white-rot basidiomycetes fungi. They are well-characterised

(Pérez *et al.,* 2002). LiP is one of the most effective peroxidases, as it oxidises phenolic and non-phenolic compounds, amines, aromatic ethers, and other polycyclic aromatics.

1.8.3.3.4 Manganese Peroxidase (MnP)

MnP (EC.1.11.1.13) is a manganese-dependant enzyme that uses Mn^{2+} as a preferred electron donor or substrate for its catalytic reactions and converts the phenol radicals to phenoxy radicals. It is a high molecular weight (45-60 kDa) glycosylated protein with a catalytic cycle similar to the LiP, with a ferric enzyme that forms reactive intermediated compound I and Compound II (Kishi *et al.,* 1994). High concentrations of H_2O_2 affect the activity of MnP as it creates a catalytically inactive Compound III, which reverses the activation of the enzyme (Wariishi et al. 1988). It is also known that malonate, along with a synthetic preparation of Mn^{3+}, mimics the MnP activity (Moilanen *et al.,* 1996).

1.8.3.3.5 Versatile Peroxidase (VPO)

VPO (EC.1.11.1.6) are non-specific oxidative enzymes that oxidise compounds with high-low redox potential and can catalyse the reaction in a dependent and independent environment (Knop, Yarden and Hadar, 2015). Versatile peroxidases (VPO) have been reported to be found in *Pleurotus Sp.*, *Bjerkendera sp*., *Physisporinus sp*. and a few other genera (Knop, Yarden and Hadar, 2015). Unlike laccase, VPOs possess a unique combination of catalytic properties, enabling them to oxidise a wide range of substrates (Kong *et al.,* 2017). Hence, VPOs can cleave the highly condensed lignin structures as well. Also known as a hybrid Mn-peroxidase, VPOs can oxidise Mn^{2+} to Mn^{3+} and oxidise the non-phenolic compounds. Enhanced VPO activity can be achieved in the presence of Mn^{2+} (Taboada-Puig *et al.,* 2011).

1.8.3.4 Enzyme Induction

For optimisation of the lignin degradation process, the augmentation of ligninolytic enzyme production and activity are critical factors. For an efficacious process the right combination of microorganism, culture media and inducers are essential. A range of various synthetic lignocellulosic substrates have previously been applied for the induction of ligninolytic enzymes (Govumoni *et al.,* 2015; Nandal, Ravella and Kuhad, 2013). The cultures medium of *Stereum Ostrea* and *Trametes versicolor* when supplemented with lignosulfonic acid and lignosulfonate in low dosage, induced laccase and MnP enzymes (Govumoni *et al.,* 2015; Nandal, Ravella and Kuhad, 2013). The addition of industrial lignin to a malt extract medium resulted a in complementary effect for the increased production of laccase. Induction of LiP enzyme in *Streptomyces viridosporus* by the addition of lignosulfonate lignin can be explained as a microbial stress response to the additive whose structure is similar to anti-microbial agents, which triggered the secretion of ligninolytic enzymes (Eggert, Temp and Eriksson, 1996). A better understanding of the environment of the lignin-based media is critical when studying enzyme induction and optimisation. However, the use of such synthetic inducers adds more cost to the process. Additionally, to obtain the same level of ligninolytic enzyme induction by microorganisms growing on lignin is a limiting factor.

The presence of phenolic compounds that are low molecular weight has been shown to affect the ligninolytic enzyme activity in fungal cultures. The cultures of *Botryosphaeria* exhibited an inhibitory effect on laccase production due to the presence of phenolic compounds in the medium. By contrast, laccase was induced in *Phomopsis liquidambari* as a result of the addition of ferulic acid as a carbon source (Xie and Dai, 2015).

Phenolic compounds such as vanillin, ferulic acid and veratric acid, when supplemented in the culture media of *Trametes versicolor*, *Cerrena unicolor* and *Ganoderma lucidum,* induced MnP in the cultures (Elisashvili *et al.,* 2010)**.** The involvement of veratryl alcohol

was reported to induce manganese peroxidase in white-rot fungi- *Nematoloma frowardii and Clitocybula dusenii* (Scheel *et al.,* 2000). Selective and simultaneous induction and inhibition of ligninolytic enzymes are also reported in cases of ligninolytic systems of *Phanerochaete chrysosporium* caused by the addition of veratryl alcohol affecting the manganese peroxidase and lignin peroxidase enzymes (Candeias *et al.,* 1994).

1.9 Challenges in the Existing Biological Treatment Systems

Conventional wastewater treatment process often applies the primary clarification process followed by the activated sludge process. However, most of these processes have not been effective in degrading lignin and other organic compounds (Balcıoğlu *et al.,* 2007). The most commonly used activated sludge method faces limitations such as "bulking", which is poor settling time, causing operational problems (Thompson *et al.,* 2001). The sludge produced during aerobic wastewater treatment, or biosludge, can be produced between 5% to 40% of the total pulp produced (Meyer and Edwards, 2014). The downstream process for biosludge includes dewatering followed by dumping the dewatered sludge into landfills or incinerated for energy production. This is not economical as the cost can contribute to half the total wastewater treatment cost. Other factors, such as low control over the sludge-forming microorganism population and poor settling and shock load sensitivity, present problems to the process (Meyer and Edwards, 2014). Furthermore, low control over the sludge-forming population and sensitivity shock loading leads to undesirable results.

The prolonged time required for the biological treatment of lignin, in addition to the stress imposed on the microorganism when imposed to black liquor hinder the process efficiencies and limits its industrial application. To address the biodegradation of lignin, some researchers have used batch and semi-batch reactors to maximise lignin degradation in black liquor (Hooda, Bhardwaj and Singh, 2015). Novel bioreactors have also been tested in an attempt to

implement as an industrially feasible process. However, these systems have been limited to bacterial remediation of lignin (Singh *et al.,* 2021).

In comparison to the aerobic method, anaerobic processes in wastewater treatment are more advantageous, such as reduced production of sludge volumes, production of methane as an energy carrier, simple operation and low costs involved and application at different scales (Ekstrand *et al.,* 2013; Zwain *et al.,* 2013). The anaerobic processes are considered economically beneficial due to the formation of valuable biogas; however, these processes have features which have been considered disadvantages, such as the rate of pollutant removal by microbes is very slow, and so is the rate of microbial growth (Rittmann and McCarty, 2001). Furthermore, to compensate for the slow pace of microbial growth, the sludge retention time needs to be lengthened, which adds further maintenance efforts to the process. Several authors have achieved high lignin removal rates in sequential treatment by fungi in the first step, followed by treatment with bacteria (Thakur, 2004). Integrated aerobic and anaerobic systems are being investigated as well (Chan *et al.,* 2009). A combination of an anaerobic and aerobic treatment was applied as a secondary treatment of kraft process wastewater by *Pseudomonas ovalis* (bacterial) and *Aspergillus fumigate* (fungal) (Singh *et al.,* 2011).

Enzymatic treatment can be an alternative to microbial processes, but isolating large industrial-scale production of enzymes is expensive (Toczyłowska-Mamińska, 2017). The enzyme system used by wood-degrading fungi is unique. It is characterised by a specific catalytic group of peroxidase enzymes that enable lignin degradation and other pollutants with structural similarity to lignin (Martínková *et al.,* 2016). However, the low level of fungal extracellular enzyme production does not meet the industrial-scale treatment requirement.

Though a broad spectrum of microorganisms can utilise lignin as energy and carbon sources for growth, pollutants in high concentrations do not support the growth of suspended microbial cultures (Thompson *et al.,* 2001). Additionally, difficulties arise at higher concentrations of lignin due to increased toxicity, which results in cellular lysis (Thompson *et al.,* 2001).

Immobilisation is a technique where microorganisms are entrapped by physical restraints such as encapsulation or chemically bound to carrier material (Bayat, Hassanshahian and Cappello, 2015). The immobilisation systems can be entirely artificial or natural by allowing microbial aggregates to form cellular aggregates called biofilms. Compared to planktonic growth, where the cells are freely suspended in the growth media, microbial growth in biofilms or immobilisation is beneficial as the cells are more resilient to high pollution load (Zahmatkesh, Spanjers and van Lier, 2018). In these microbial aggregate structures, high microbial biomass enhances gene expression, resulting in increased production of enzymes, which play an essential role in pollutant degradation (Singh, Paul and Jain, 2006). Artificial immobilisation is a complex process where the synthetic matrix is used as a carrier for microorganisms, adding extra costs to the treatment process. Additionally, the microorganisms suffer from mass transfer and nutrient transfer limitations, loss of biological activity, high-pressure drops and membrane fouling in immobilized-cell membrane bioreactor systems.

To protect biomass from the detrimental effects of high concentrations of pollutants, a technique known as cell immobilisation in hollow fibre membranes is explored (Radjenović *et al.,* 2015). The immobilized-cell membrane bioreactor systems face other limitations such as diffusion, deprivation in biological activity, pore-clogging in membranes and high-pressure drop (Buchholz, Kasche and Bornscheuer, 2012). Natural biofilm formation is a more sustainable and eco-friendly process where microorganisms form self-assembling

aggregates on surfaces (Rosche *et al.,* 2009). The organisms in biofilm structures can withstand extreme environmental conditions as they are protected in the self-secreted extracellular matrix that acts as a fortress (Flemming *et al.,* 2016).

1.10 Fungal Biofilms in Wastewater Treatment

Biofilms are ubiquitous life forms of microorganisms embedded in a self-produced extracellular biopolymeric matrix (ECM) by collectively organising at interfaces and forming a film-like composite structure (Flemming and Wingender, 2010). The highly hydrated ECM includes a matrix acting as an adhesive to keep biofilm cells together. The cohesive matrix comprises extracellular enzymes, polysaccharides, nucleic acids, and lipids and provides mechanical strength by immobilising biofilm cells (Flemming and Wingender, 2010). Cells growing within a biofilm are found to be more successful alternatives to planktonic microorganisms as cells within the biofilms adapt well to different environmental conditions and have higher chances of their subsequent survival in challenging environments (Costerton *et al.,* 1995). Compared to suspended cell cultures, biofilm communities provide a beneficial structure to the microorganisms and protection from the chemical stress in the surrounding environments. Biofilms consists of cells of microorganism embedded in an extra-cellular biopolymeric matrix (ECM), which is self-secreted, providing durability and structure together with a wide range of structural and metabolic characteristics. These attributes of biofilms make these microbial structures an appealing option for biofilm-based remediation solutions (Edwards and Kjellerup, 2013; Aravinda Narayanan and Ahmed, 2019).

Natural biofilms are formed by *Fusarium species, Candida albicans, Acremonium, Neocosmopora and Penicillium rubrum* are examples of fungal species that can create biofilms (Saini *et al.,* 2023). *P. chrysosporium* is a widely studied white-rot fungus famous as an efficient lignin degrader. Fungal biofilm-based bioreactors such as trickle bed reactors, moving bed reactors and rotating contactors have been applied by artificially entrapping

biomass, spores, or enzymes for the degradation of target pollutants. Biofilm systems explored for lignin degradation have mainly used *P. chrysosporium* due to biodegradation capabilities and toxicity resistance. The lignin degradation capabilities of white rot fungi as attached growth systems were explored by growing *P. chrysosporium, Lentinus edodes, Trametes versicolor, Pleurotus ostreatus,* and S22 individually on porous plastic media, resulting in 71% lignin degradation in pulp mill wastewater (Wu, Xiao and Yu, 2005). Another more studied species, *N. crassa*, has served as an organism of interest for studies on the bioconversion of lignocellulosic biomass into ethanol (Dogaris, Mamma and Kekos, 2013). The same organism has also been studied for the degradation of phenolic compounds in membrane-immobilized biofilms to continuously build a sustainable system effective for four months (Luke and Burton, 2001).

Innovative biofilm systems were investigated by growing *P. chrysosporium* on silicon membranes to support biofilm growth and minimise shear in the reactor (Venkatadri and Irvine, 1993). In another study, polysulphone capillary membranes were used to immobilise spores of *P. chrysosporium*, allowing fungal growth as a biofilm on the surface of the membrane where nutrient-rich media was available, which allowed the fungal secretion of lignin and manganese peroxidase enzymes (Mahdinia and Demirci, 2020). Though these biofilm systems are sustainable and renewable and have proven efficient in lignin degradation, they face other challenges. Limitations such as mass transfer limitations, artificial immobilisation generated loss of biological activities, fouling of membranes used for immobilisation and pressure drops affect the entire system negatively.

1.11 Gaps in Existing Research

Biological processes are more beneficial than physiochemical ones for treating lignin-rich wastewater produced by pulp and paper mills. This is because they are cheaper and more sustainable. For the complete removal of lignin from paper mill effluents, the conventional

wastewater treatment processes are rarely efficacious. Biofilm-based methods are promising for treating highly concentrated wastewater; however, they have limitations, as discussed previously. A sustainable and robust approach for treating wastewater rich in lignin is not currently available.

The potential of filamentous fungi in pollution mitigation has been investigated in high numbers in high-impact scientific reviews (Ferreira, Varjani and Taherzadeh, 2020; Sankaran *et al.,* 2010; Chu *et al.,* 2021). However, their role in treating lignin-rich wastewater is limited to the more studied white rot fungi (Gold and Alic, 1993). The range of fungal strains identified based on morphology is around 50,000 species, with ascomycetes being the largest class of fungi with about 30,000 species (Rittmann and McCarty, 2001). Despite knowing that ascomycetes fungi can assimilate, detoxify, and degrade lignin, only a few species are studied (Prenafeta-Boldú, De Hoog and Summerbell, 2018) . This highlights the need to explore the lignin degradation ability of other groups of lignin degraders.

The immobilized-cell membrane bioreactor systems have been explored to solve the problems of freely suspended microbial processes (Wu, Xiao and Yu, 2005). Biofilm technology, which uses entrapped cells, has been widely used in wastewater treatment. However, using natural biofilms to treat wastewater generated by pulp and paper mills has not yet been explored. There is a need for more research to identify and optimise the process factors that can adjust the functionality of natural fungal biofilms.

The fungal wastewater treatment processes suffer from low levels of ligninolytic enzyme production and slow rates of lignin degradation (Rüttimann-Johnson *et al.,* 1993). The functions of fungal ligninolytic enzymes are associated with the activation of metabolic processes. There are reports on chemicals that can be added to enhance the activity of ligninolytic enzymes. Still, these chemicals can be more polluting and increase the cost of the

treatment process (Prasad *et al.,* 2005). Natural inducers can be used as additives to make the process more economically viable and efficient. Also, the slow growth adds lag in lignin degradation efficiency, which can be investigated to further the process improvisation.

In the last two decades, mycoremediation has been used successfully in bioremediation, but fungal biofilm methods have yet to be developed for treating industrial wastewater at a large scale. The biological process can be used as a single-step wastewater treatment method for industrial processes at an industrial scale. The fungal wastewater treatment is limited to investigations carried out in sterile conditions, done at laboratory and pilot scale only. Despite all the potentialities in wastewater treatment, fungal processes are only sometimes applied at the industrial level.

1.12 Research Questions and Aim

Based on the gaps identified in the literature, as discussed in the previous section, the following research questions were investigated:

Q 1. Can fungal biofilms be used for the degradation of lignin in wastewater?

Q 2. What are the critical process parameters and optimal requirements to tune the fundamental properties of biofilms, such as enzyme production and lignin degradation, for application in wastewater treatment?

Q 3. Can in-process lignin degradation products act as inducers to increase enzyme activity in fungal biofilms, resulting in a more efficient process?

Q 4. Can fungal biofilms be used efficiently in a continuous process for wastewater treatment?

Q 5. Are processes based on fungal biofilms scalable?

The aim of the project was to develop and optimise the potential of *Neurospora discreta* biofilms in lignin and COD removal in wastewater generated by pulp and paper mills.

1.13 Rationale

This project attempts to address the gaps mentioned in section 1.11, as there is a need to find a cost-effective, eco-friendly method to be adopted as an efficient cleaning technology. Biofilms formed by the non-pathogenic fungus *Neurospora discreta* have been shown to degrade lignin and are used to develop renewable, biodegradable, and sustainable wastewater treatment processes (Pamidipati and Ahmed, 2017). *N. discreta* has been an organism of choice in several previous studies, owing to its ability to biodegrade lignin and the presence of ligninolytic enzyme machinery that enables lignin degradation. Though the organism has been reported to degrade lignin, the studies have been limited to agricultural residues, such as sugarcane bagasse and cocopeat. Ascomycetes fungus growth in waterlogged areas is preferred and is known to degrade lignin degradation phenolic compounds more efficiently than the basidiomycetes (Katiyar, Srivastava and Kushwaha, 2020). A previous study found that *N. discreta* was 1.5 times more efficient in lignin degradation than the more widely studied *P. crysosporium* in submerged cultures than solid cultures (Pamidipati and Ahmed, 2017). The distinctive property of this fungus to form biofilms enables the cells to grow on the air-liquid interface of the wastewater being treated (Aravinda Narayanan and Ahmed, 2019). These properties of *N. discreta* allow the biodegradation of the pollutant and enable easy harvest of the biofilm post-treatment. The use of biofilms can eliminate the need for extensive downstream separation of biomass from the wastewater. Furthermore, biofilms are more resilient to high pollutant loads in wastewater than suspended microorganisms due to the extracellular matrix, which acts as a barrier, protecting them from pollutants and enabling longer process life.

In this project, the biofilms formed by *N. discreta* were viewed as potential bio-membranes for efficiently treating wastewater generated from pulp and paper mills. The biofilms formed by *N. discreta* are viscoelastic material thick enough to be harvested. These biofilms have a high water-retention value, which means they can hold large amounts of water owing to the highly porous microstructure formed due to the meshwork of filaments. The extracellular matrix contains the extracellular enzymes which enable the treatment of pollutants adsorbed by the biofilms. These featured were utilised to develop a one-step treatment process to treat pulp and paper mill wastewater.

1.14 Objectives

Objective 1 - To identify and optimise key process levers for effective lignin degradation.

Hypothesis - The process conditions including pH, trace elements, and copper sulphate can be used to influence lignin degradation, enzyme activities, and biofilm properties.

A comprehensive study was carried out for the optimisation of growth conditions of biofilm using a design of experiments (DOE) to evaluate various process conditions to enhance enzyme activity and lignin degradation. Taguchi is a statistical DOE that enables evaluating multivariable interactions in a set number of orthogonal arrays of experiments. The DOE aimed to optimise the biofilm process considering the enzymatic activity and lignin degradation as the responses to variation in process parameters. Taguchi's experiment design was applied to grow biofilms on synthetic lignin-rich media to investigate the effect of pH, trace elements and copper sulphate concentrations on lignin degradation and enzyme activity. These factors were tested at three levels based on the previous results from studies conducted to test one variable at a time. The lignin degradation efficiency and enzyme activity were

analysed to identify the most significant parameter for enhanced degradation and enzyme activity.

Objective 2 - To evaluate the effect of adding lignin degradation intermediates on lignin degradation during fungal treatment

Hypothesis - The addition of naturally formed in-process lignin degradation intermediates to the fungal wastewater treatment cultures of the process will increase enzyme activity significantly, resulting in an efficient lignin degradation process.

This study used in-process lignin degradation products as additives to enhance ligninolytic enzyme activity and enhance lignin removal efficiency. The industrial wastewater, rich in lignin, from pulp and paper mills was used as media for fungal biofilm growth. This objective comprised two sets of experiments: in the first set of experiments, the spent media treated with *N. discreta* biofilms was collected from different time points of wastewater treatment. The next stage of the investigation had the fresh wastewater media inoculated with *N. discreta* spores, and cultures were fed with spent media from the first experiment. The spent media collected in the first experiment was autoclaved to denature any enzymes present in the spent media before the spent media was fed into the fresh cultures. Residual lignin concentrations were analysed using RP-HPLC and UV spectroscopy. COD removal was also measured, and the results were correlated with the lignin removal concentrations. An in-depth analysis of lignin degradation products was done using RP-LCMS, and the compounds present in the spent media were identified.

Objective 3 - To evaluate the repeated batch process for lignin degradation in pulp and paper mill wastewater using biofilms formed by *N. discreta.*

Hypothesis - Repeated-batch treatment of industrial wastewater using mature biofilms will accelerate the rates of lignin and COD removal by eliminating the cellular lag phase involving biofilm formation.

The biofilm treatment of wastewater was investigated in a repeated batch process where industrial wastewater was treated in three treatment cycles with increasing lignin concentrations. The two conditions that were compared were selectively supplemented with sucrose. First, the biofilms were grown by spore inoculation in the first cycle. During the second cycle, the biofilm biomass was transferred to fresh wastewater media with one experiment set supplemented with sucrose and the other deficient sucrose. The biofilm biomass was transferred to new wastewater media containing the same nutrient conditions during the third cycle. The spent media from all the cycles were analysed for residual lignin and COD concentrations using RP-HPLC and UV-spectroscopy methods. The biofilms were imaged using SEM and analysed using Image J. The biofilm microstructure was studied as well.

Objective 4 - To evaluate process scalability in a repeated batch process.

Hypothesis - Scaling up will result in similar or improved biofilm properties, enzyme activities and lignin and COD removal rates.

The repeated batch treatment process was scaled-up in non-sterile conditions at room temperature with a treatment volume of 800 ml in tray reactors. The wastewater was treated in three cycles, comparing two experiment sets with the difference in the composition of initial growth media used during the first cycle. The biofilm biomass was retained in the tray reactors at the end of each process. Fresh wastewater media with increasing order of lignin concentration supplemented with sucrose was added. The HRT of each cycle was the same as in the previous study. The performance of the operation was monitored concerning lignin

removal and measuring COD. The lignin concentration in wastewater will be analysed using a UV–Vis spectrophotometer and HPLC. The biological performance of the process was monitored by the activity of enzymes, and harvested biofilms were characterised after gathering on the last day of the process.

1.15 Thesis Organisation

This chapter presents an overview of the project with details about the research questions, gaps, aims and objectives. A detailed literature review on wastewater characteristics, existing methods used for treatment, biological treatment of wastewater rich in lignin and lignin-derived pollutants, and role of biofilms and enzymes secreted in the extracellular matrix is provided in Chapter 1. Chapter 2 details the methods used during the experiments for the standardised techniques. Chapter 3 describes the optimisation of critical process variables using the design of an experiment to enhance laccase production and, subsequently, the degradation of lignin. Chapter 4 details the addition of in-process lignin degradation intermediates for an efficient process. Chapter 5, describes studied involving the repeated batch mode in the presence and absence of sucrose. Finally, Chapter 6 describes the scalability of the process. Chapters 3 to 6 correspond to the thesis objectives mentioned in section 1.14. Chapter 7 details the conclusion and future work.

2. Materials and Methods

2.1 Background

This chapter presents the details of all the methods used during the experiments. The fungal cells and biofilm growth in liquid cultures were maintained aseptically unless mentioned otherwise. For quantification of residual lignin concentrations in spent media, UV-visible spectroscopy and reverse-phase high-performance liquid chromatography techniques were used (RP-HPLC). Mass spectroscopy was used to identify the lignin degradation intermediate products. Enzyme activity analysis is critical for a successful, robust, efficient biological, biofilm-based wastewater treatment system. The methods used for evaluating the enzyme activities are listed for all the ligninolytic enzymes produced by *N. discreta* during treatment. For the characterisation of pollutant parameters in wastewater, Chemical Oxygen Demand (COD) was quantified before and after biological treatment (Federation and Aph Association, 2005). For biofilm characterisation, methods used to quantify wet weight, dry weight, water retention value, and inoculum density are also presented. The biofilm microstructures were studied using scanning electron microscopy, and the images were analysed using ImageJ software.

2.2 Fungal Strain Maintenance

Neurospora discreta is a filamentous fungus isolated from the Subabul tree in India and used to produce biofilms (Pamidipati and Ahmed, 2017). *N. discreta* cells were maintained on a solid medium made by solidifying potato dextrose agar (PDA) procured from Sigma Aldrich. The filamentous fungus grows as an orange-pink fluffy layer of healthy filaments and spores (Figure 2.1). The cells are maintained by periodic streaking of PDA plates and incubating them at 30° C for 3-4 days. The plates were then stored at 4° C for further use.

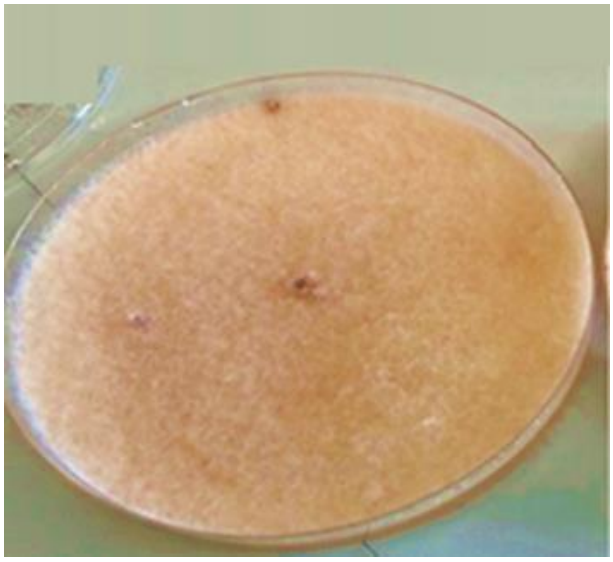


Figure 2.1 - *Neurospora discreta* growing on PDA plates

2.3 Minimal Media and Wastewater Composition

For the initial studies (Objective 1) the biofilms were grown in 250 ml Erlenmeyer flasks containing 150 ml of synthetic wastewater prepared in Vogel's media containing lignin and sucrose as additional carbon sources. For the preparation of lignin-rich synthetic wastewater, kraft lignin (Sigma-Aldrich) was added to Vogel's media. The liquid cultures for the growth of biofilms were prepared by using Vogel's minimal medium recipe (Vogel, 1964)**.** Vogel's medium included 5 g of potassium dihydrogen phosphate, 2.5 g of trisodium citrate dihydrate, 0.2 g magnesium sulphate heptahydrate, 2 g ammonium nitrate, 0.1 g calcium chloride dihydrate and 0.1 ml of trace element solution. All the salts mentioned above were dissolved in 1 L of deionised water, which was then autoclaved for sterilisation. When the solution cooled down, 1 ml of biotin was added before the media was inoculated.

The solution for trace elements was made by adding and dissolving 5 g of citric acid monohydrate ($C_6H_8O_7.H_2O$), 5 g zinc sulphate heptahydrate ($ZnSO_4.7H_2O$), 1 g ammonium iron (II) sulphate hexahydrate ($Fe(NH_4)_2(SO_4)_2.6\ H_2O$), 0.05g manganese sulphate ($MnSO_4.1H_2O$), 0.25 g copper sulphate pentahydrate ($CuSO_4.5H_2O$), 0.05 g sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$), 0.05g anhydrous boric acid (H_3BO_3), in de-ionized

water (100 ml). The biotin solution was prepared by adding 5 mg of biotin to 50 ml of water and was stored in cold conditions (4^{o} C).

2.4 Wastewater Media

For the studies done on real wastewater (Objective 2 - 4) the wastewater was provided by a paper-making company, Mondi Frantschach. The wastewater provided was a filtrate from the pulp-bleaching plant. The details of the wastewater composition are provided in Table 2.1. To grow the fungus on lignin-rich wastewater, the media was supplemented with Vogel's media components and sucrose as a more readily used carbon source, as mentioned above (Kirk and Farrell, 1987). The kraft treatment process was used for the pulp-making and brightening process; hence, the wastewater contained kraft lignin. To encourage fungal growth in the wastewater, it was supplemented with nutrients to match Vogel's media composition, as mentioned in section 2.3. The wastewater was intensely coloured and had a high pH of 10.5 due to the kraft process that results in the degradation of polymeric nonpolar lignin in more polar lignin degradation products.

2.5 Inoculation of Reactors

In liquid cultures, the spore count in the inoculum, called the seeding density (cells per ml of inoculum), directly affects the growth of the fungus, affecting the production of extracellular enzymes (Nandal, Ravella and Kuhad, 2013). An independent study was carried out to standardise the inoculum size and to evaluate the effect of inoculum spore count on the characteristics of the biofilms formed by *N. discreta*.

Table 2.1- Characteristics of wastewater provided by the pulp and paper-making company. The wastewater used was the filtrate from bleaching plant - during pulp brightening period. The asterisks (*) represent the concentration with the sample Diluted 1 to 200.

Components	**Concentration**
Suspended fines (mgL^{-1})	16.6
Ash - suspended fines (%)	17.9
COD (mgL^{-1})	13527
Colour - UV/VIS 450 nm & 600 nm	344
Lignin (mgL^{-1})	2934
Chloride (mgL^{-1})	57,1839 *
Sulfate (mgL^{-1})	277,2057 *
Thiosulfate (mgL^{-1})	83,6177 *
Phosphate (mgL^{-1})	4,2118 *
Sodium (mgL^{-1})	1380
Calcium (mgL^{-1})	65.4
Potassium (mgL^{-1})	254
Magnesium (mgL^{-1})	8.56
Iron (mgL^{-1})	0.115
Manganese (mgL^{-1})	2.86

The final spore count used for all the studies was 1×10^7 spores per ml. The fungus forms spores on healthy filaments when grown on agar (PDA). The filaments and spores formed on PDA were dislodged by scraping off the fungus from the plates, and the cells were added to 20 ml of Vogel's media devoid of sucrose. To obtain a homogenous spore suspension, this was then filtered through a sterile muslin cloth. A haemocytometer was used to count cells, and the spore counts were adjusted to 1 X 10^7 spores per ml. The reactors were inoculated using an aseptic technique. The flasks were plugged with foam bungs covered with aluminium foil and left in an incubator at 30^o C for all the studies except for the last (Figure 2.2). The reactors were sampled periodically throughout the experiment.

Figure 2.2 - Fermentation set up for wastewater treatment in a stationary incubator.

2.6 Sample Preparation for Lignin Estimation

Lignin estimation using the spectrophotometric method overestimates the lignin content due to media interference. This is due to absorption interference created by the presence of carbohydrate-degraded products, such as hydroxymethylfurfural and furfural, which can absorb light strongly at the characteristic wavelength ranges in which lignin absorbs light (Liu *et al.*, 2009). This overestimation is common in media containing low lignin content (Hatfield and Fukushima, 2005). Additionally, proteins absorb light at 280 nm, contributing to the overestimation of lignin at low lignin con(Hatfield and Fukushima, 2005; Bruce, 1998; Asina *et al.*, 2017)(Hatfield and Fukushima, 2005; Bruce, 1998; Asina *et al.*, 2017). For the lignin estimation in treated samples and comparison with untreated samples, the precipitation protocol by Laboratory Analytical Procedures from the National Renewable Energy Laboratory is applied (Sluiter *et al.*, 2008). The samples are treated to remove biomass after raising the pH to 10.5, followed by centrifuging at 5000 RCF for 30 mins. The supernatant is

retained, and the biomass pellet is discarded. The samples (3ml) are hydrolysed by using 2M H_2SO_4 to drop the pH 1-2 for lignin to precipitate, followed by centrifugation at 5000 RCF for 30 mins. The supernatant is removed from the isolated lignin, also known as Klason's lignin, which was given two acid washes with acidified water. The precipitated lignin was dried in an oven at 30^{o}C till constant weight was achieved. The dried lignin is redissolved in alkaline water at pH 10.5 and analysed further using reverse-phase high-performance liquid chromatography (RP-HPLC) and UV spectroscopy.

2.7 pH Estimation

The pH of the liquid supernatant was monitored using a HI-1083B pH electrode pH meter at regular intervals.

2.8 Lignin Estimation

2.8.1 Ultraviolet-visible Spectrometry

One of the easiest-to-use analytical techniques for lignin quantification is UV-vis spectroscopy, previously reported for lignin quantification in pulp paper effluent and wood. Though there are two notable adsorption bands in the lignin spectrum, first at 200 to 230 nm and second at 260 to 280 nm, lignin quantification of the macromolecule typically relies on the adsorption at 280 nm (Lee *et al.,* 2013; Skulcova *et al.,* 2017)**.** For the current study, lignin quantification was measured at the characteristic decreasing absorption band of lignin at 278 nm characteristic wavelength for grass lignin (Siqueira *et al.,* 2011; Ramadhani, Kanti and Sudiana, 2019). A Thermo Scientific spectrophotometer was used for this study. Lignin samples for the UV- spectrometry were prepared by periodically drawing treated wastewater from the reactors. For the standard curve, standard kraft lignin (Sigma-Aldrich) was dissolved in Vogel's minimal media to get concentrations of 10 – 80 mg L^{-1}. At absorbance 278 nm, the

A_{278} lignin standard curve (Figure 2.3) shows a linear relationship with the correlation coefficient R^2 value of 0.9964.

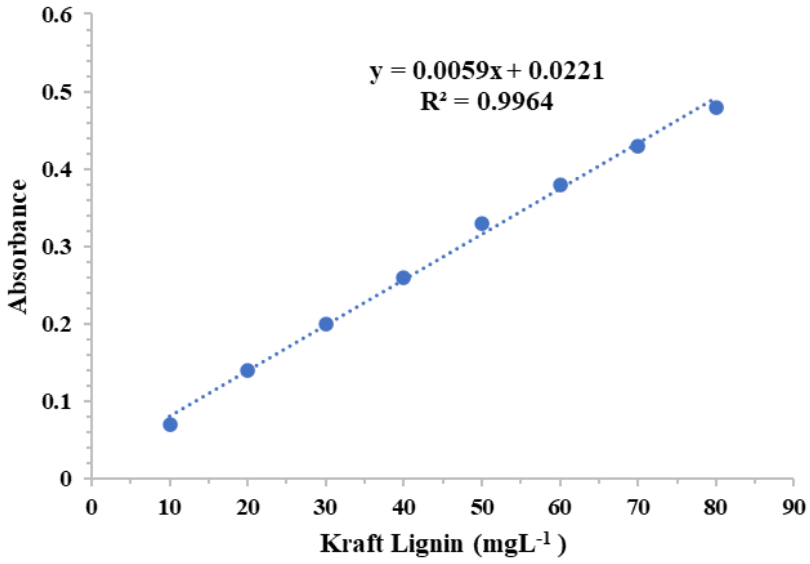


Figure 2.3 - Standard curve for kraft lignin at different concentrations.

2.8.2 Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

Liquid chromatography is commonly used to separate, identify, and quantify lignin components in liquid samples. HPLC involves the separation of the molecules based on the hydrophobicity. In normal phase HPLC, the stationary phase is polar, while in reverse phase RP-HPLC, the stationary phase is nonpolar, and the mobile step is polar. This technique involves interaction of dissolved organic matter with the hydrophobic stationary phase (C-18 RP-HPLC resin), eluting with a polar solvent and is helpful in quantifying lignin content in wastewater. The fungal biofilm treated wastewater samples were analysed using Thermo Scientific BDS Hypersil C-18 column (4.6 mm ID × 250, 5 µm particle size) as stationary phase. The neat sample (25 µL) is injected into the column with a flow rate of 1 ml per minute during the loading of the sample, and the elute is monitored using a UV detector at 254 nm.

The mobile phase is a mixture of HPLC grade water (A) and 100% acetonitrile (B) with a total run time of 45 minutes for kraft lignin samples (Pamidipati and Ahmed, 2017; Pamidipati and Ahmed, 2020). An elution gradient with the concentrations of solvent B as follows was used: at 0 min - 0 %; 5 min - 3 %; 10 mins - 6 %; 15 min - 10% and 20 min - 20 %; 25 min - 40%; and 35 min - 80%. For the analysis of lignin content in the samples, standard kraft lignin is used for concentrations 1000 mgL^{-1} to 5000 mgL^{-1}. As a result, in RP-HPLC, the polar component is eluted first and non-polar eluted later. According to the previously reported HPLC method, lignin degradation intermediates are polar in nature and form a peak at a retention time (RT) of 2.8 minutes (Pamidipati and Ahmed, 2017). The lignin component shown as peak at RT 16.5 min is nonpolar in nature (Figure 2.4 a).

A modified HPLC method was used to estimate lignin during the treatment of synthetic and industrial wastewater, as mentioned in the following studies. A guard column (apHera™, I.D. 1cm × L4.6 mm × 5µm particle size) was attached to the C18 column. The standard curves were created by plotting lignin concentration against the chromatogram peak area for kraft lignin samples. Adding a column guard reduced the nonpolar peak retention time to 16.5 minutes for kraft lignin standards. The lignin standard curve used to estimate lignin in the industrial wastewater provided by the paper-making company, Mondi Frantschach, was also prepared by precipitating the wastewater by adding sulphuric acid to drop the pH below 2. The precipitated and dried lignin was redissolved in DI water to prepare concentrations from 5000-1000 mgL^{-1}. These standard solutions were injected (25µL) in the HPLC column to get chromatograms. The peak area from the chromatograms was used to create a standard curve. Only one major polar peak was seen in the chromatogram at 2.4 minutes RT (Figure 2.4 b). The lignin removal efficiency (%) was calculated using equation (2.1).

$$Lignin\ Removal\ Efficiency(\%) = \frac{Lignin\ conc.(start) - Lignin\ conc.(end)}{Lignin\ conc.(start)} \times 100 \qquad (2.1)$$

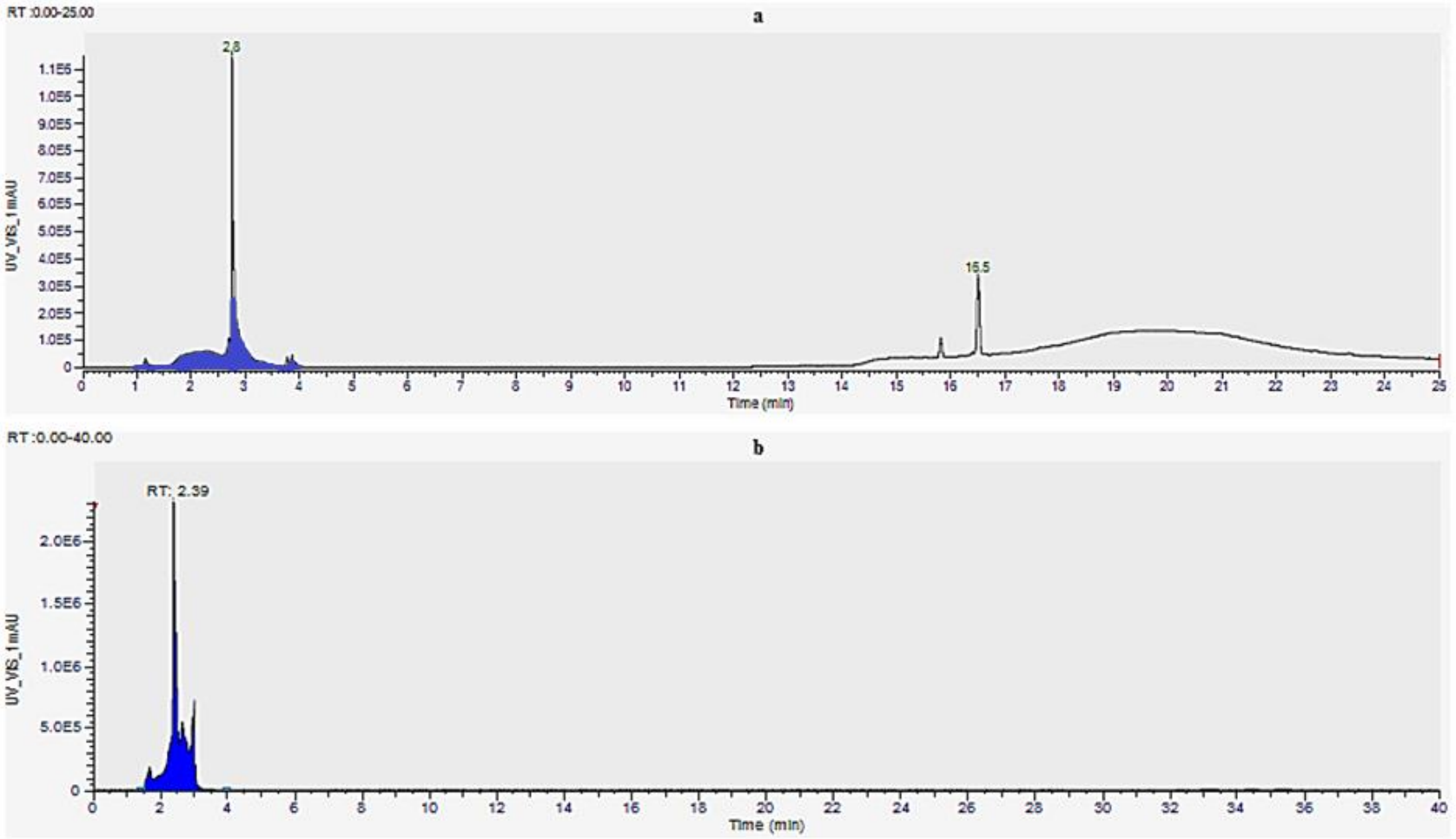


Figure 2.4 - Chromatogram profile of lignin standard (a) synthetic wastewater samples showing polar and nonpolar peaks for kraft lignin standard (b) wastewater from paper and pulp mill showing the polar peak representing the lignin degradation products in wastewater.

2.9 Chemical Oxygen Demand

The Chemical Oxygen Demand (COD) was estimated using the COD/2000 kits from Palintest. The wastewater samples were first treated to remove the fungal biomass, for which the samples were centrifuged at 10000xg for 10 minutes. The supernatant was collected diluted 200-fold, and 2 ml of the resulting solution was added to the reagent tubes, which were shaken well to mix. The heat block was set at 150^{o} C, and the sample tubes were left in the heated block for 2 hours to digest. After the digested samples cooled to room temperature, the tubes were inserted in the photometer to obtain the COD values in mgL^{-1}. The COD was measured at the beginning and end of the fungal treatment process, and the COD removal efficiency was calculated using equation (2.2).

$$COD\ Removal\ Efficiency\ (\%) = \frac{COD\ (initial) - COD(final)}{COD\ (initial)} \times 100 \qquad (2.2)$$

2.10 Biofilm Growth and Harvest

The biofilms formed on the liquid-air interface by *N. discreta* were harvested using a long spatula under sterile conditions. The fully formed biofilms are rich in water. To drain excess water, the biofilms were placed on a water-absorbent paper for a few minutes. Fresh samples were used to calculate wet weight, water retention value, dry biomass and extracellular matrix components. The biofilm samples were sprayed with isopropyl alcohol to halt growth and then stored at 4°C for analysis.

2.11 Water Retention Value

Water retention value (WRV) is the capacity of a material to hold water. It is a material property that highlights the ability to swell by absorbing water or its hydrophilic nature, due to which it repels water. Under standard conditions, the capacity of a material to hold water after centrifugation is the measure of water retention value (Cheng *et al.*, 2010b). Biofilms formed by *N. discreta* are highly porous and viscoelastic. The cells in the biofilm are

embedded in the self-secreted extracellular matrix, composed of carbohydrates, lipids, proteins, and other substances. Carbohydrates are hydrophilic and attract water molecules, resulting in biofilms loaded with water forming more than 90% of the total weight in the porous meshwork.

The standard method, Tappi UM 256, was used to quantify the WRV; in this case, a slightly modified protocol was applied (Cheng *et al.*, 2010b). WRV is defined by Cheng et al., 2010 as "the per cent ratio of water contained in the sample after centrifuging at a certain force and time relative to the dry weight of the sample." Here, the known amount of sample was centrifuged for 15 min at 1217 g after being soaked in tap water for an hour. The weight of the biofilm after centrifuging ($Weight_{wet}$) was noted as the wet weight of the biofilm. The sample was dried at 103^{o} C to gain a constant weight ($Weight_{dry}$). The WRV was calculated as the below equation (2.3).

$$WRV\ (\%) = \frac{Weight(wet) - Weight(dry)}{Weight\ (wet)} \times 100 \qquad (2.3)$$

The WRV of biofilms formed by *N. discreta* depend on the complexity of the carbon source provided, with sucrose being the more readily degradable carbon source and lignin being more complex. As is reported and established by (Pamidipati and Ahmed, 2017), as the carbon source complexity in the growth medium increases, the WRV of *N. discreta* biofilms decreases. On the other hand, the dry weight of the biofilms grown on sucrose showcased the lowest numbers of normalised dry weight than lignin gown biofilms (Pamidipati and Ahmed, 2017). To measure the water-holding ability of the biofilms grown on different concentrations of kraft lignin, the harvested biofilm was soaked for an hour in tap water and weighed, after which the water was drained in a known amount of biofilm by centrifuging at 1217g. The centrifuged biofilm was transferred to weighed crucibles and dried at 103.5^{o} C in an oven till a constant weight was achieved.

2.12 Extracellular Polymeric Substances

A known amount of biofilm, with excess water drained, was soaked in a 25 ml solution of sodium chloride (8.5 %) and formaldehyde (0.22 %). The biofilms were soaked for 3 hours at 4^{o}C and centrifuged for 10 minutes at 10,000 g. A syringe filter (0.25 µm) was used to filter the supernatant saved for carbohydrate and protein assays (Ahmed, Narayanan and Veni, 2020). The method used to quantify the carbohydrate content in the EPS dinitro salicylic acid (DNS) reagent was used. Dinitro salicylic acid (2.5 g) procured from Across Organics was dissolved in deionised water (125 mL) at 80^{o} C. Sodium hydroxide (2N) and potassium sodium tartrate hydrate (75 g) were added to the solution at room temperature. The total volume was brought up to 250 mL by topping the solution with deionised water. The sample (1 mL) was hydrolysed by adding 0.25 mL of concentrated sulphuric acid and heated for an hour in a water bath at 95^{o}C. After cooling the hydrolysed samples, 1 mL of 10 N sodium hydroxide was added to each sample. In 96 microwell plates, 75 µL DNS and 75 µL of samples were mixed and kept in a preheated oven at 100^{o} C for 30 minutes. To stop the reaction, 75 µL of ice-cold deionised water was added, and plates were refrigerated at 4^{o} C for 15 minutes. A MULTISKAN Go spectrophotometer measured the absorbance at 540 nm (Gonçalves *et al.,* 2010). For protein quantification in the EPS, a protein kit number 77371 from Sigma, UK, was used per the manufacturer's instructions.

2.13 Activity of Lignin Degrading Enzymes

2.13.1 Laccase Activity

Laccase oxidised phenols and polyphenols and their activity can be measured by using the method of Roy-Areand and Archibald (Roy-Arcand and Archibald, 1991) in which the oxidation of 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) as substrate in citrate buffer (Bourbonnais and Paice, 1990; Baldrian and Šnajdr, 2006). Dark green cations

are produced because of the oxidation of ABTS by laccases, and the spectral time scan is measured spectrophotometrically at 420 nm wavelength. For the reaction, ABTS (3mM,0.6ml) is added to 1.8 ml of sample drawn from the treated flasks. Defining enzyme activity is the amount of green cation formed per unit volume per unit time at assay condition, and its unit is M-sec^{-1}. The enzyme activity (1 Unit) is quantified using the extinction coefficient (ε420) 36000 M^{-1} cm^{-1} (Wang *et al.*, 2016; Saito *et al.*, 2003).

2.13.2 Polyphenol Oxidase Activity

Polyphenol oxidase (PPO) activity was performed in microwell plates using pyrocatechol as a substrate (Ascacio-Valdés *et al.*, 2014). The pyrocatechol solution (0.2 M) was prepared in citrate buffer (0.05 M) at pH 5. The samples (100 µL) in the microwell plate were left in an incubator at 30^{o}C for 30 mins. The pyrocatechol solution (200 µL) was added to the sample, and the absorbance was read at 420 nm at 30^{o}C for an hour. An increase in absorbance by 0.001 min^{-1} was defined as one unit activity measured in M.min^{-1}.ml^{-1}. One unit of enzyme was defined as the amount of enzyme that forms 1 mol of product minute^{-1}.

2.13.3 Versatile Peroxidase Activity

Versatile peroxidase (VPO) activity was determined by oxidation on reactive black dye (RB5) in sodium tartrate buffer 50 mM at pH 3 (Ravichandran *et al.*, 2018). To the 100 µL sample, 50 µL RB5 (6 µM) was added to the microwell plate. Freshly prepared hydrogen peroxide (0.6mM, 50 µL) was added to the reaction mix. A decrease in absorbance was noted at 598 nm at an extinction coefficient of ε_{598} 24000 M^{-1} cm^{-1}. One unit of enzyme activity is defined as the amount of enzyme that transforms 1mol of substrate consumed.

2.14 Microscopy

2.14.1 Scanning Electron Microscopy

Biofilms formed by *N. discreta* have an intricate meshwork of filaments with interspersed pores. The cells are embedded in the self-produced extracellular matrix. Scanning Electron Microscopy Hitachi TM4000 plus was used to study the microstructure of biofilms. Figure 2.5 shows the SEM image of a harvested biofilm. A section of the harvested biofilm was washed with water to remove IPA and left to dry on the microscopic slide for 24 h in an incubator at 25^{o} C. The slides were imaged when the biofilms were completely dry. The SEM accelerating voltage used was 15kV, magnification of 1000-1500X in the backscattered electron (BSC) mode. The microstructure characteristics such as filament length, diameter, pore size and area were calculated by analysing the SEM images using ImageJ software.

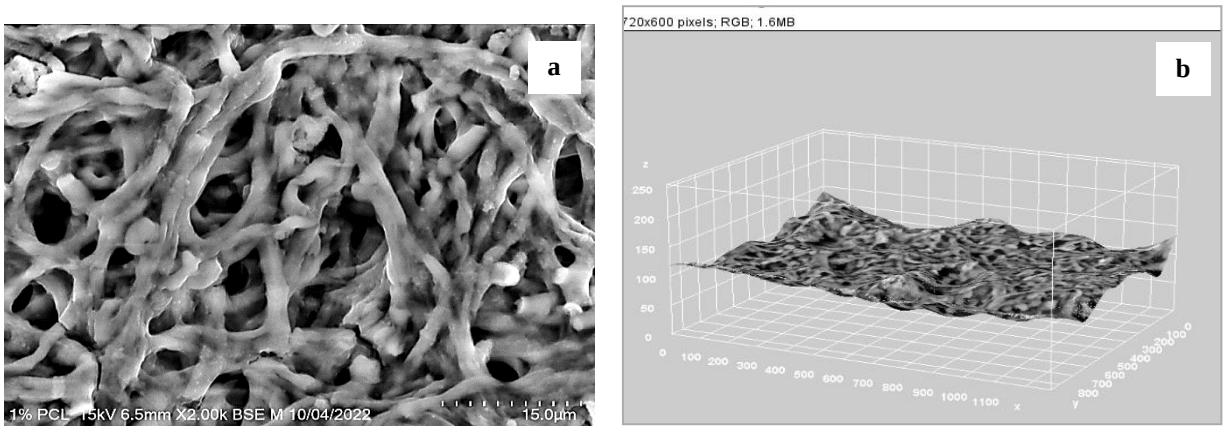


Figure 2.5 - Biofilm microstructure (a) SEM (2D) image of biofilm formed on wastewater showing intricate meshwork of filaments and pores 1000x magnification (b) 3D surface plot of the SEM biofilm

2.14.2 Environment Scanning Electron Microscopy

To get images of biofilms in native condition, without drying them, the imaging was done using a scanning electron microscope Thermofisher Quanta 650 ESEM with a Tungsten filament. The biofilms were imaged at the University of Nottingham (Nanoscale and

Microscale Research Centre). Samples were mounted on a vertical stub held by wrapping an aluminium tape. The stub was fitted onto the Peltier stage cooled to 2^{o}C, and the chamber was pumped to 5.3 Torr following two purge cycles to flush with water vapour. The imaging mode was ESEM using the gaseous secondary electron detector (GSED) at 1000 x magnification.

2.14.3 Image Analysis

ImageJ software (US National Institutes of Health) was used to measure the filament thickness, length and area of areal pores in bright field settings to analyse SEM images (Huang and Wang, 1995). The magnification of SEM images was 1500 × and 2500 x magnification, at a scale of 9.27 pixels per micron (μm). The segmented line function of ImageJ was used to manually measure the length, thickness and diameter of the filaments (Abràmoff et al., 2004). Images were converted from bright field images into 8-bit to get the total area of the visible pores and the thresholding algorithm was applied for each biofilm (Figure 2.6) (Huang and Wang, 1995).

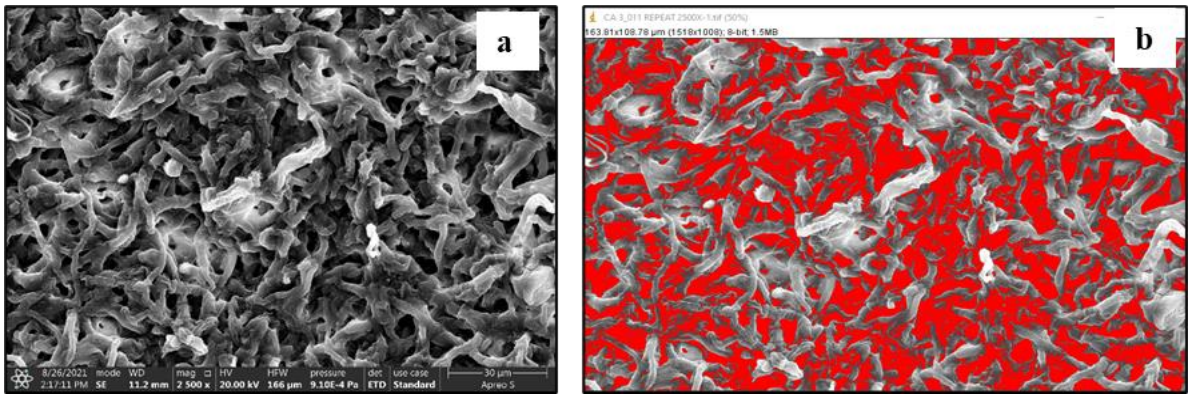


Figure 2.6 - SEM image of biofilms grown in normal conditions showcasing the intricate network of filaments and interspersed with pores (a) magnification at 2500 (b) image converted into 8-bit with thresholding algorithm applied in red.

3. Optimisation of Process Conditions for Treatment of Lignin Wastewater Using Novel Biofilm Formed by *Neurospora discreta*

3.1 Introduction

Lignin is a large heterogenous, complex biopolymer found in plants and is responsible for imparting strength and protection to the plant parts (Rajesh *et al.*, 2019). Second to cellulose, lignin is the most abundantly found biopolymer on earth. Lignin is relatively recalcitrant as the macromolecule is a highly ramified structure having β-*O*-4 ether bonds, 5-5′ carbon–carbon bonds and β-5 bonds that are difficult to break (Sun, Tomkinson and Bolton, 1999). Lignin is a key component of the lignocellulosic biomass used as raw material in paper making and is removed from the biomass during the pulping process. This results in the generation of large quantities of lignin-rich wastewater from paper and pulp industries. Discharge of untreated wastewater rich in lignin and phenolic compounds can lead to serious risks to human health, aquatic life, and plants (Hutchins, 1901; Oikari and Nakari, 1982). The environmental impact of the pulp and paper mill effluent is a result of its chemical composition which makes it highly coloured and turbid, with a high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) and low biodegradability owing to the presence of lignin and compounds derived from lignin (Christov and Van Driessel, 2003; Srivastava, Mall and Mishra, 2005).

The activated sludge process is a commonly used biological treatment method for pulp mill effluent, although it is associated with challenges, including poor settling and low control over the microbial population within the sludge (Thompson *et al.*, 2001). Another challenge of this process is the low tolerance of the sludge microorganisms for high concentrations of lignin (Radjenović *et al.*, 2015). *Neurospora discreta*, a pink ascomycete, has been reported to degrade lignin in agricultur(Pamidipati and Ahmed, 2017)(Pamidipati and Ahmed, 2017).

This fungus also has the ability to form strong biofilms naturally on the air-liquid interface (Ahmed, Narayanan and Veni, 2020).

Recently, these biofilms have been studied to remove nitrogen and phosphorous from synthetic wastewater as a one-step method (Tabraiz *et al.,* 2022). The present work is based on these novel characteristics of *N. discreta* that can be used to treat lignin-rich wastewater in a biofilm-based process. Being housed in biofilms enables the cells to tolerate higher levels of contaminants and can allow prolonged retention times leading to higher removal efficiencies (Mishra *et al.,* 2022). Additionally, the downstream processing of biofilm-based processes is less challenging as the biofilms can easily be harvested to remove the microbial cells from treated wastewater (Ahmed, Narayanan and Veni, 2020). In this process, the biofilms were formed on the air-liquid interface and did not require any external structures or matrices to support the biofilms.

In this work, key process parameters were optimised to enhance the lignin degradation and enzyme activities of *N. discreta* during the treatment of synthetic lignin-rich wastewater that mimics pulp mill effluent. In order to study the relative interactions between parameters and their effect on lignin degradation and enzyme activity, as well as biofilm properties, the Taguchi design of experiments (DoE) method was used for the study. Taguchi is a statistical method that can be applied to study any given process with multiple independent parameters used as variables, also known as factors, over different levels of interest (Prasad *et al.,* 2005). Taguchi is a useful method for process optimisation as it can identify the effects of individual parameters as well as the interactions between variables, which can play a critical role in biological processes (Chenthamarakshan *et al.,* 2017). The Taguchi method has an easy execution and applies a shorter fractional factorial design in comparison to more explored methods such as response surface methodology (RSM) and the Plackett-Burman design (Rao

et al., 2008). Taguchi is also twice as quick as RSM (Rao *et al.,* 2008). This method has been employed in a number of biochemical process studies to augment ligninolytic enzyme function (Parveen *et al.,* 2022; Nandal, Ravella and Kuhad, 2013). However, the application of Taguchi in optimising the treatment of lignin-rich wastewater is not well explored.

The factors studied in this study were initial culture pH, $CuSO_4$ as an enzyme inducer, and trace elements in the wastewater media at three different levels. This study examines the influence of process parameters on lignin removal and enzyme activities during the treatment of lignin-rich wastewater by *N. discreta* biofilms. Additionally, it evaluates how these parameters affect biofilm properties, including microstructure and composition and discusses the relationship between biofilm properties and lignin removal efficiencies. The novelty of this work lies in the following key aspects: (1) the implementation of Taguchi Design of Experiments (DOE) to optimise a novel biofilm-based lignin degradation process and (2) the utilization of high-strength wastewater with significantly higher lignin concentrations compared to those documented in the existing literature, to reflect the concentrations of lignin in real effluents from the paper and pulp industry.

3.2 Materials and Methods

3.2.1 Fungus Maintenance and Inoculation

Neurospora discreta cells were maintained on potato dextrose agar (PDA) plates. The plates were stored at 4^o C and were used to collect the spores for inoculation. Vogel's minimal media, containing no sucrose, was used to flood the plates and the fugal filaments were gently scraped off to collect spores suspended in the media (Vogel, 1964). The spore suspension was filtered through a sterile muslin cloth and then used for inoculation. For the spore count a haemocytometer was used and the spore suspension was adjusted to 1×10^7 spores m L^{-1}. The reactors were inoculated using 1 mL of the spore suspension.

3.2.2 Wastewater Composition and Fermentation Setup

Synthetic wastewater was prepared by adding 17.5 g L^{-1} of water-soluble kraft lignin (Sigma-Aldrich, 4710003) to Vogel's minimal media containing 2.5 g L^{-1} sucrose. The wastewater was designed to match the lignin concentrations of effluents from the paper and pulp industry bleaching plants and to provide the essential nutrients required for fungal growth. The experiments were set up in Erlenmeyer flasks (250 ml), plugged with foam bungs containing 120 ml of media. The pH was adjusted and other variables, $CuSO_4$ and trace elements, were altered as per the experimental design discussed in section 2.3. Each of the nine experiment conditions was set up in triplicates.

A negative control was set up with an uninoculated reactor containing 17.5 gL^{-1} kraft lignin (KL, Sigma-Aldrich) in Vogel's media containing 2.5 gL^{-1} sucrose. The media was autoclaved at 121^o C for 15 minutes. Each flask was inoculated under sterile conditions with 10^7 spores per mL. The process was run for 14 days, and samples were drawn on days 3, 7, 10 and 13 after inoculation.

3.2.3 Design of Experiments

In the DoE study culture pH, $CuSO_4$ as an inducer of ligninolytic enzymes and trace elements were the three factors tested (Moshtaghioun *et al.*, 2017). The experiment was constructed using an L9 (3^4) orthogonal array, as the Taguchi design structure, with three levels of variation for all factors, was tested using MINITAB. The experimental results obtained from the 9 experiments were analysed to identify the most important factor accountable for enhanced degradation of lignin. Considering the optimum pH requirements for the ligninolytic enzymes to remain active and the lignin to remain dissolved in wastewater, three pH levels of 5, 6 and 7 were chosen to design the experiments. $CuSO_4$ at

three levels, 1 mgL^{-1}, 4 mgL^{-1} and 8 mgL^{-1}, was selected as a ligninolytic enzyme inducer. Three different volumes of trace elements solution (v/v), 0.05 ml L^{-1}, 0.20 ml L^{-1} and 0.30 ml L^{-1}, containing citric acid, zinc, $CuSO_4$, boric acid, ammonium iron (II) sulphate, sodium molybdate and manganese sulphate were added to the culture media.

3.2.4 Reversed-Phase High-Performance Liquid Chromatography

A Thermo Scientific™ LTQ XL™ RP-HPLC with UV-detector was used to analyse the lignin degradation products in the wastewater before and after treatment with *N. discreta* biofilms. A C18 column (250 x 4.6 mm ID 5 μm particle size) attached to a guard column (apHera™, 5μm particle size, L × I.D. 1 cm × 4.6 mm) was used. The compounds were detected at 254 nm using a photodiode array detector. A two-solvent gradient elution process made up of HPLC-grade water (A) and 100% acetonitrile (B) was used. The solvent B concentration used to establish a gradient was: 0 min - 0%, 5 min - 3%, 10 min - 6%, 15 min - 40% and 20 min – 80% till 25 mins. The sample injection volume was 25 μL and a flow rate of 1 ml min^{-1} was maintained. For data integration, the Xcalibur FreeStyle application was used. Before analysis, samples from the last day of the fermentation (day 14) were precipitated to remove the spent media. The precipitated lignin was redissolved in water at pH 10.5, and samples were analysed on RP- HPLC. Kraft lignin (Sigma-Aldrich) dissolved in DI water at varying concentrations was used to generate a standard curve. The lignin degradation percentage was calculated based on the total peak areas of the polar and non-polar peaks (equation 3.1).

$$\text{Lignin degradation } \% = \frac{Lignin\ conc.(start) - Lignin\ conc.(end)}{Lignin\ conc.(start)}\ x\ 100 \qquad (3.1)$$

3.2.5 Activities of Lignin-Degrading Enzymes

Polyphenol oxidase (PPO) activity was determined using pyrocatechol as a substrate and the increase in absorbance was noted for the formation of p-quinone, which absorbs light at 420 nm as mentioned in section 2.7 (Bending and Read, 1997). One unit of enzyme activity calculated was defined as the increase in absorbance by 0.001 min^{-1} and expressed in $M.min^{-1}ml^{-1}$ (Ascacio-Valdés *et al.*, 2014).

Versatile peroxidase (VPO) activity was determined by the oxidation of reactive black dye (RB5) in 50 mM sodium tartrate buffer at pH 3 (Ravichandran *et al.*, 2018). A decrease in absorbance was noted at 598 nM at an extinction coefficient of ε598 24000 M^{-1} cm^{-1}. One unit of enzyme is defined as the amount of enzyme that transforms 1mol of substrate consumed.

Laccase activity was quantified spectrophotometrically by quantifying the oxidation kinetics of the substrate 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Bourbonnais, Leech and Paice, 1998). To 100 µL centrifuged samples from each reactor, 100 µL of 0.1 M citrate buffer at pH 5 was added to the microwell plate. The mixture was left to equilibrate at room temperature for 30 minutes. To the mix, 100 µL of 0.3 M ABTS was added, and the spectral scan at 420 nm was started immediately. The increase in the concentration of blue-green cation radicals formed due to the oxidation of ABTS is correlated to enzyme activity using the extinction coefficient ε420=36,000 $M^{-1}cm^{-1}$. Enzyme activity was expressed in units defined as the amount of product formed per unit volume per unit time under the assay conditions (Saito *et al.*, 2003).

3.2.6 Biofilm Composition

To determine the biochemical composition of biofilms, the 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 left for 3 h at 4^{o} C and then centrifuged at 10,000 x g for 10 min. The supernatant was filtered through a 0.45 μm filter and was saved for further analysis. Polysaccharides in EPS were quantified with the dinitro salicylic acid (DNS) reagent method (Gonçalves *et al.,* 2010). The EPS was hydrolysed in glass test tubes by mixing 1 ml of extracted EPS with 0.25 mL of 98% sulphuric acid and heated in a water bath for 1h at 95^{o} C. After cooling to room temperature, the solution was neutralised by adding 10 N sodium hydroxide. The reaction mixture was prepared in 96-well plates by adding 25 μL of DNS solution with 25uL of EPS solution. The 96-well plate, with the lid on, was heated in an oven at 100^{o}C for 10 mins and immediately placed on ice followed by adding 250 μL of ice-cold water. The absorbance was measured at 540 nm using a MULTISCAN GO spectrophotometer. The 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) using the manufacturer's protocol.

3.2.7 Biofilm Microstructure

Harvested biofilms were placed on an absorbent paper for about an hour to drain excess water. For microscopy, a section of the biofilm was washed with tap water to remove lignin particles and media, then placed on a microscopic slide. The slides were left to dry in an incubator at 25^{o} C for 2 days. The dried biofilms were then imaged using a scanning electron microscope, and investigations were performed at 1500X and 2500X magnification. The images were further analysed using ImageJ software (US National Institute of Health) to

measure the filament length, filament thickness and total pore surface area (Huang and Wang, 1995). To determine the aereal porosity of each biofilm, the bright field SEM images were converted into 8 bits images followed by applying a threshold algorithm (Huang and Wang, 1995). The pore area was calculated using the method described previously (Ahmed, Narayanan and Veni, 2020) The filament dimensions, length, and thickness were measured manually using the segmented line function (Abramoff, Magalhães and Ram, 2004) .

3.2.8 Statistical Analysis

Enzyme activity and lignin degradation were used in a two-way analysis of variance test (ANOVA) at $p\text{-}value \leq 0.05$, i.e., 95% confidence limit. ANOVA for lignin degradation, polyphenol oxidase activity and versatile peroxidase activity as a response is reported in the results section. The p-value shows the significance of factors influencing the responses, where any factor that showed a p-value of less than 0.05 was considered significant, and the null hypothesis was rejected. The efficacy and significance of the individual factors were validated using probability analysis.

3.3 Results and Discussion

3.3.1 Effect of Process Parameters on Lignin Degradation Efficiency

Lignin degradation efficiency was computed from the RP-HPLC chromatograms as discussed in Section 2.4. The characteristic lignin profile of the standards, as well as the untreated wastewater, consisted of a large polar peak at retention time (RT) 2.8 ± 0.1 minutes retention time along with smaller relatively non-polar peaks eluting between RT 15.8 ± 0.1 and 16.5 ± 0.1 minutes. The wastewater treated with *N. discreta* biofilms showed a clear decrease in peak areas under all conditions. Figure 3.1 a shows a representative set of chromatograms obtained before and after treatment at pH 5, 6, and 7. At pH 5, under all concentrations of

$CuSO_4$ and trace elements, the degradation of the polar peak was between 68 % and 71% with the peak at 15.8 minutes being undetectable after treatment (Figure 3.1 b). The degradation of the 16.5-minute peak was much higher at pH 5, ranging from 67 to 83%. In the case of pH 6, the highest lignin degradation was seen at 4 mg L^{-1} $CuSO_4$ and 0.3 ml L^1 trace elements. At pH 7, the overall degradation was significantly lower compared to the acidic pH conditions, and the difference was particularly stark for the polar components (Figure 3.1 b).

The assignment of variables, parameter levels, and outputs for the L9 design are summarised in Table 3.1. The relative effects of the three factors on lignin degradation were determined by the analysis of variance (ANOVA) method and the contribution of each factor was calculated using the ratio between the pure sum to the total sum of squares (SS) adjusted mean square values (Table 3.2). Of all the factors, pH had the most significant effect (68.91% contribution, p-value 0.003) on the lignin degradation efficiency. Trace elements ranked second although the contribution was much lower than that of pH. The higher lignin degradation at lower pH can be attributed to better fungal growth at lower pH (Taboada-Puig *et al.,* 2011; Rousk, Brookes and Bååth, 2009), as well as the increased activity of ligninolytic enzymes such as peroxidases at low pH conditions. Reports have shown increased activity of fungal peroxidases on aromatic compounds at pH levels as low as 3 (Heinzkill *et al.,* 1998; Singh *et al.,* 2021) . The optimum pH for ligninolytic enzyme activity was found to be 4.5 - 5.5 for *P. chrysosporium,* with a decrease in lignin degradation levels reported above pH 5.5 (Garg and Modi, 1999).

The overall effects of the three factors are shown in the main effects plot (Figure 3.2). To evaluate the combined effects of the three factors on lignin degradation, 3D interaction plots

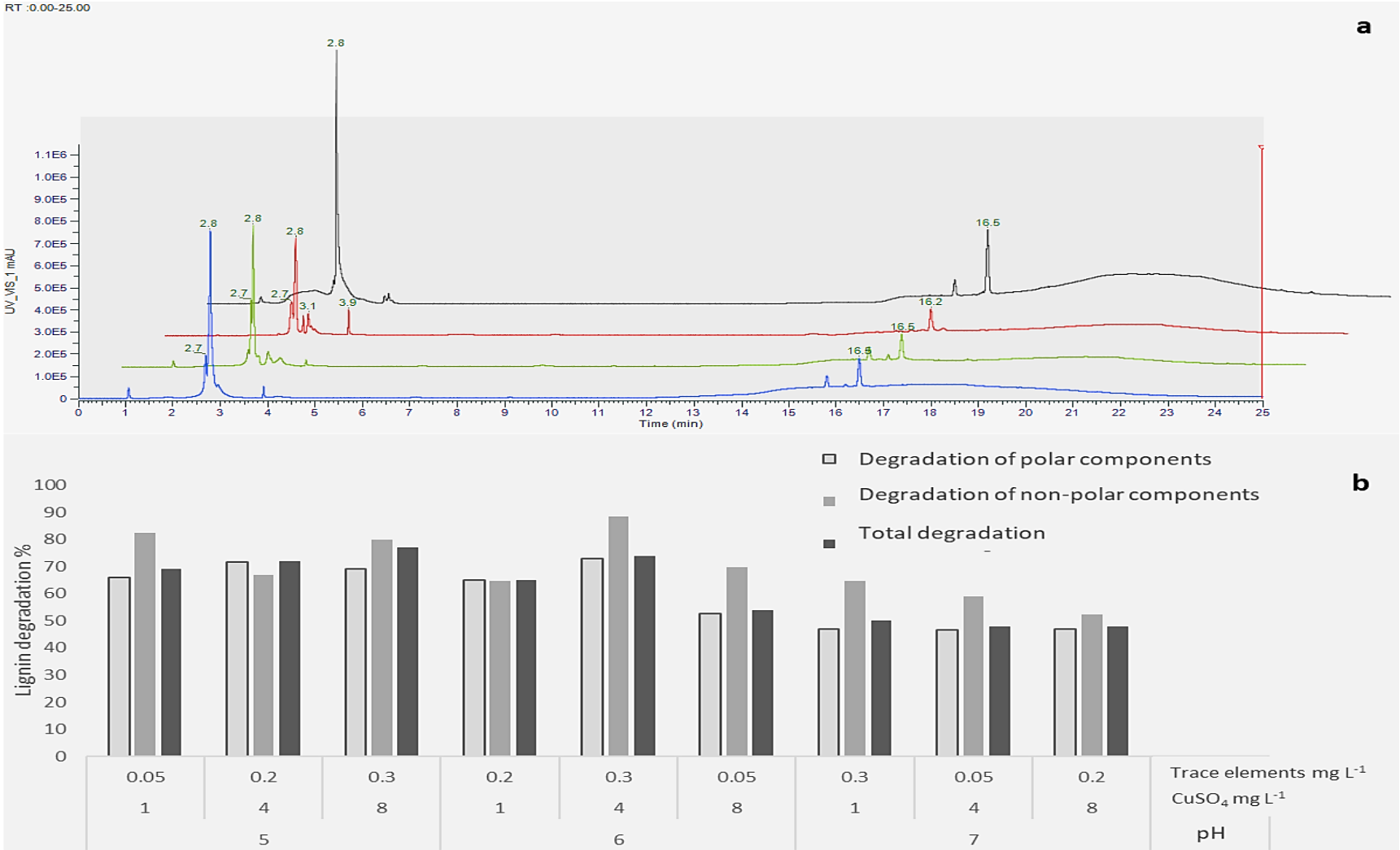


Figure 3.1 - (a) Reversed-Phase High-Performance Liquid Chromatography profiles for untreated wastewater sample from day 0 (black), pH-5; $CuSO_4$-1 mgL^{-1}; TE-0.05 mlL^{-1} (red), pH-6; $CuSO_4$-8 mgL^{-1}; TE-0.05 mlL^{-1}(green), pH-7; $CuSO_4$-1 mgL^{-1}; TE-0.3 mlL^{-1} (blue), (b) Degradation of relatively polar and non-polar components of lignin with varying pH, $CuSO_4$ and TE.

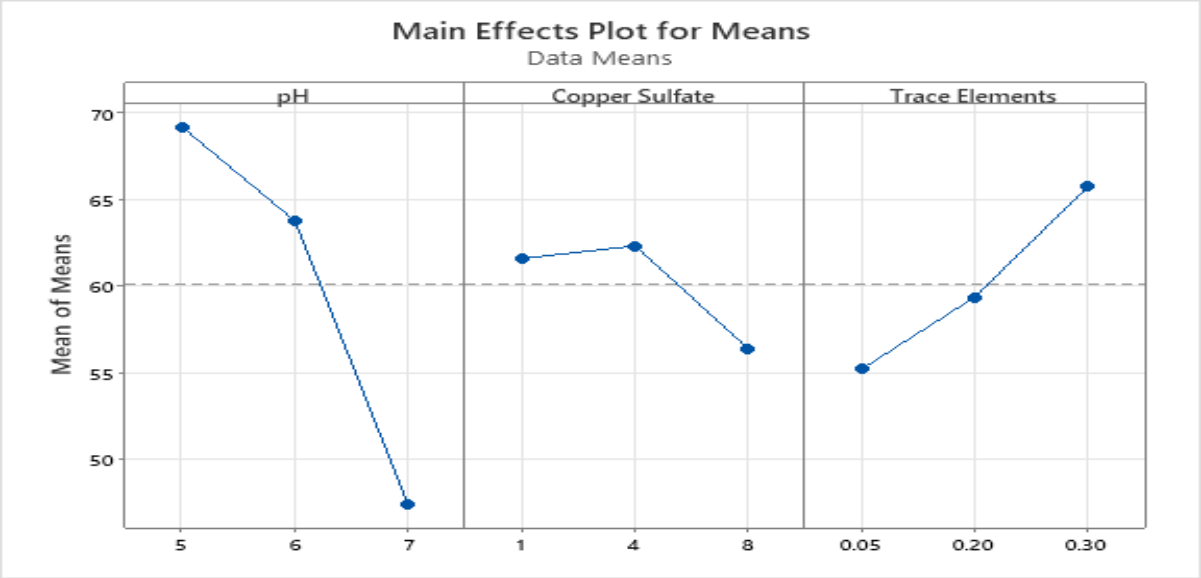


Figure 3.2. Main effects plot showing the effects of pH, $CuSO_4$ and Trace Elements on lignin degradation efficiency.

Table 3.1 -Assignment of variables and parameter levels using L9 design.

S. No.	pH	Copper Sulphate (mg L^{-1})	Trace Elements (ml L^{-1})	Lignin Degradation Efficiency (%)	Polyphenol Oxidase Activity (U L^{-1})	Versatile Peroxidase Activity (U L^{-1})
1	5	1	0.05	67.89	10.00	4.20
2	5	4	0.20	68.27	5.50	5.10
3	5	8	0.30	71.50	3.50	7.50
4	6	1	0.20	64.85	3.00	5.50
5	6	4	0.30	73.73	2.89	4.96
6	6	8	0.05	52.74	2.27	3.05
7	7	1	0.30	52.08	2.00	3.00
8	7	4	0.05	45.00	1.57	2.50
9	7	8	0.20	47.75	0.67	1.80

Table 3.2- Two-way Analysis of Variance (ANOVA) for lignin degradation efficiency (%) ($\alpha = 0.05$).

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value	Delta	Rank
Regression	3	922.47	88.68%	922.47	307.49	13.05	0.008		
pH	1	716.79	68.91%	716.79	716.79	30.43	0.003	21.86	1
Copper Sulphate	1	45.23	4.35%	45.23	45.23	1.92	0.224	5.92	3
Trace Elements	1	160.45	15.42%	160.45	160.45	6.81	0.048	10.56	2
Error	5	117.77	11.32%	117.77	23.55				
Total	8	1040.24	100.00%						

were constructed (Figure 3.3). At pH 5, varying the $CuSO_4$ and trace element levels did not affect the lignin degradation significantly. However, at higher pH levels, particularly at pH 7, increasing the $CuSO_4$ concentration decreased the lignin degradation. This is further discussed in the following sections on enzyme activity. Increasing trace element levels at pH 6 and pH 7 increased the lignin degradation efficiencies, although this effect was not significant at pH 5. At the highest level of trace elements (0.3 mL L^{-1}), increasing the $CuSO_4$ increased the lignin degradation levels. Although the lignin degradation levels reported here are comparable to those seen in the literature, it should be noted that the present study was conducted at significantly higher lignin concentrations, demonstrating a much more efficient process. For instance, most studies report initial lignin concentrations ranging from 0.05 $g.L^{-1}$ to 0.1 $g.L^{-1}$ (Vashi, Iorhemen and Tay, 2018), whilst the present study was conducted at 17.5 $g.L^{-1}$ of lignin to reflect the concentrations found in effluents from the pulping and bleaching processes. This demonstrates the ability of the biofilms to tolerate and treat high levels of lignin in wastewater.

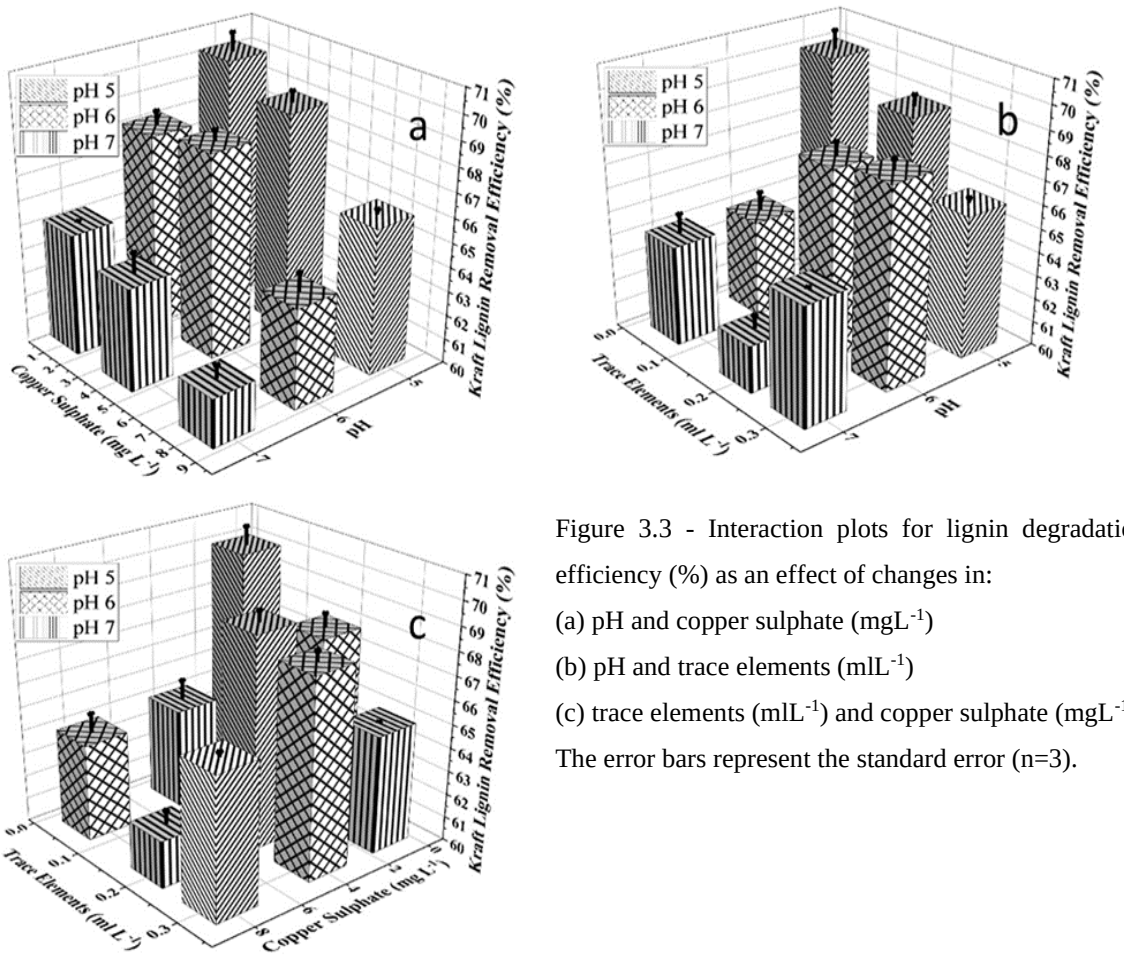


Figure 3.3 - Interaction plots for lignin degradation efficiency (%) as an effect of changes in:
(a) pH and copper sulphate (mgL^{-1})
(b) pH and trace elements (mlL^{-1})
(c) trace elements (mlL^{-1}) and copper sulphate (mgL^{-1})
The error bars represent the standard error (n=3).

3.3.2 Activities of Ligninolytic Enzymes

Cultures in nutrient-limiting conditions transition to a secondary metabolic state during which ligninolytic enzymes are known to be produced (Baldrian, 2006). Enzyme activity was noted for PPO and VPO under all conditions described in the following sections. Enhanced enzyme activity in acidic cultures was noted from day 3 onwards, once the fully formed biofilms were seen on the surface of the wastewater. 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 act as inhibitors of laccase as shown in previous studies (Pamidipati and Ahmed, 2020).

3.3.2.1 Polyphenol Oxidase Activity

The highest polyphenol oxidase (PPO) activity was obtained at the lowest pH (5) and lowest $CuSO_4$ (1 mg L^{-1}) level (Figure 3.4 a, Table 3.1). The mechanism and action of the fungal PPO are dependent on copper which is essential for PPO activity as the removal of copper from the enzyme results in inactive forms which can be restored 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 1000 µM of $CuSO_4$ strongly induced the production of laccase but PPO in *Pleurotus ostreatus* is induced at much lower levels (150 µM) of $CuSO_4$ (Palmieri *et al.,* 2000; Levin, Forchiassin and Ramos, 2002). However, higher levels of copper have also been reported to inhibit fungal growth and PPO activity in *Trametes trogii* (Levin, Forchiassin and Ramos, 2002). In this study, an increase in $CuSO_4$ concentration resulted in lower PPO activity in *N. discreta* biofilms, indicating an inhibitory effect of copper ions on PPO at high concentrations. This explains the effect of $CuSO_4$ on lignin degradation efficiency as discussed in the previous section.

An increase in pH caused a significant and dramatic decrease in PPO activity. PPO is a growth-associated enzyme with an optimum pH that lies in the acidic range, and most fungi prefer acidic conditions. Hence, it can be concluded that these conditions enabled enhanced PPO activity in acidic cultures (Palmieri *et al.,* 1993). As trace elements did not have a significant effect on PPO activity, the interaction between pH and trace elements (Figure 3.4 b) was strongly influenced by pH. As seen in Figure 3.4 c, the highest PPO activity was seen at the lowest levels of $CuSO_4$ and trace elements.

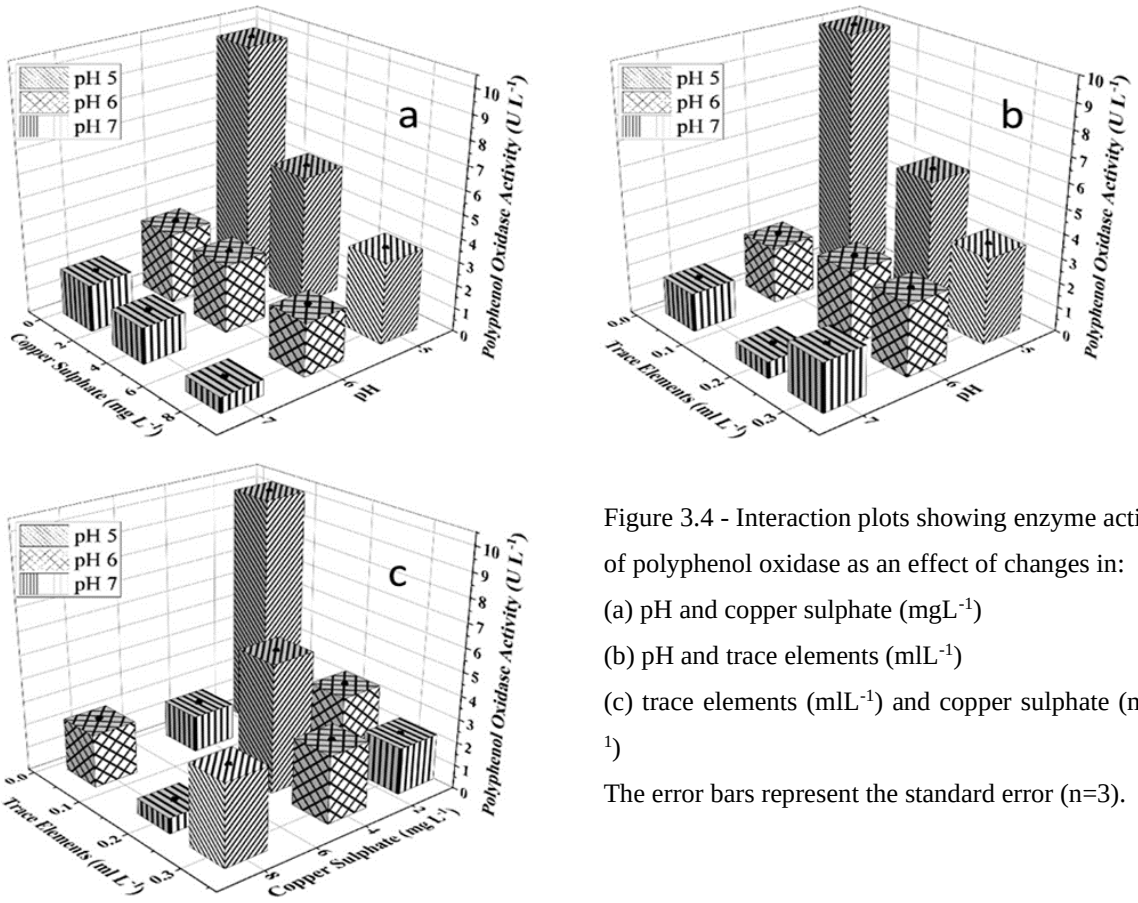


Figure 3.4 - Interaction plots showing enzyme activity of polyphenol oxidase as an effect of changes in:
(a) pH and copper sulphate (mgL^{-1})
(b) pH and trace elements (mlL^{-1})
(c) trace elements (mlL^{-1}) and copper sulphate (mgL^{-1})
The error bars represent the standard error (n=3).

ANOVA analysis for PPO activity is presented in Table 3.3. The initial pH and addition of $CuSO_4$ had a significant effect on lignin degradation (p-value = 0.048); however, trace elements had no significant effect (p-value < 0.05). Additionally, ANOVA was used to find the percentage contribution of factors towards PPO activity as a response, which was the maximum for pH (58.23%), followed by $CuSO_4$ (19.14%) and trace elements contributing the least (8.57%). As in the case of lignin degradation, pH was concluded to be the most important factor for PPO activity followed by copper sulphate. Trace elements had the lowest rank as there was no significant difference noted between the PPO activity at the three levels across the conditions tested.

Table 3.3- Two-way Analysis of Variance (ANOVA) for polyphenol oxidase activity (α = 0.05)

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value	Delta	Rank
Regression	3	53.635	85.94%	53.635	17.878	10.19	0.014		
pH	1	36.342	58.23%	36.342	36.342	20.71	0.006	4.922	1
Copper Sulphate	1	11.944	19.14%	11.944	11.944	6.81	0.048	2.856	2
Trace Elements	1	5.349	8.57%	5.349	5.349	3.05	0.141	1.814	3
Error	5	8.772	14.06%	8.772	1.754				
Total	8	62.407	100.00%						

3.3.2.2 Versatile Peroxidase Activity

Versatile peroxidases (VPO, E.C.1.11.1.16) have been reported to be found in *Pleurotus Sp.*, *Bjerkendera sp*. and *Physisporinus sp*. and a few other genera (Knop, Yarden and Hadar, 2015). This is the first time VPO (Luke and Burton, 2001) is reported in *Neurospora sp.* which has more commonly been reported to produce laccase and PPO (Luke and Burton, 2001). There is also limited evidence of VPO production from biofilm structures in the literature (Sridhar, 2016). In the present study, VPO activity was significantly affected by pH (Figure 3.5, Table 3.4). At a pH of 5, increasing $CuSO_4$ and trace elements led to increasing VPO activity. This is the opposite of the trend observed for PPO activity and shows that the opposing effects cancelled each other out, resulting in the overall lignin degradation remaining nearly the same under all levels of $CuSO_4$ at pH 5. At higher pH conditions, the opposite trend was observed with increasing $CuSO_4$ concentrations causing a decrease in VPO activity, although at pH 7 the VPO activity remained low irrespective of $CuSO_4$ concentrations.

ANOVA analysis for VPO activity (Table 3.4) shows that pH was the most significant factor for VPO activity (contribution - 59.2%, p-value = 0.012). The VPO activity as a response is represented as a delta which is the difference between the VPO activity at the highest and lowest level. Similar to PPO activity, pH ranks the highest in all three factors. However, unlike PPO, a variation in $CuSO_4$ concentration did not have a significant effect (p-value < 0.05) on VPO activity.

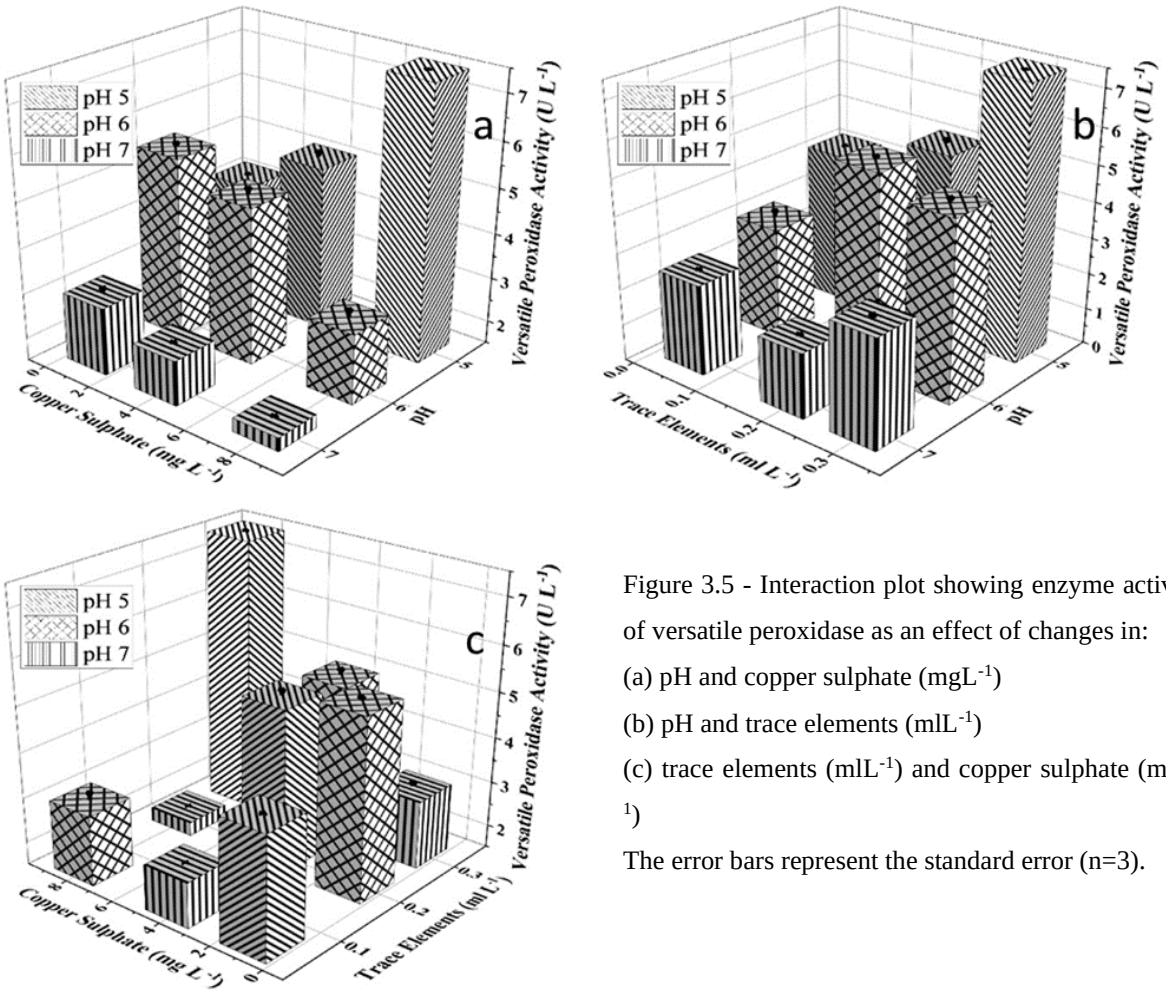


Figure 3.5 - Interaction plot showing enzyme activity of versatile peroxidase as an effect of changes in:
(a) pH and copper sulphate (mgL^{-1})
(b) pH and trace elements (mlL^{-1})
(c) trace elements (mlL^{-1}) and copper sulphate (mgL^{-1})
The error bars represent the standard error (n=3).

Table 3.4- Two-way Analysis of Variance (ANOVA) for versatile peroxidase activity ($\alpha = 0.05$)

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value	Delta	Rank
Regression	3	20.3736	80.29%	20.3736	6.7912	6.79	0.033		
pH	1	15.0417	59.27%	15.0417	15.0417	15.03	0.012	3.167	1
Copper Sulphate	1	0.0207	0.08%	0.0207	0.0207	0.02	0.891	0.117	3
Trace Elements	1	5.3113	20.93%	5.3113	5.3113	5.31	0.069	1.903	2
Error	5	5.0025	19.71%	5.0025	1.0005				
Total	8	25.3761	100.00%						

3.3.3 Biofilm Growth, Water Retention Value and Composition

Biofilms were observed on the air-liquid interface of all reactors about 24 hours after inoculation, after which their thickness increased each day with the formation of new layers on the surface. A strong positive correlation between lignin removal efficiency and the water

retention value (WRV) of the biofilms was obtained using a 2-tailed test of significance with a Pearson's correlation coefficient (R) of 0.854 and an associated p-value of 0.003. This indicates that the properties of biofilms are closely related to their ability to degrade lignin. A higher WRV indicates a higher water holding capacity and can facilitate mass transfer, thereby influencing the lignin degradation efficiencies.

Biofilm wet and dry weights and water retention values, as factors of pH and $CuSO_4$, are shown in Figure 3.6 a. Compared to $CuSO_4$, pH had a more significant effect on biofilm thickness, which was visibly observed to be higher at pH 5 across all conditions. Biofilm wet weights and water retention values decreased with increasing pH across all levels of $CuSO_4$. However, the normalised dry weight increased with an increase in pH which is likely due to the greater availability of lignin for cell growth, due to its higher solubility at pH 7.

The chemical analysis of EPS extracted from the biofilms shows higher polysaccharide and protein concentrations in biofilms grown in more acidic conditions (Figure 3.6 b). As polysaccharides are hydrophilic in nature, the biofilms grown in acidic conditions absorbed more water (Ahmed, Narayanan and Veni, 2020), resulting in better lignin degradation. Copper has been known to influence biofilm formation in both bacterial (Vargas-Straube *et al.,* 2020) and fungal (Gomes, Simões and Simões, 2020) biofilms with increased copper levels inhibiting EPS production. In the present study, increasing levels of $CuSO_4$ resulted in decreasing polysaccharide and protein content, particularly at the lower pH levels (Figure 3.6 b), indicating that copper ions affected biofilm growth as well as EPS composition.

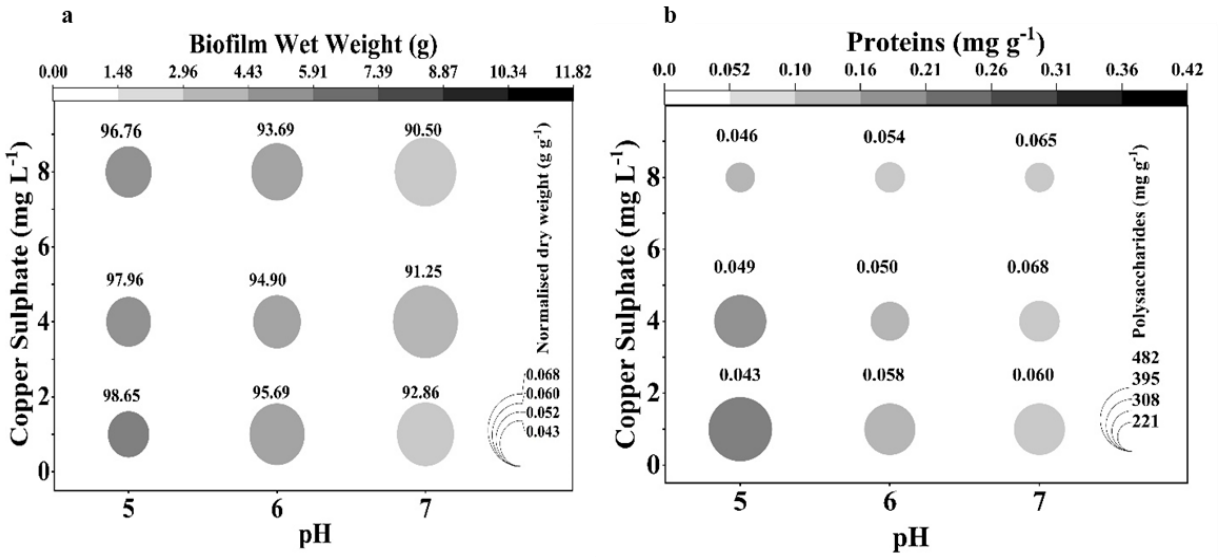


Figure 3.6- Bubble plot comparing (a) biofilm wet weight (colour intensity; g), dry biomass (bubble size; gg^{-1}) and water retention value (label; %) (b) biofilm normalised dry weight (label, gg^{-1}), polysaccharides (bubble size, mgg^{-1}) and proteins (colour intensity, mgg^{-1}).

3.3.4 Biofilm Microstructure Using Scanning Electron Microscopy

The scanning electron microscopy (SEM) images (Figure 3.7 a, b, c) of the harvested biofilms were analysed to measure the filament length, filament diameter and area of the pores using the Image J application in order to establish the relationship between microstructural features of the biofilms and lignin removal. Culture pH is known to affect fungal morphology as the increase in pH results in a decrease in hyphal length and an increase in hyphal thickness (Papagianni, 2004). The biofilms grown at pH 5 had longer and thinner hyphae (filaments) compared to those grown under neutral conditions, which had a more globular appearance (Figure 3.7 a, c). As the solubility of lignin is lower in acidic conditions (Norgren and Edlund, 2014), lower pH of the wastewater leads to lower availability of lignin to the cells, causing them to grow longer, seeking out nutrients as seen in previous studies (Ahmed, Narayanan and Veni, 2020). The lignin removal efficiency and pore area were correlated using a 2-tailed test of significance that resulted in a Pearson's

correlation coefficient of 0.7807 and p-value of 0.013 showing a significant correlation between lignin removal and total pore area of biofilms.

Additionally, the total pore area decreased with an increase in pH (Figure 3.7 d). These results agree with the inverse correlation between filament diameter and total pore area in previous studies (Ahmed, Narayanan and Veni, 2020). The pore area reported for biofilms grown in neutral pH conditions was much lower resulting in thinner and less water-absorbing biofilms. Higher biofilm porosity is desirable in biofilms as it allows more wastewater to flow through the water channels in biofilms, allowing exposure to more filament surface area.

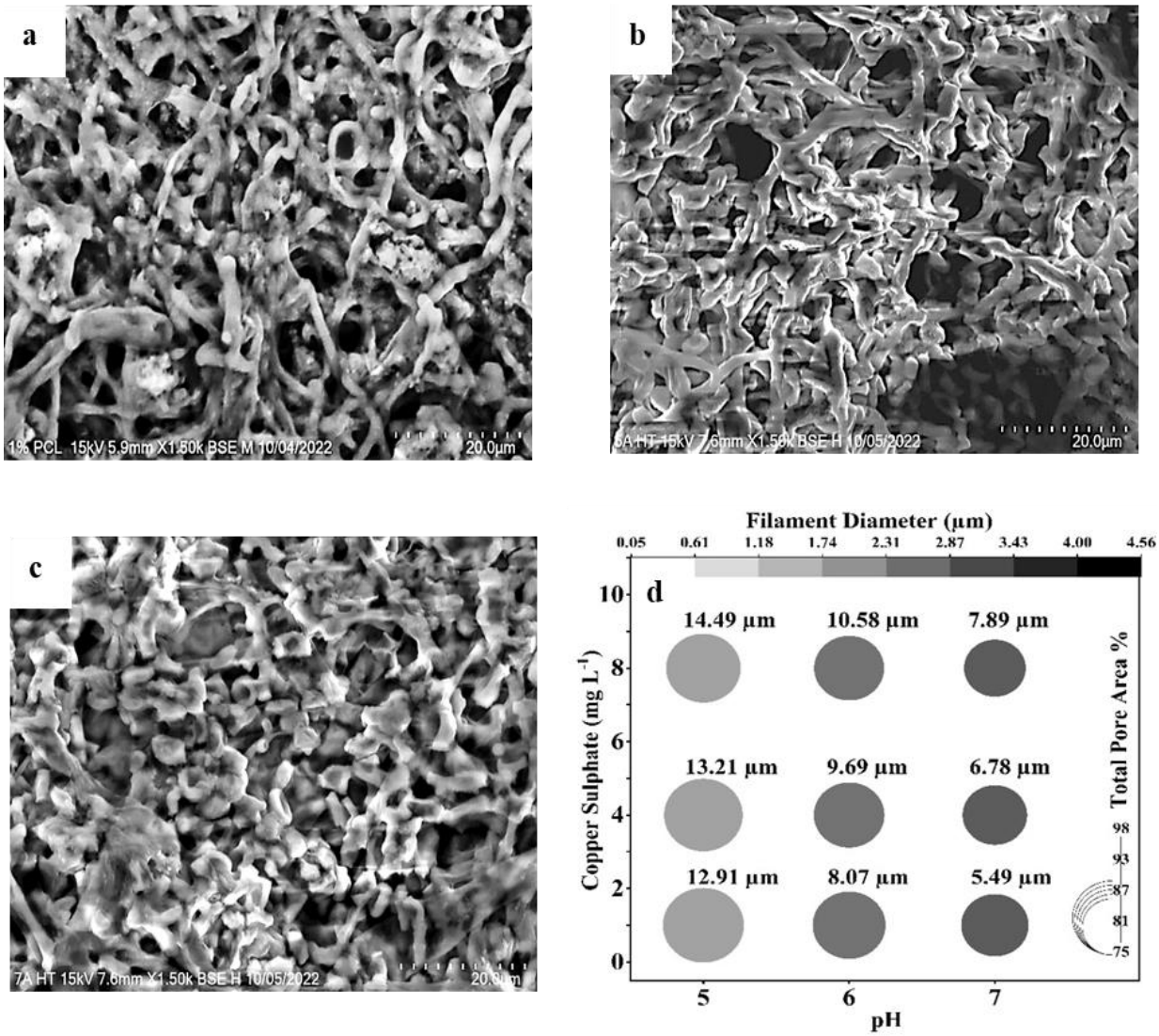


Figure 3.7 - Representative Scanning Electron Microscopy images of biofilms grown at (a) pH 5, (b) pH 6, (c) pH 7, (d) Comparison of biofilm filament length (labels; µm), thickness (colour; µm), and total pore area (size; pixels). SEM accelerating voltage 15kV, magnification-X 1.50 k, Backscattered electron (BSE) mode.

3.4 Conclusion

The key process parameters and their optimal values for lignin degradation in high-strength wastewater were identified using the Taguchi DoE approach. Wastewater pH was the most significant factor affecting the lignin degradation efficiency of *N. discreta* biofilms as well as enzyme activities. At pH 5, the other factors did not significantly influence the overall lignin degradation. Interestingly, at this pH, increasing the $CuSO_4$ concentration increased the versatile peroxidase activity but decreased the polyphenol oxidase activity resulting in the overall lignin degradation remaining nearly the same irrespective of $CuSO_4$. This shows that the overall lignin degradation is a factor of both enzyme activities and that the factors affect the enzyme activities in different ways. Additionally, this study showcases how the efficiency of lignin degradation in biofilms is correlated with various biofilm properties, such as microstructure and biochemical composition. This work proposes a novel biofilm-based process for the treatment of wastewater containing relatively high lignin concentrations .

---

4. Addition of Lignin-Degradation Intermediates for Enhanced Lignin and COD Removal from Industrial Wastewater in a Biofilm-Based Process

4.1 Introduction

The aromatic structure of lignin can be oxidised only by a small group of peroxidases secreted by ligninolytic fungi, which can cleave the more abundant ether bonds and C-C bonds (Pamidipati and Ahmed, 2017). The degradation of lignin by fungi depends largely on ligninolytic enzymes, which act as biocatalysts for sustainable mitigation of the polymer (Singh *et al.,* 2021). Lignin degraders, such as white-rot fungi, mineralise lignin for their growth and produce enzymes as a secondary metabolite along with other metabolites during the idiophase of the growth (Sigoillot *et al.,* 2012; Asina *et al.,* 2016). The process, however, suffers from slow degradation rates due to various reasons depending on multiple factors. In one study, lignin degradation slowed as white-rot fungi treatment progressed due to enzyme production instability in biofilms (Singh and Chen, 2008). Another study found a correlation between enzyme activity and lignin loss during the fermentation process by white rot fungi (Gupte, Gupte and Patel, 2007). In the same study, the highest lignin removal efficiency was observed at the beginning of the process when the laccase activity was increased. However, lignin removal efficiency was also reduced as the enzyme activity decreased. The low rate of lignin degradation is due to the difficulty in maintaining the optimum catalytic activity of an enzyme in controlled conditions to treat large volumes of wastewater is a difficult task (Asina *et al.,* 2017). To develop an efficacious lignin degradation process, it is essential to find ways to increase enzyme activity in fungal treatment processes (Vrsanska *et al.,* 2015).

Enzyme activity levels can vary due to different reasons. One of the reasons that may affect enzyme activity is the variation in enzyme expression levels, which is dependent on the

expression of specific genes (Knop, Yarden and Hadar, 2015). Enzymes are affected by their environment, and certain molecules can act as inhibitors, leading to enzyme inactivation (Wu, Xiao and Yu, 2005). Conversely, specific aromatic molecules can act as inducers, prompting organisms to synthesise the inducible ligninolytic enzymes (Suryadi *et al.,* 2022). The inducer is normally a substrate of the enzyme, and the level of enzyme induction depends on the presence of these compounds (Raghukumar et al., 2008). Enzyme regulation is a critical parameter for optimising the process for efficient and rapid lignin degradation (Asina *et al.,* 2017).

The addition of certain compounds in the fungal treatment process has proven to induce the synthesis of ligninolytic enzymes in Ascomycetes and Basidiomycetes. The inducers can be synthetic in nature, such as 2,5-xylidine, guaiacol, ferulic acid, 2,6- dimethoxyphenol (Vrsanska *et al.,* 2015). However, these compounds are expensive and add cost to the process. On the other hand, natural inducers, such as wood, straw, fruit, etc., are preferred over synthetic compounds (Singh Arora and Kumar Sharma, 2010). These inducers act as substrates for the given enzyme and result in increased enzyme activity (Vrsanska *et al.,* 2015).

Lignin and its model compounds have been found to increase the activity of ligninolytic enzymes, such as peroxidases and laccases, in extracellular fluid cultures (Cancel et al., 1993; Viswanath et al., 2014). The lignin polymer, being bulky in structure, cannot enter the fungal cells. Comparatively, lignin-degradation products are phenolic fragments of the polymer and are hydrophilic so dissolve readily in water (Fabbri *et al.,* 2023). Consequently, it is reported in literature that the oligo-monomers or lignin degradation intermediates, which have small molecular weight, trigger the enzyme induction (Znameroski *et al.,* 2014).

The water-soluble compounds help in biological treatment as the fungal cells can readily metabolise them using them as substrates (Dhagat and Jujjavarapu, 2022). A variety of water-soluble aromatic-lignin degraded products have been reported to induce extracellular secretion of peroxidase enzymes and further enhanced degradation of lignin by *P. chrysosporium* (Eriksson *et al.,* 1990). Compounds derived from lignocellulosic substrates were reported to induce the production of ligninolytic peroxidases (Shanthi Kumari et al., 2019). Lignin derivatives and intermediates of lignin degradation, such as chlorogenic acid, veratryl alcohol, vanillic acid, veratraldehyde and veratric acid, are reported to induce and enhance the production of ligninolytic activity (Cho, N. et al., 2006).

There is contradictory evidence in the literature for the function of lignin-derived compounds to act as inducers for the enhanced production of peroxidases in lignin-degrading fungi (Pamidipati and Ahmed, 2017; Cancel, Orth and Tien, 1993; Keyser, Kirk and Zeikus, 1978). In one of the studies using *Neurospora discreta* biofilms, the in-process lignin degradation intermediates formed by fungal treatment of lignocellulosic biomass were reported as a potential laccase inhibitor (Pamidipati and Ahmed, 2020). In the previous study (Chapter 3), no laccase activity was noted, and the other enzymes, polyphenol oxidase (PPO) and versatile peroxidase (VPO), showed increased activity as the process progressed. This could be due to the formation of low-molecular-weight lignin degradation intermediates, which selectively induce (VPO and PPO) or inhibit other ligninolytic enzymes such as laccase.

The most suitable inducers for lignin degradation enzymes expressed by filamentous fungi are naturally occurring plant materials such as cellulose, hemicellulose, and lignin (Znameroski *et al.,* 2012). However, natural lignin is insoluble in water and is not ideal for aqueous processes (Wang *et al.,* 2022). Hence, it was proposed that the lignin degradation intermediates from different time points from inoculation can be used as additives in the

subsequent cultures. Several studies have reported using chemicals added exogenously to enhance ligninolytic enzyme activity. However, in-process lignin degradation intermediates have never been explored as additives for enhanced ligninolytic enzyme activity. As ligninolytic enzymes are produced extracellularly, adding water-soluble in-process lignin degradation products could increase the enzyme activity, resulting in a more efficient process.

It was hypothesised that adding naturally formed in-process lignin degradation products during the fungal wastewater treatment cultures will increase the ligninolytic enzyme activity, resulting in efficacious lignin degradation. These natural additives can act as simulators to enhance enzyme production, resulting in a sustainable and efficient biological treatment process (Singh and Chen, 2008). The previous chapter reported the optimum culture conditions for growing *N. discreta* biofilms and enhancing kraft lignin degradation. Although the *N. discreta* biofilm cultures did not produce any laccase, the peroxidases-polyphenol oxidase and versatile peroxidase activity were reported. In this chapter, we investigated the effect of feeding in-process lignin degradation intermediate products to the industrial wastewater culture medium. For the feeding purpose, the spent media containing lignin degradation intermediates from different time points of wastewater treatment by *N. discreta* biofilms was selected and fed into fresh cultures.

4.2 Materials and Methods

4.2.1 Wastewater Composition

The wastewater used in this study was provided by a paper and pulp-making company, Mondi Frantschach. The filtrate provided was from the bleaching plant using the kraft process during the pulp brightening period. The characteristics of the wastewater were supplied by the company. The lignin concentration in the wastewater was 1500 mg L^{-1}, as confirmed by the

UV-visible spectroscopy method, and the media was supplemented with 1000 mg L^{-1} sucrose. The wastewater was supplemented with components of Vogel's minimal media (recipe mentioned in Section 2.3) to support fungal growth and subsequent formation of biofilms. The wastewater (1 L) was supplemented with 2.5 g of trisodium citrate dihydrate, 5 g of potassium dihydrogen phosphate, 2 g ammonium nitrate, 0.2 g magnesium sulphate heptahydrate, 0.1 g calcium chloride dihydrate and 0.1 ml of trace element solution. All the compounds mentioned above were dissolved in the wastewater, which was autoclaved for sterilisation at 121^{o} C for 15 mins. When the solution cooled down, 1 ml of biotin was added before the media was inoculated. The solution for trace elements was made as described in section 2.3. The solution for biotin was made by adding 5 mg of biotin to 50 ml of water and was stored in cold conditions at 4^{o} C.

4.2.2 Inoculum

The inoculum was prepared following the methods mentioned in section 2.5. The *N. discreta* grew on potato dextrose agar plates (PDA) by streaking the cells and incubating the leaves for 3 days at 30^{o} C. The fungal spores and filaments scraped off the plates and suspended into synthetic media (30 ml) containing no sucrose. The suspension was filtered through a sterile muslin cloth to remove the filaments. The spore count was adjusted to 1 x 10^{7} spores per mL, and cultures were inoculated by adding 1 ml of the spore suspension.

4.2.3 Fermentation Setup

The experiments were carried out in 250 ml Erlenmeyer conical flasks, plugged with foam bungs, containing 100 ml. After inoculation, the flasks were incubated at 30^{o} C in static conditions for 19 days. Uninoculated flasks containing wastewater media were run in parallel

as a negative control, and another set of triplicate flasks inoculated with *N. discreta* spores, but unfed was run as a positive control.

4.2.4 Feeding

The experiment was conducted in two cycles, where the spent wastewater media from different time points of the first cycle of the experiment was fed into the reactors in the second cycle of experiment (Table 4.1). The wastewater composition in both experiment cycles remained the same. The spent wastewater media was collected by harvesting a flask each from days 7, 10, 13 and 15 from inoculation. The spent media was heat treated by autoclaving at 121^{o} C for 15 mins to denature the pre-existing enzymes in the media. The second cycle of reactors were set in triplicates, with 100 ml wastewater media. Each group of triplicate reactors was fed on day 6 with 15 ml of spent wastewater media collected from the flask harvested from the previous cycle. The reactors were sampled before and after feeding, followed by regular sampling till biofilms were harvested on day 19 from inoculation.

Table 4.1- Spent media collection in Cycle 1 of fungal wastewater treatment process to be fed in Cycle 2

Cycle 1 **Spent Media Collection**	**Cycle 2** **Reactor**
-	**1 (Unfed Control)**
Day 7	**2**
Day 10	**3**
Day 13	**4**
Day 15	**5**

4.2.5 Sample Preparation for UV-Based Lignin Estimation

The samples were treated according to the process mentioned in Section 2.7. The precipitation protocol by Laboratory Analytical Procedures was applied (Sluiter *et al.*, 2008). For the precipitation procedure, the samples were treated to remove biomass after raising the

pH to 10.5, followed by centrifuging at 5000 RCF for 30 mins. The supernatant was collected, and the biomass pellet was discarded. The samples (3ml) were hydrolysed by using 2M H_2SO_4 to drop the pH 1-2 for lignin to precipitate, followed by centrifugation at 5000 RCF for 30 mins. The supernatant was removed from the isolated lignin, also known as Klason's lignin, which was given two acid washes with acidified water. The precipitated lignin was dried in an oven at 30^o C till a constant weight was achieved. After the precipitation procedure, the dried lignin was redissolved in water at pH 10.5, and lignin was quantified spectrophotometrically at a characteristic 278 nm wavelength (Seesuriyachan *et al.,* 2015; Janshekar, Brown and Fiechter, 1981). The lignin content was measured and analysed before and after treatment of lignin-rich wastewater using HPLC to calculate the lignin removal efficiency (%) according to equation 4.1.

$$Lignin\ Removal\ Efficiency(\%) = \frac{Lignin\ conc.(start) - Lignin\ conc.(end)}{Lignin\ conc.(start)} \times 100 \qquad (4.1)$$

4.2.6 Lignin Estimation Using Reverse Phase Liquid Chromatography

The redissolved lignin samples prepared after precipitation, as mentioned in the section above, were subjected to RP-HPLC to estimate the lignin degradation and products formed using a BDS C-18 column from Thermo Scientific (5 μm particle size, 250 x 4.6 mm ID). A guard column (apHera™, 5 μm particle size, L × I.D. 1cm × 4.6 mm) was attached to the C18 column. A two-solvent system up of HPLC grade water (A) and 100% acetonitrile (B) was used with the gradient: 0 min - 0%, 5 min - 3%, 10 min - 6%, 15 min - 10% and 20 min – 20%,25 min - 40%; and 35 min - 80%. For comparison, the lignin standard was prepared by precipitating lignin from the wastewater and was used to plot a calibration curve ranging over the concentration of 1000 mgL^{-1} to 5000 mgL^{-1}. The compounds were detected at a 254 nm UV detector. The data from chromatograms was integrated using a Freestyle analyser.

4.2.7 Identification of Lignin Degradation Compounds

A polyphenolic acids and alcohol standard mixture kit from MetaSci (Catalog- MSIPAA050, Batch- ZAA) generated standard chromatograms for 50 lignin degradation products. The lignin degradation compounds were identified at 220 nm using a C18 column described above. The mobile phase was 0.1% formic acid in HPLC-grade water, and mobile phase B was 0.1% formic acid in acetonitrile. The two solvent gradient was established using the following percentage of solvent B as follows: 0 min - 5%, 2 min - 5%, 15 min - 12%, 35 min - 55%, 36 min - 100%, 38 mins -100%, 39 min - 5% and 40 min - 5% with a flow rate of 2 ml min^{-1}.

4.2.8 Chemical Oxygen Demand

The chemical oxygen demand (COD) was determined photometrically according to the method mentioned in section 2.10, using a Palintest COD 2000 test kit. The user instructions were followed as was provided. The samples were diluted 20 times, and 2 ml was added to the COD reagent tubes. The blank and the sample tubes were heated at 150^{o} C for 2 hours. The photometric readings for samples were taken and compared with the blank, and COD removal efficiency (%) was calculated using equation 4.2, given below.

$$COD\ Removal\ Efficiency\ (\%) = \frac{COD\ (initial) - COD(final)}{COD\ (initial)} \times 100 \qquad (4.2)$$

4.2.9 Water Retention Value and Dry Weight

To calculate the water retention value (WRV), a standard protocol from TAPPI UM 256 was used, as mentioned in section 2.1 (Cheng *et al.*, 2010b). The dry weight noted was subtracted from the wet weight to find a ratio with the wet weight. This gave the WRV, as a percentage, for every sample according to equation 4.3 given below. Normalised dry weight

was calculated by dividing the dry weight by the wet weight of the biofilm section. Biofilm biomass per unit volume is calculated by multiplying the normalised dry biomass with the growth area of the wet biofilm.

$$WRV\ (\%) = \frac{Weight(wet) - Weight(dry)}{Weight\ (wet)} \times 100 \qquad (4.3)$$

4.2.10 Enzymes Activity

The enzyme activity was determined for laccase, polyphenol oxidase and versatile peroxidase according to the protocol mentioned in section 2.13. Laccase activity was measured using 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) as substrate in citrate buffer (Bourbonnais and Paice, 1990; Baldrian and Šnajdr, 2006). Dark green cations are produced because of the oxidation of ABTS by laccases, and the spectral time scan is measured spectrophotometrically at 420 nm wavelength. The enzyme activity (1 Unit) is quantified using the extinction coefficient (ε420) 36000 M^{-1} cm^{-1} (Wang *et al.*, 2016; Saito *et al.*, 2003). The polyphenol oxidase activity was measured using a pyrocatechol solution (0.2 M) prepared in citrate buffer (0.05 M) at pH 5. One unit of enzyme was defined as the amount of enzyme that forms 1 mol of product per minute. The oxidation of reactive black dye (RB5) in sodium tartrate buffer 50 mM at pH 3 measured versatile peroxidase activity. One unit of enzyme is defined as the amount of enzyme that transforms 1mol of substrate consumed.

4.2.11 Extracellular Polymeric Substances Extraction

The extra cellular matrix (ECM) was extracted from the biofilms to estimate the carbohydrate and protein content. Pre-weighed biofilms were soaked in 5 ml of 8.5% sodium chloride and 0.22% formaldehyde for 2 hours at 4^{o} C. The biofilms were then centrifuged at

10,000 g for 10 mins. The EPS extracted was filtered using a 0.45 μm pore size syringe filter and was saved for further analysis (Liu and Fang, 2002; Ahmed, Narayanan and Veni, 2020).

4.2.12 Polysaccharides and Protein Estimation in ECM

Carbohydrates in ECM were quantified by using the dinitro salicylic acid (DNS) reagent method, as mentioned in section 2.13 (Gonçalves *et al.,* 2010). The polysaccharide concentrations were calculated using a glucose standard curve. For the preparation of the DNS reagent, dinitro salicylic acid (2.5 g, Acros Organic) was dissolved in 125 ml of deionised water and left at 80^{o} C. After cooling to room temperature, sodium hydroxide (50 ml, 2N) was added to the solution. A total volume of 250 ml was achieved by adding 75 g of sodium titrate tetrahydrate. The protein concentration was measured using a protein quantification kit (77371, Sigma Aldrich, UK) with standard reactive blue dye and albumin.

4.3 Results and Discussion

4.3.1 Lignin Estimation

4.3.1.1 UV-Vis Spectroscopy

The spent media from different time points was analysed to calculate the residual lignin concentrations. The UV-spectroscopy analysis of spent media from all the unfed and fed spent media showed that the cultures fed were more efficient in removal of lignin. The fed cultures showed better lignin degradation than the unfed cultures. The cultures fed with spent media from day 10 of fungal treatment showed a significant ($p < 0.05$) increase with 1.3-fold higher lignin removal efficiency than the unfed culture. Figure 4.1 a shows that lignin removal was better in cultures fed with spent media compared to unfed cultures. The unfed cultures showed an overall 56 % lignin removal. The reactors fed spent media containing lignin-derived products from days 7, 13 and 15 of fungal treatment resulted in lower lignin

degradation close to unfed cultures of 62.89 %, 63.55 % and 61 %, respectively. The results indicated that adding lignin degradation intermediate products improved lignin removal efficiency.

Figure 4.1 b shows a day-wise comparison between residual lignin concentrations in unfed and culture fed with day 10 spent media. The cultures fed with spent media from day 10 of fungal treatment showed a linear reduction in residual lignin. On the last day of treatment (Day 19), there was a significant difference between the residual lignin in the cultures that were unfed and fed ($p < 0.05$).

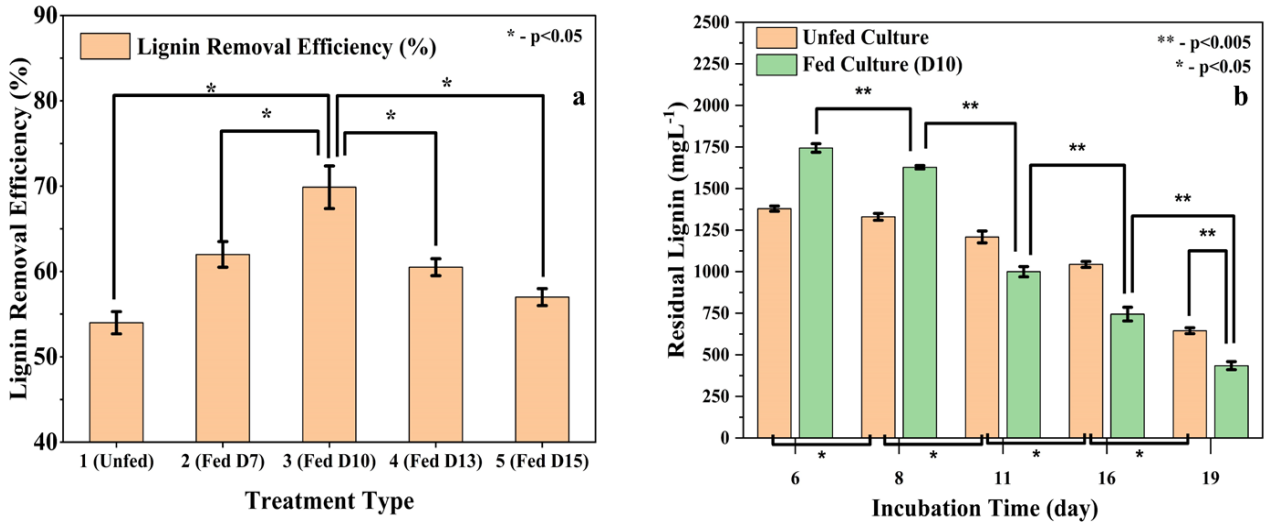


Figure 4.1 - (a) Comparison of lignin degradation efficiencies on day 19 of inoculation in unfed and culture fed spent media (b) Day-wise comparison of residual lignin concentration on days 6, 8, 11, 16, and 19 post-inoculation, in unfed and cultures fed with day 10 spent media. A statistically significant difference ($p<0.05$) between the residual lignin concentration in unfed and fed cultures was determined using one way ANOVA and tukey test is denoted by an asterisk (*).

4.3.1.2 Reverse-Phase Liquid Chromatography

Figure 4.2 shows the chromatogram profile of lignin precipitated from the untreated pulp and paper mill wastewater. The standard kraft lignin chromatogram profile exhibits a polar peak at RT 2.7 and a nonpolar peak at RT 16.5 +/- 0.3 minutes, as section 2.9.2 shows. On the other hand, the wastewater samples from the pulp and paper mill showed only one prominent

polar peak at RT 2.89 +/- 0.3 minutes. This is because the kraft process in every pulp and paper mill process varies, and the wastewater provided by the mill had lignin degradation phenolic polar products only.

The chromatogram peak area comparison showed the same trend in lignin removal as is reported by using the UV-spectrometry analysis. This provides evidence that feeding the cultures with spent media improved lignin removal efficiency. On comparison of the chromatogram profiles of samples drawn on the last day (day 19) of the treatment, it was confirmed that all the fed cultures showed increased lignin degradation in contrast to the cultures that were not fed. The cultures fed with day 10 spent media showed significantly high lignin degradation compared to the other fed and unfed cultures.

The chromatogram overlay in Figure 4.3 exhibits a complete chromatogram profile (a) and the enlarged polar peak area (b) of lignin content in untreated samples compared with the treated samples. Figure 4.4 shows the chromatogram overlay of control spent media compared to the unfed and culture fed with day 10 spent media. On comparing the manually integrated peak area, it was noted that the degradation in this particular fed culture was 70.47 %, and the degradation in the unfed culture was estimated to be 57%. The cultures fed spent media from day 7 showed the second-highest lignin degradation of 63%. The cultures fed spent media from the 13 and 15 showed lesser degradation of 60.23% and 59.40%.

The significant increase in lignin removal was due to the addition of lignin intermediates formed during the initial stages of fungal treatment. However, the cultures fed with spent media from the latter stages (days 13 and 15) did not show a significant difference, as the lignin degradation intermediate products responsible for the enzyme induction may have been consumed and absent in the spent media fed in those cultures.

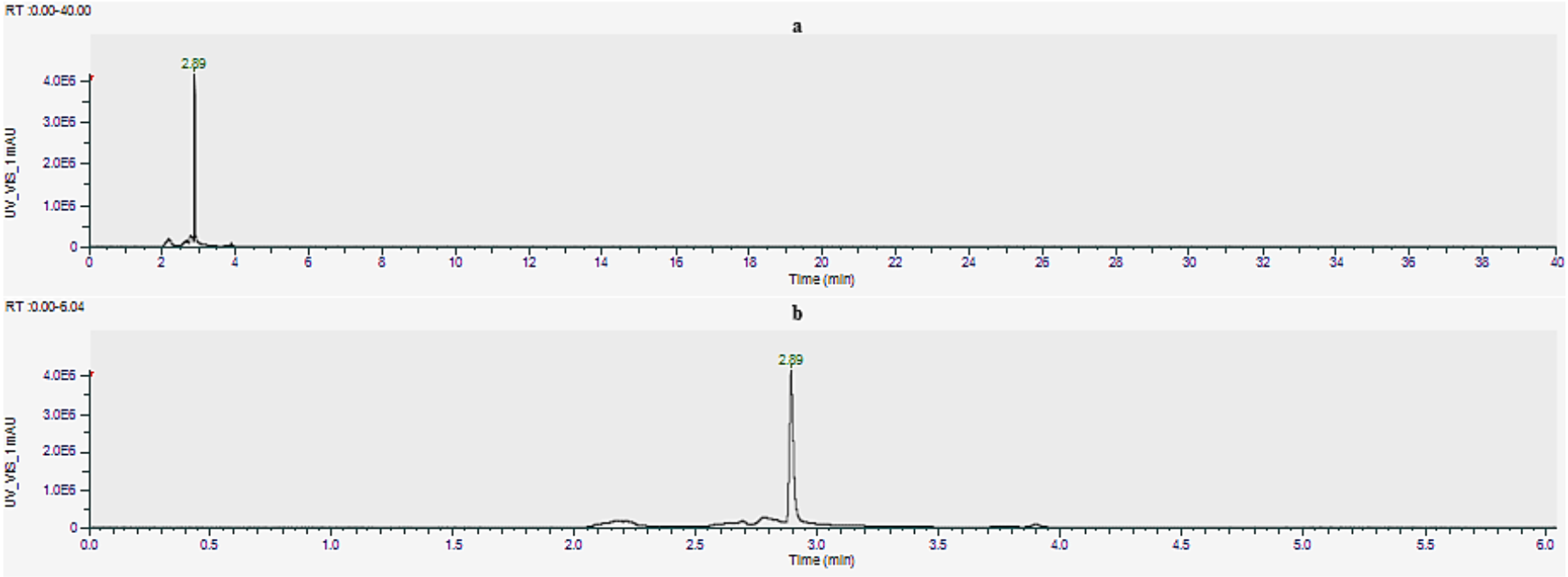


Figure 4.2 - Chromatograms profile of untreated wastewater sample showing (a) Complete profile with only a significant polar peak formed at 2.89 minutes (b) Enlarged polar peak area

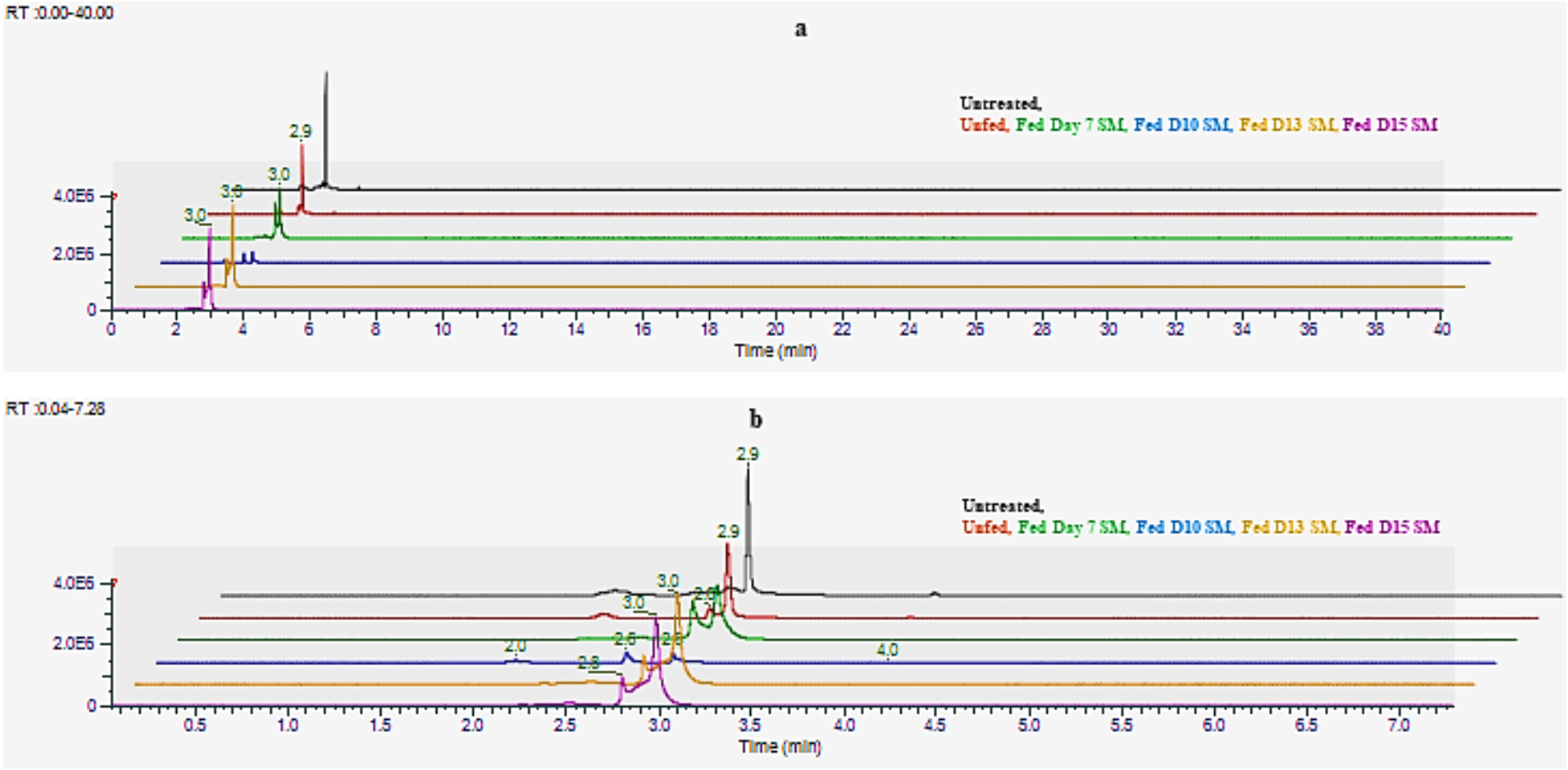


Figure 4.3 - Chromatograms overlay of unfed and fed cultures from day 19 of fungal treatment in comparison with the untreated samples. (a) complete profile representing the untreated sample (black line), unfed (red line) and cultures fed with spent media from day7 (green line), 10 (blue line). 13 (yellow line) and 15 (purple line) (b) enlarged polar peak area shown in Figure 4.3a.

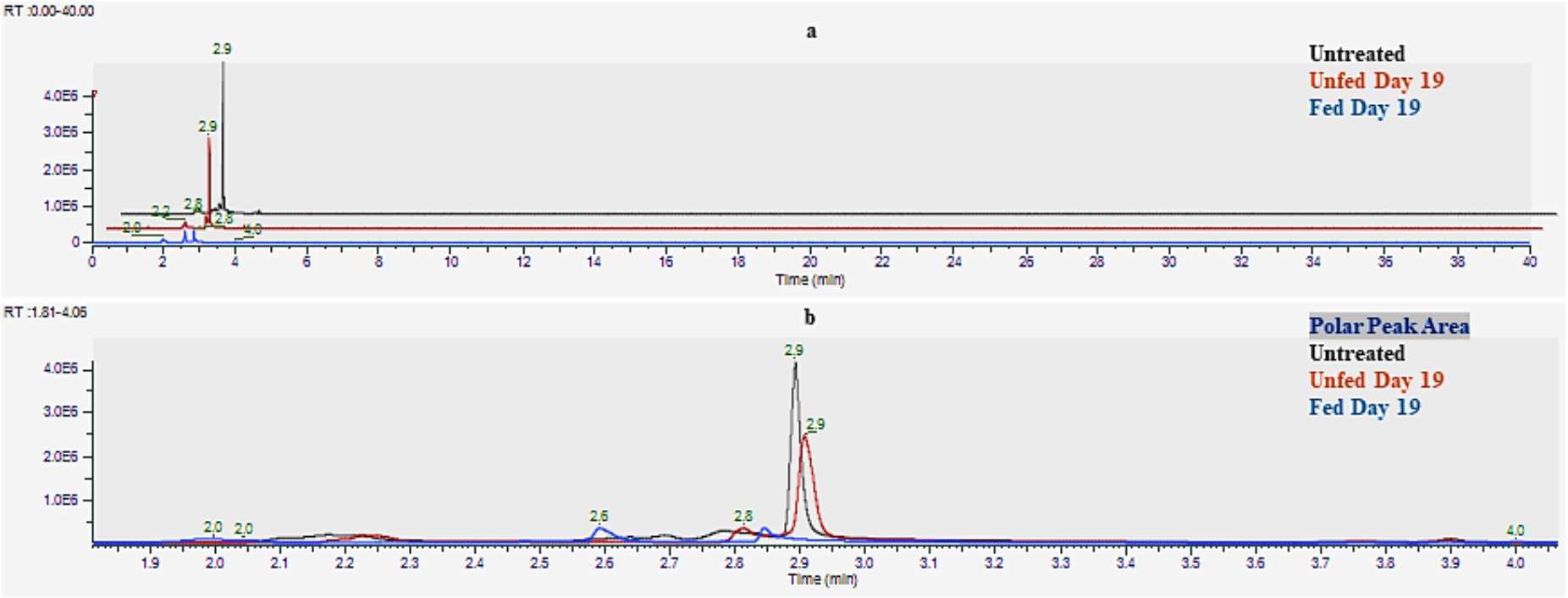


Figure 4.4 - Chromatograms overlay of wastewater media samples comparing lignin content in untreated samples with fungal treated cultures (a) chromatogram profile showing only a significant polar peak (b) enlarged polar peak area.

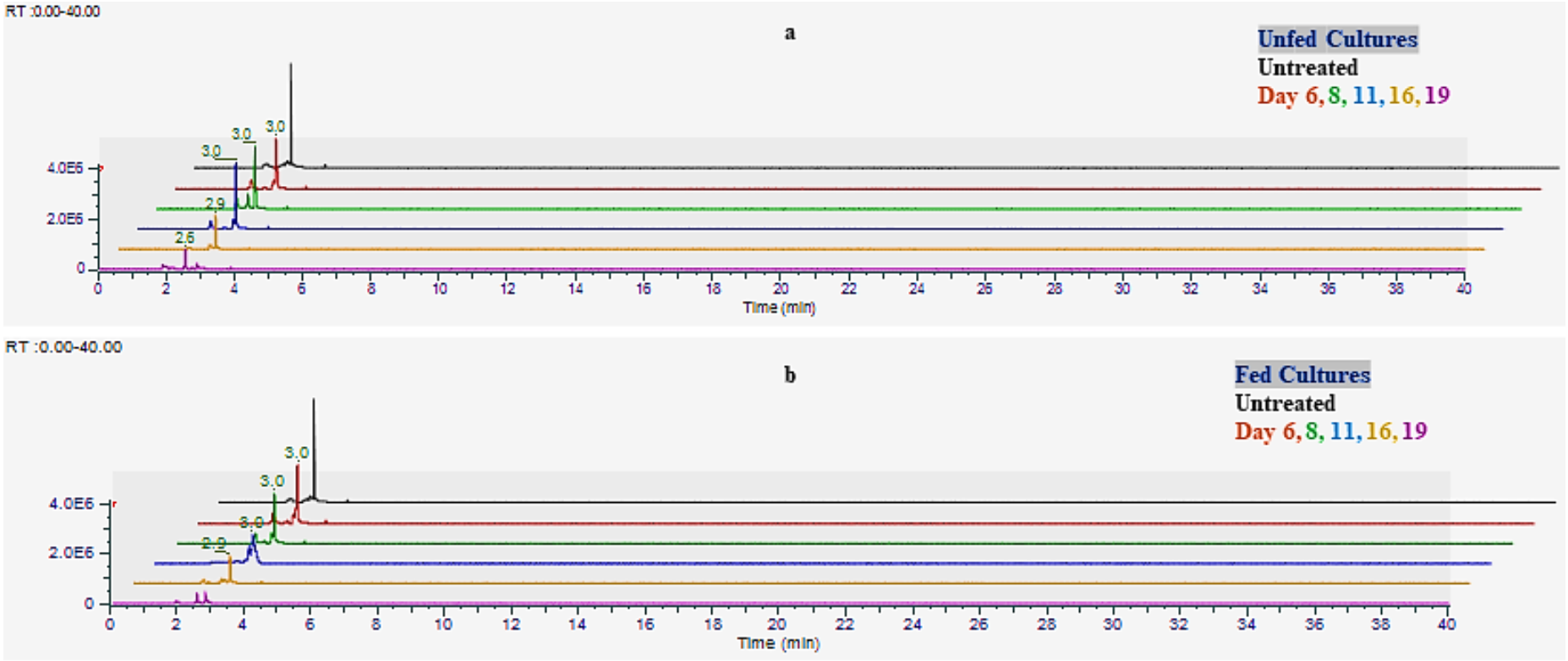


Figure 4.5 - Chromatograms overlay of wastewater media comparing day-wise residual lignin content in untreated samples with fungal treated cultures which were fed and not fed. The comparison shows residual lignin in spent media on day 6 (red line), 8 (green line), 11 (blue line), 16 (yellow line) and 19 (purple line) of fungal treatment. (a) unfed and fed cultures.

Figure 4.5 shows the chromatogram overlay of samples drawn on days 6, 8, 11, 16 and 19 from unfed (4.5 a) and fed (4.5 b) cultures, respectively. The unfed cultures (Figure 4.5 a) establish a non-linear trend with slow degradation till day 11, followed by linear degradation whereas fed cultures (Figure 4.5 b) show a linear trend in lignin removal untill day 19

4.3.2 Chemical Oxygen Demand Removal Efficiency

The removal of COD as a function of feed addition is shown in Figure 4.6. The fungal-treated cultures from the fed reactors showed higher COD removal than the unfed culture. Compared to the unfed cultures, a significantly high COD removal ($p < 0.05$) was noted for the culture fed with the spent media from day 10 of fungal treatment outperforming other fed and unfed culture efficiencies. A significant correlation was revealed between the lignin degradation and COD removal efficiency (R - 0.853, $p < 0.05$), except for reactor 5 cultures, where the COD removal efficiency was lower than the unfed cultures. This could be due to the addition of spent media from day 15 to rector 5 cultures, which were rich in organic matter caused by the decomposition of biofilms (Ntougias *et al.,* 2015). In all the other reactors, the addition of spent media was beneficial in improving the COD removal efficiency.

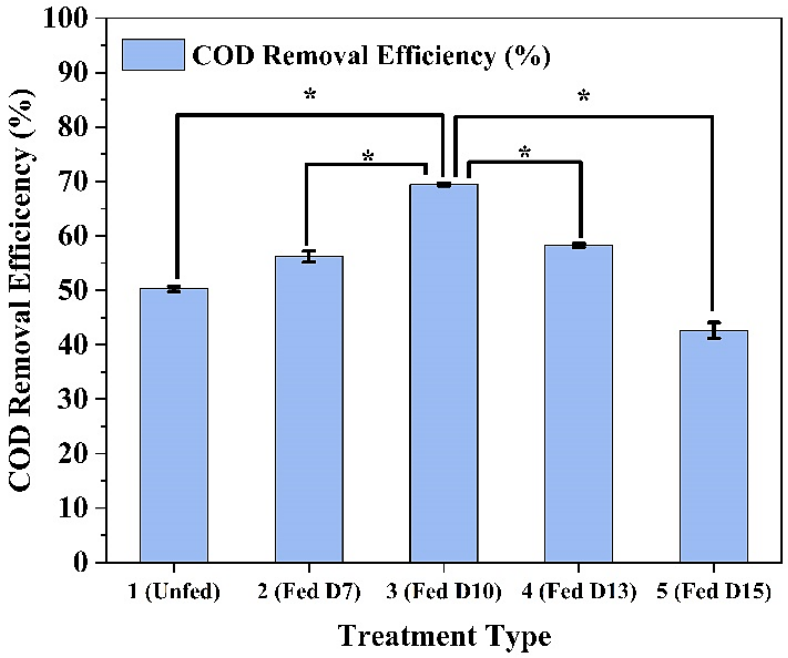


Figure 4.6 - Comparison of COD removal efficiencies in unfed and spent-media-fed cultures on day 19 of incubation. A statistically significant difference ($p<0.05$) between the residual lignin concentration in unfed and fed cultures was determined using one way ANOVA and tukey test is denoted by an asterisk (*).

4.3.3 Activity of Lignin-Degrading Enzymes

The enzyme system of *N. discreta* is dominated by polyphenol oxidase (PPO) and versatile peroxidase (VPO). Figures 4.7 a and b show the enzyme activity for unfed cultures compared to fed cultures. A significant positive correlation (R-0.84, $p < 0.05$) was observed between the PPO enzyme activity, lignin degradation, and COD efficiencies, indicating its significant involvement in lignin remediation. Higher PPO activity was observed in spent-media-fed cultures compared to unfed cultures. A 2.24-fold increase in PPO activity was noted in the culture fed with day 10 spent media compared to a 1.33-fold increase in unfed culture. Although there was an increase in PPO enzyme activity for all the cultures fed with spent media, only the cultures fed with spent media from the day 10 exhibited a significant increase in lignin degradation. The PPO activity was noted to show a 2.4-fold increase from day 6. In addition, these cultures also showed a 4.7-fold higher PPO activity than unfed cultures on the

last day of treatment. While all the other cultures that were fed showed an increased PPO activity, they did not exhibit a significant increase in lignin degradation.

Figure 4.7 b shows the comparison of the activity of the VPO enzyme in unfed and fed cultures. For the fed cultures, the VPO activity started to drop after day 6 after being fed. But showed an increase towards the end of the process on day 19. Compared to the unfed cultures, all the cultures fed with spent media showed more pronounced VPO activity. For the cultures fed, spent media from day 10 of fungal treatment, they also exhibited maximum degradation in addition to a 4.5-fold increase in VPO activity. The unfed cultures showed a 2-fold increase in VPO activity from the initial activity on day 6. In contrast, a more pronounced increase was noted in a 2.30-fold increase for culture showing significant lignin degradation. In general, laccase is a preferred ligninolytic enzyme for lignin biodegradation; its activity in white-rot fungi had previously been reported to drop too low to be detected during the biofilm treatment (Asif *et al.*, 2017). Similarly, no laccase activity was seen in the *N. discreta* cultures in any conditions. Similar reports were previously presented for other ascomycetes grown on lignin-rich wastewater for treatment (Barapatre and Jha, 2017).

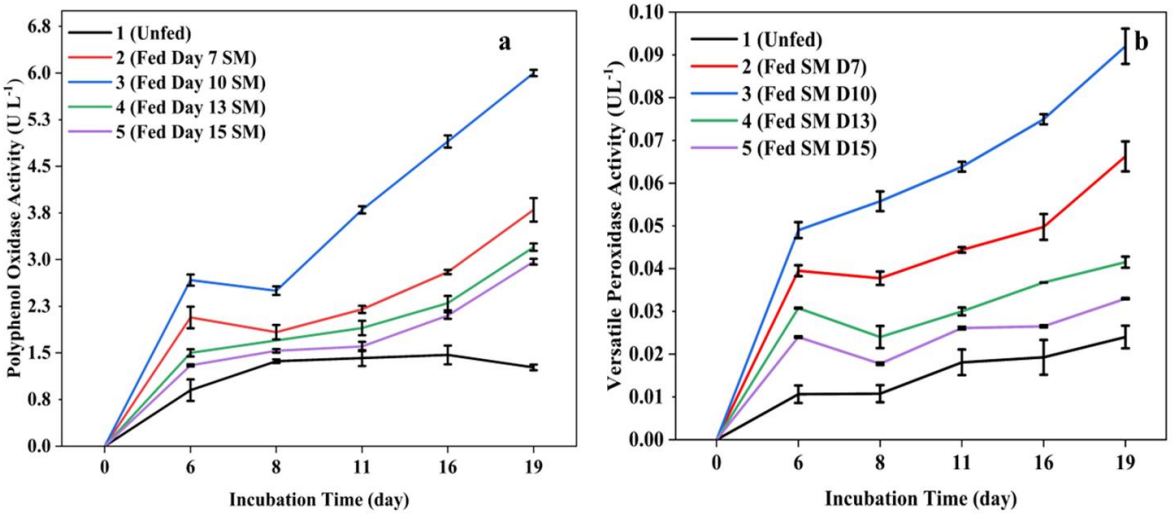


Figure 4.7- Enzyme activity in cultures fed with lignin degradation intermediates in comparison with unfed culture (a) Polyphenol oxidase (b) Versatile peroxidase.

4.3.4 Identification of Compounds Fed for Enhanced Degradation

The lignin degradation was the most efficient for cultures fed with spent media from day 10 of fungal treatment. As stated previously, there was an increase in enzyme activity for all the cultures fed with spent media; it was only in this particular set of cultures, fed with spent media from day 10, that the lignin and COD removal efficiencies were significantly higher compared to unfed and other fed cultures. It was necessary to identify the lignin degradation intermediates present in the spent media to determine the difference in the spent media fed into the cultures. The chromatogram profile of each fed spent media was compared to investigate the differences among the profiles. Figure 4.8 shows the chromatogram overlay of all the spent media from different time points. It was found that the spent media from day 10 (red line) had 4 distinct peaks, which were not found in the spent media from other time points. In day 10 spent media, the new peaks were seen at RT 32.43, 32.81, 33.81 and 34.41 minutes. The peak at RT 33.44 minutes was common in spent media from all time points, including the untreated samples. The chromatograms of day 10 spent media revealed new lignin degradation intermediate compounds formed due to fungal treatment, likely contributing to enhanced lignin degradation.

Figure 4.9 a shows the chromatogram profile of the untreated and spent media from culture fed with day 10 spent media . The chromatogram profile of the phenolic standard mixtures was used to identify lignin degradation intermediate products seen as new peaks in the spent media. Figure 4.9 b shows the chromatogram profile obtained from the spent media from day 10 of treatment, overlaying in the chromatograms obtained from the standard mixture of phenolic acids and alcohol standard mixture showing common peaks for sinapic acid at 32.43 min, Resveratrol at 32.81 min and trans-cinammic acid at 33.41 minutes RT. The peaks formed at 33.81 and 34.38 minutes were not identified. The phenolic acid and alcohol

standard mixture was used as polyphenols are highly sought-after plant-derived compounds representing the largest chemical groups and are also known for their antioxidant properties (Pandey and Rizvi, 2009). The polyphenols are classified as phenolic acids, flavonoids, stilbenes and lignans (Nićiforović and Abramovič, 2014). The plant material used for pulp and paper production is treated to extract cellulose, and the effluent generated is rich in secondary metabolites produced as polyphenols in plant biomass (Waye *et al.,* 2014). The release of untreated wastewater rich in lignin also contains plant-derived metabolites, which are reported to harm aquatic life as they disrupt the reproductive and metabolic processes (Waye *et al.,* 2014).

Sinapic acid is 3,5-Dimethoxy-4-hydroxycinnamic acid (structure shown in Figure 4.10 a), also known as hydroxycinnamic acid, is found in guava, rye, purple lavers, lupins and significant amounts are found in cereal grains (Nićiforović and Abramovič, 2014). Sinapic acid is a precursor of the syringyl unit of lignin in plants and is reported as an intermediate during the degradation of lignin by the fungal polyphenol oxidase enzyme (Lacki and Duvnjak, 1998). Hydrolysis of sinapic acid results in sinapyl alcohol and is a common constituent of phenolic wastewater (Mukherjee *et al.,* 2006) (Yamauchi, Yasuda and Fukushima, 2002). Compared to the *p*-coumaryl and coniferyl alcohol, the sinaply alcohol fragment as a lignin degradation product does not undergo repolymerisation due to the presence of a methoxy group (Zakzeski *et al.,* 2010). This explains the enhanced lignin degradation, which may have been due to the prevention of repolymerisation of lignin degradation intermediate products due to the formation of sinapyl alcohol.

Sinapic acid and trans-cinnamic acid are also reported to mediate and induce the ligninolytic enzyme activity in white-rot fungi for the removal of poly-hydrocarbon treatment (Cañas and Camarero, 2010; Camarero *et al.,* 2008). Fermentation of rapeseed meal with *Trametes versicolor*, a white rot fungus, resulted in a 60% reduction of sinapic acid content due to

significantly enhanced extracellular enzyme activity (Żuchowski *et al.*, 2013). There are reports of sinapic acid improving laccase production in the filamentous fungus *Paecilomyces variotii* (Mukherjee 2006). Degradation of sinapic acid by endophytic fungus *Phomopsis liquidambari* is also reported (Xie *et al.*, 2016). *Trametes versicolor*, a white rot fungus, could degrade sinapic acid by enhancing polyphenol oxidase activity (Lacki and Duvnjak, 1996). Additionally, these lignin degradation aromatic compounds are grouped as value-added products and find use in the food industry and are known to enhance ligninolytic enzyme activity in fungal-treated biomass processes.

Resveratrol is 3,4',5-trihydroxystilbene (Structure shown in Figure 4.10 b) is a stilbene formed from lignin during the kraft pulping process (McAndrew *et al.*, 2016). It is also produced in plants as a secondary metabolite and is known for its anti-ageing properties. It is commonly found in red wine and enhances the life span of eukaryotic organisms such as mice and yeast (Wang, Chen and Yu, 2010). The effect of resveratrol on the production of a novel phenol oxidase was studied in *Scytalidium thermophilum*, a thermophilic fungus, and was found to enhance the production of the enzyme (Yuzugullu, 2015).

In the same process, resveratrol showed neither a growth-promoting nor an antifungal effect but resulted in a significant 2.5-fold increase in phenol oxidase activity. Resveratrol was tested as an inducer for laccase in a white-rot-basidiomycetes, *Stereum Ostrea* and resulted in enhanced enzyme activity (Elisashvili, Asatiani and Kachlishvili, 2020). Resveratrol produced as a phytoalexin in grapevine was reported to be metabolised by fungal oxidases induced in *Botryosphaeriaceae* fungi, a plant pathogen (Labois *et al.*, 2021). Both compounds have been reported to enhance ligninolytic enzyme activity in fungal-treatment of lignin.

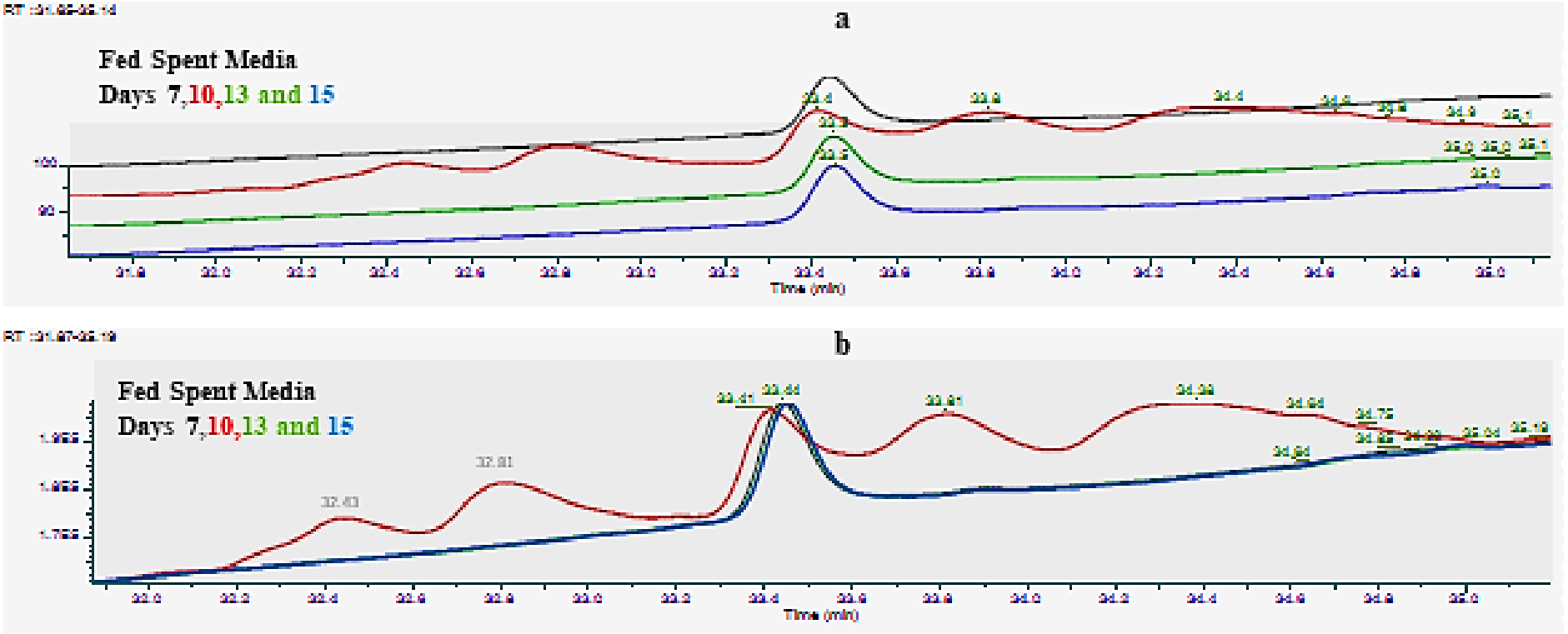


Figure 4.8- Chromatograms overlay of fed spent wastewater media comparing showing the skewed chromatogram profile showing only new peaks seen in fed spent media from day 10 of treatment.

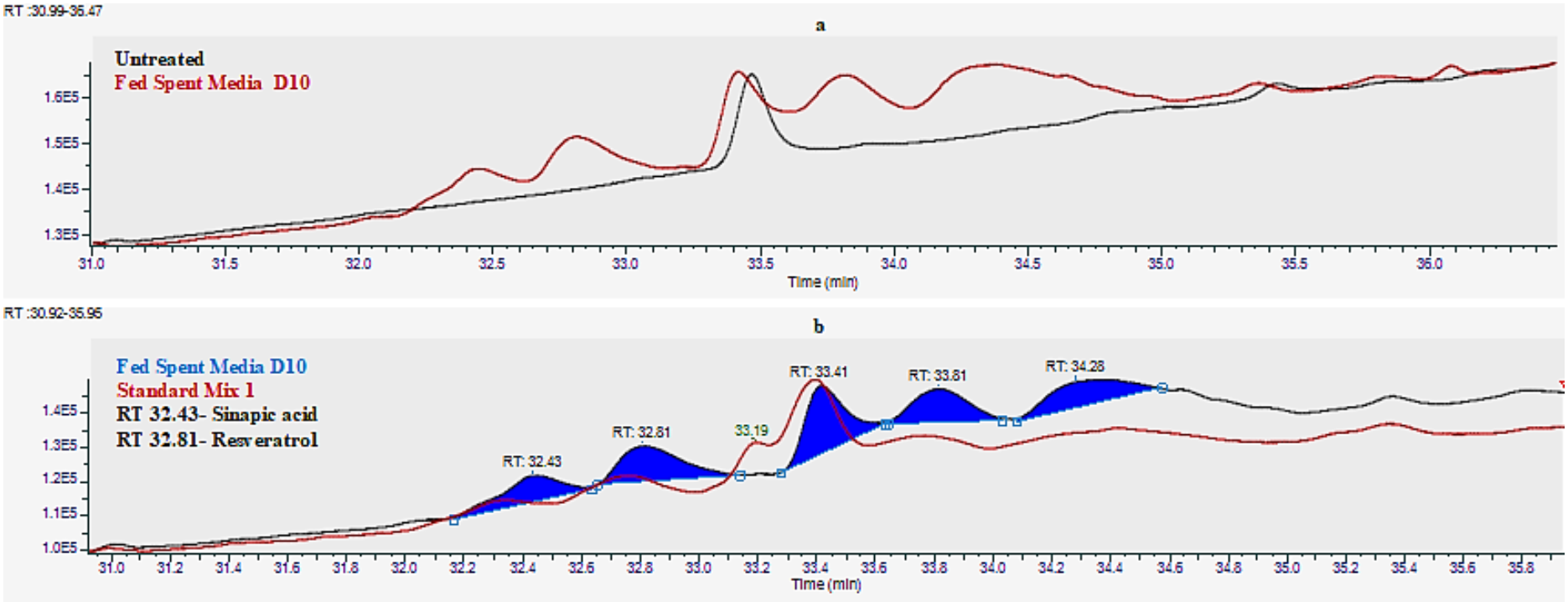


Figure 4.9 - Chromatograms of spent wastewater media comparing lignin degradation intermediates on day 10 (blue peaks) (a) with untreated samples (black line peaks) (b) standard mix (red line peaks) identified as sinapic acid (RT-32.43) and resveratrol (RT- 32.81), trans-cinnamic acid (RT- 33.41).

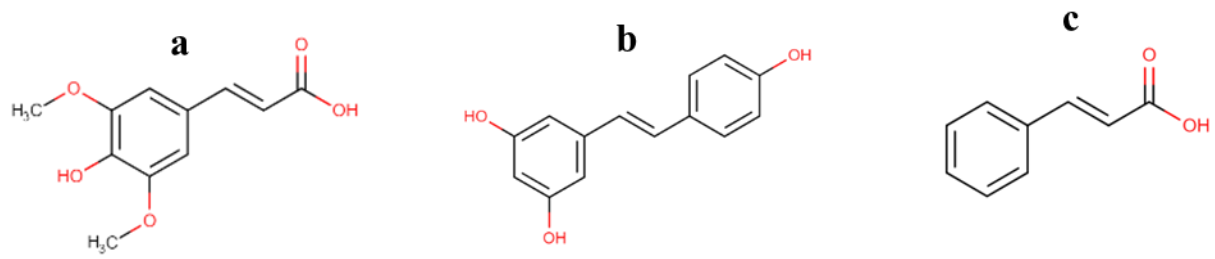
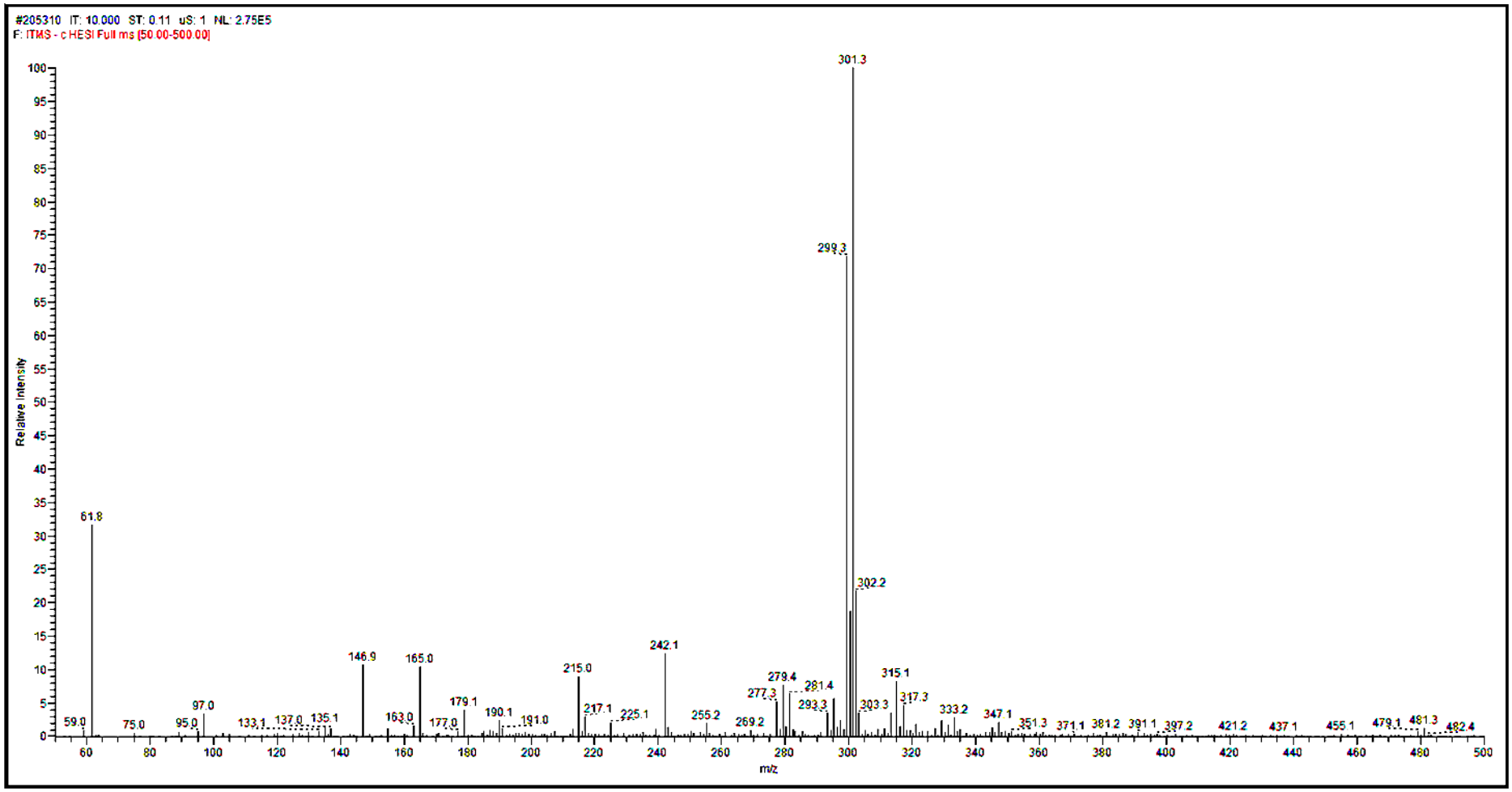


Figure 4.10- Structure of (a) Sinapic acid (b) Resveratrol (c) trans-Cinnamic acid

Microbial depolymerisation of lignin was reported to result in the breakdown of polymer into low-molecular-weight aromatic compounds, and trans-cinnamic acid is one of them (Raj, Reddy and Chandra, 2007; D'annibale *et al.*, 1996). During pulp and paper mill production, Cinnamic acid is present in the alkali lignin fraction as it is present in lignocellulosic biomass, connecting the lignin and cellulose (Yamada *et al.*, 1981; Hernandez *et al.*, 1997). The presence of cinnamic acid indicated the oxidation of the sinapyl alcohol (S unit) subunit of lignin monomer (Raj *et al.*, 2007). The negative ESI-MS/MS mode with 100 % relative intensity at 301.3 m/z, as shown in the mass spectra obtained from the spent media samples (Figure 4.11 a) (Wang, Chen and Yu, 2010). The compound was identified as Nordihydroguaiaretic acid (NDGA), also known as masoprocol, with the mass spectra (Figure 4.11 b).


#205310 IT: 10.000 ST: 0.11 uS: 1 NL: 2.75E5
F: ITMS - c HESI Full ms [50.00-500.00]
Relative Intensity
m/z
301.3
299.3
302.2
61.8
315.1
242.1
146.9
165.0
215.0
279.4
281.4
277.3
317.3
179.1
97.0
333.2
59.0
75.0
95.0
133.1
137.0
135.1
163.0
177.0
190.1
191.0
217.1
225.1
255.2
269.2
293.3
303.3
347.1
351.3
371.1
381.2
391.1
397.2
421.2
437.1
455.1
479.1
481.3
482.4

h

cultures

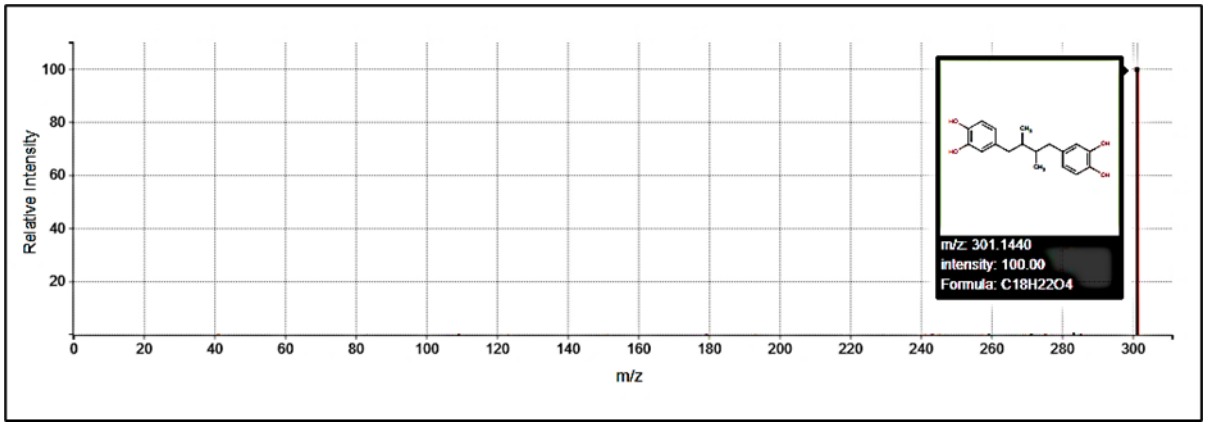


Figure 4.11 b - Mass spectra of Nordihydroguaiaretic acid showing peak formed at 301.14 m/z with 100% intensity under the negative ionization mode. (https://products.metasci.ca/spec/MSIPAA050/PR22LC1001/GE3/plus/198/1338).

NDGA is a naturally occurring tetrahydroxy lignin, a phenylpropane dimer known for its antioxidant activity (Manda *et al.*, 2020). The role of PPO in the biosynthesis of NDGA was studied due to the enhanced PPO activity of the enzyme (Cho *et al.*, 2003). *Phanerochaete chrysosporium* was used in a study to degrade plant leaves, resulting in the extraction of NDGA as a lignin degradation product (Martins, Teixeira and Mussatto, 2013). Other compounds identified by applying LC-MS/MS using predicted spectra from www.metasci.ca are trans-cinnamic acid (146.9 m/z), sinapic acid (179.1 m/z), resveratrol (135.1 m/z), protocatechuic acid (153.01 m/z), 2-pyrocatechuic acid (155.03 m/z), vanillic acid (167.03 m/z) and m-coumaric acid (163 m/z).

4.3.5 Biofilm Microstructure and Composition

The harvested biofilms were studied under the scanning electron microscope, and the images produced were analysed using the ImageJ application (Figure 4.12). The SEM image analysis of biofilms showed that the biofilm microstructure - filament length, filament thickness and pore area did not show significant differences. This concludes that there was no change in the biofilm microstructure due to the feeding, indicating that the fed spent media was responsible for the enhanced production of enzymes.

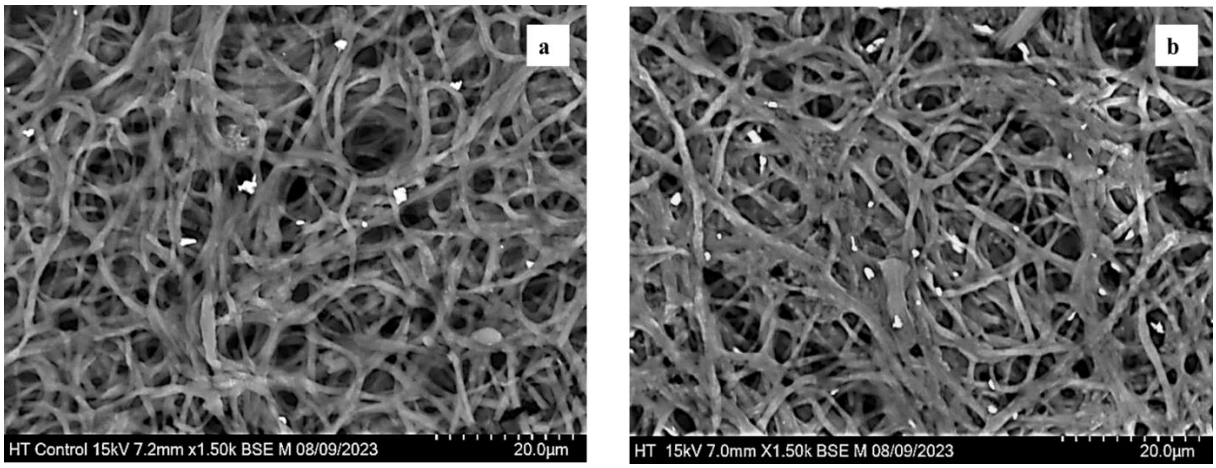


Figure 4.12- Scanning electron microscope images showing biofilm structure of biofilms grown in cultures that were (a) Unfed (b) Fed with spent media from day 10.

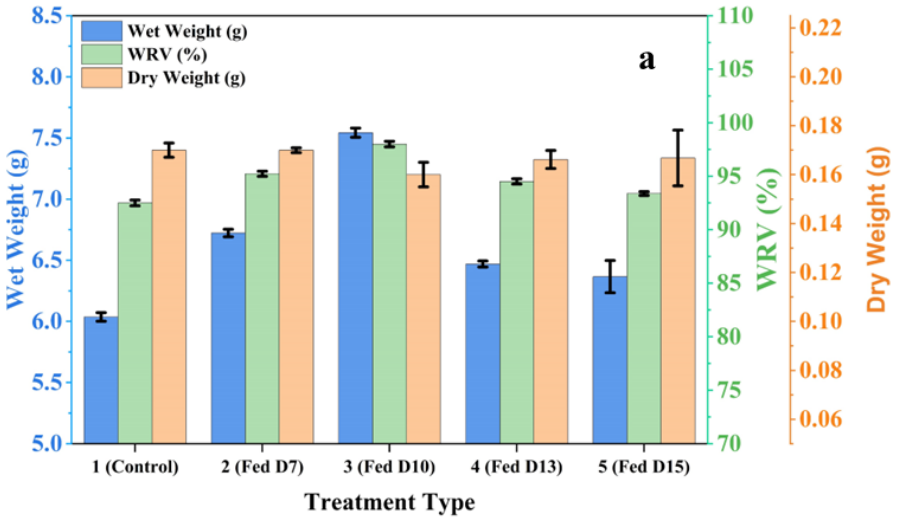
Table 4.2 - Quantification of biofilm characteristics by analysing SEM images with ImageJ software. The filament length, diameter, and pore size of biofilms grown in unfed and cultures fed with spent media from day 10 of fungal treatment.

Treatment Type	**Filament length (µm)**	**Filament thickness (µm)**	**Pore Size diameter (µm)**
Unfed Culture	9.25 +/- 0.75	1.34 +/- 0.85	5.67 +/- 0.65
Fed Culture (D10 SM)	10.55 +/- 0.55	1.45 +/- 0.56	6.23+/- 0.75

The biofilm composition and microstructure of harvested biofilms from unfed reactors and those fed were evaluated (Figure 4.13 a, Table 4.2). Compared to the whole biofilm wet weight, the biofilms in fed cultures were heavier than those formed in unfed cultures. The dry biomass of all the biofilms did not show any significant difference. However, the water retention value (WRV) in cultures fed with early stages of spent media (day 7 and day 10) showed a significantly higher WRV than the unfed cultures. This suggests the variation in wet weight of all the biofilms was because of more water held in the biofilms and not due to the biomass. This was evidenced by the water retention values that represents the water

holding capacity. The WRV in biofilms formed in culture fed with day 10 spent media was 1.06 times more than the unfed cultures (fed - 98.48 +/- 2 %, unfed- 92.65 +/- 1.35 %). These results point to the composition of the extracellular matrix of the biofilms that is also responsible for the water-holding attribute of the biofilms (Flemming and Wingender, 2010).

The cultures that were fed showed to contain more polysaccharides and protein content in, the extracellular matrix extracted from the biofilms and also showed significantly higher WRV (Figure 4.13 b). Polysaccharides are hydrophilic, meaning they attract water. This results in increased water absorption in the extracellular matrix of biofilms formed in fed cultures (Cheng *et al.,* 2010a). The increased polysaccharide content in ECM could be attributed to spent media feeding, which caused the induction of more ECM produced in fed cultures. Furthermore, the increased enzyme activity in the fed culture suggests that the enzymes were also induced due to the addition of lignin degradation intermediates. Additionally, a more critical factor responsible for the enhanced ECM production could have been the induction of metabolites due to the addition of lignin degradation products. This strengthens the assumption that adding the spent media containing lignin degradation products was responsible for enhanced enzyme activity and increase in process efficiencies.



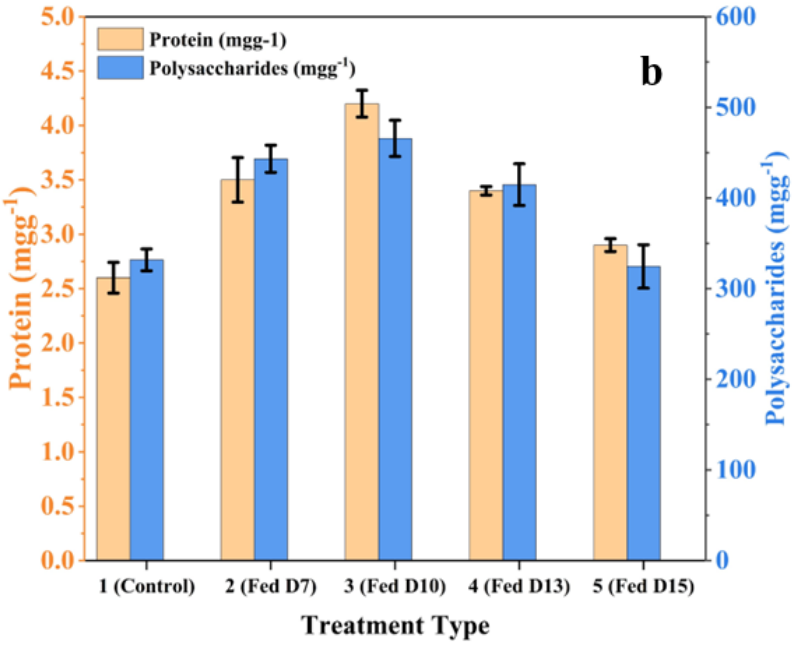


Figure 4.13 - Biofilm characteristics comparison (a) wet weight (g), water retention value (%) and dry biomass (g) (b) Polysaccharides per unit biomass (mg g^{-1}) and protein per unit biomass (mg g^{-1}) of biofilms grown on unfed and fed batch reactors.

4.3.6 Correlations Between Process Efficiencies and Biofilm Properties

The results of unfed and cultures fed with lignin degradation intermediates were analysed further to identify the correlations between the biofilm properties, enzyme activities of PPO and VPO and the process efficiencies, as these parameters seem to be interconnected. It was noted that there was a significant correlation between the lignin and COD removal efficiencies (R - 0.88, $p < 0.05$). Similarly, the enzyme activity of PPO and VPO correlated significantly with the lignin (R - 0.96, $p < 0.05$) and COD (R - 0.97, $p < 0.05$) removal efficiencies. The extracellular matrix plays an important role in pollutant degradation and is reported to be an important factor for water adsorption (Yadav *et al.*, 2023). Strong positive correlation between lignin and COD removal efficiency with polysaccharides per unit biomass (R - 0.90, $p < 0.05$), proteins per unit biomass (R-0.99, $p < 0.001$) and water retention values (R - 1, $p < 0.001$) also correlated positively and strongly showing the secretion of extracellular matrix has a strong influence on the process efficiencies. In a

previous study, the correlation between the biofilm microstructure and the process efficiencies was reported (Tabraiz *et al.,* 2022). In this study, poor correlation between the process efficiencies and biofilm biomass and properties such as filament length, diameter and pore size were noted. These results conclude that the production of ECM , including the polysaccharides and proteins, was influenced by the difference in the environmental conditions, strengthening the hypothesis that the intermediate feeding had a positive inductive effect on the process efficiencies.

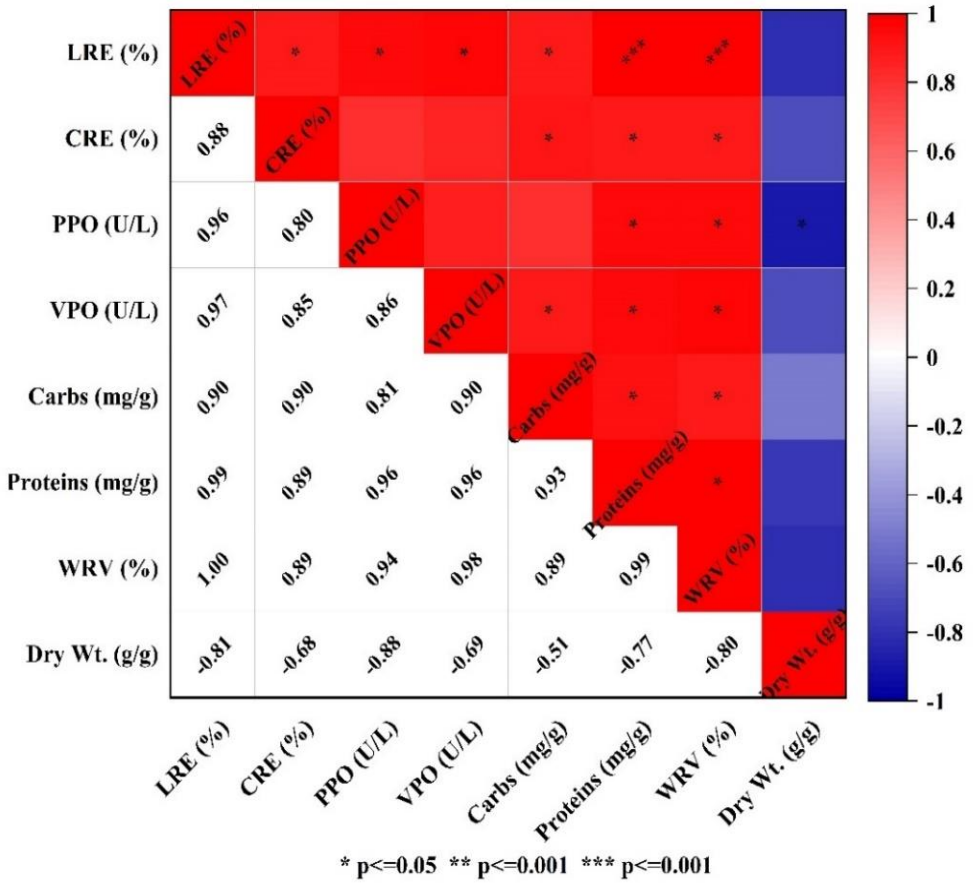


Figure 4.14 - Pearson's correlation plot showing the correlations between lignin removal efficiency (LRE, %). COD removal efficiency (CRE, %), polyphenol oxidase activity (PPO, UL-1), versatile peroxidase activity (VPO, UL-1), polysaccharides (Carbs, mgg-1), proteins (mgg-1), water retention value (WRV, %), dry biomass(g). The asterisks in the upper triangle signify the variables to have a significant correlation and the colours denote the positive (red) and negative (blue) correlation. A statistically significant difference (* p<=0.05 ** p<=0.001 *** p<=0.001). The Pearson's coefficient values (r) are presented as the labels in the lower triangle.

4.4 Conclusion

This study evaluated the positive effect of feeding wastewater medium with lignin degradation intermediate products. The fed cultures exhibited a significant ($p < 0.05$) increase in lignin and COD removal efficiencies in all the cultures fed with lignin degradation intermediates. The cultures fed with spent media from day 10 outperformed the process efficiencies noted for other fed cultures by 1.16 times. The enhanced lignin and COD removal efficiency positively correlated (R-0.853, $p < 0.05$). The RP-HPLC chromatograms were analysed by comparing the residual lignin represented as peak area with the untreated, unfed, and fed cultures and aligned with the UV-spectroscopy results confirming positive effects of feeding spent media containing lignin degradation intermediates. Additionally, the cultures fed spent media that outperformed other treatments also showed increased enzyme activity of PPO (4.7-fold increase) and VPO (4.5-fold increase) activity compared to the unfed cultures. The analysis of the EPS confirmed the production of more polysaccharides and proteins in all the fed cultures supporting the hypothesis that the feeding process was beneficial to improve enzyme efficacy. The lignin degradation products identified using RP-HPLC method with standard mixtures, were sinapic acid and resveratrol. The untargeted direct injection into MS resulted in the identification of nordihydroguaiaretic acid in addition to few other commonly detected lignin degradation intermediates. All the identified compounds have been reported as lignin degradation products in literature and a few have been evaluated as substrates for the more studies white-rot fungi. The role of lignin degradation products, sinapic acid and resveratrol, was potentially that of inducers of enzymes and enhanced lignin degradation and COD removal efficiencies. Adding spent media containing lignin degradation intermediates from the initial stages of biofilm treatment was more beneficial as the intermediate products may have been consumed towards the latter

stages and may contain cellular degradation organic matter that can contribute to an increase in COD.

---

5. Evaluation of a Repeated-Batch Process for Treating Lignin-Rich Industrial Effluent Using Biofilms Formed by *Neurospora discreta*

5.1 Introduction

In pulp and paper-making plants, the biological treatment process, as part of the secondary treatment, is used to treat lignin-rich effluent after the primary clarification process (Thompson *et al.,* 2001). Few pulp and paper mills in the UK utilise on-site biological treatment methods due to the dynamic nature of biological processes (Thompson *et al.,* 2001). The activated sludge process is the most commonly used method for biological effluent treatment, often applied in sequencing batch reactors (Dionisi *et al.,* 2001). The activated sludge process suffers from a costly sludge settlement, which is overcome in biofilm processes in which easy biomass separation is possible. In previous studies, biofilms formed by *Neurospora discreta* are effective in degrading lignin. However, there are certain drawbacks associated with fungal treatment. One of these drawbacks is the slow rate of bioconversion of the pollutant. The process duration is also extended as the fungal cells suffer from slow metabolism, and there is a lag in biomass growth from spore inoculation (Balcıoğlu *et al.,* 2007). Another drawback of fungal wastewater treatment is that it requires another more readily available carbon source, such as glucose or sucrose (Tarlan, Dilek and Yetis, 2002).

Compared to a batch and semi-batch process, the repeated-batch process used in biofilm treatment reactors is more advantageous because it allows the inoculum to be reused during different wastewater treatment cycles (Todhanakasem *et al.,* 2019). The bioconversion rate of pollutants can be increased by cell recycling, where inoculum can be retained and reused during the following batch cycles (Todhanakasem *et al.,* 2019). This technique has previously proven to be efficient in lignin (76.1%) and COD (80%) removal from pulp and paper mill

wastewater by coculturing ascomycetous fungi in a study by Tajwar et al. (Rajwar, Paliwal and Rai, 2017). Another survey by Sedighi et al. demonstrated that repeated-batch techniques using *Phanerochaete chrysosporium* resulted in consistent COD removal (48%) in each batch of textile effluent treatment, showing promise as a method (Sedighi, Karimi and Vahabzadeh, 2009). In another study, the repeated-batch treatment was carried out 12 times for 36 days, resulting in COD (40%) and colour (70%) removal that concluded to be dependent on the biomass concentration (Ma *et al.,* 2008). The technique was successfully applied to a silicone membrane reactor to produce lignin peroxidase enzyme from *Phanerochaete chrysosporium* over 5 weeks, alternating periods of growth and production (Venkatadri and Irvine, 1993). Additionally, the process operation is more stable due to high cell concentration in each fermentation batch, resulting in low chances of contamination (Ma *et al.,* 2008).

In biological wastewater treatment, targeted results such as lignin and COD removal depend on microbial growth and extracellular enzyme production (Haq, Mazumder and Kalamdhad, 2020). The repeated-batch technique is highly advantageous in fermentation processes, as it supports the maintenance of high microbial biomass density and eliminates any lag time since the microbial cells are actively metabolising at the start of the treatment cycle (Watanabe *et al.,* 2012). This technique has proven efficient in treating lignin and for longer retention of fungal biomass in industrial effluent (Ma *et al.,* 2008; Rajwar, Paliwal and Rai, 2017). Though the repeated-batch technique has been successfully investigated for processes involving fungal biofilm, the biofilms studied in the process till now were grown by artificially entrapping fungal cells grown on support material (Khiyami, Pometto and Kennedy, 2006; Venkatadri and Irvine, 1993). Processes applying naturally formed biofilms, such as those formed by *N. discreta*, have not been explored yet under repeated-batch

conditions. Such methods can potentially be profitable applications at an industrial scale as the same biomass can be reused in treating multiple cycles of wastewater.

During the fungal treatment, nutrient-limiting conditions are preferred for producing extracellular enzymes as secondary metabolites (Fulekar *et al.*, 2012). However, as the fungal cultures approach the nutrient-limiting conditions, the biomass concentration ceases to grow, and eventually, the process comes to a halt (Mir-Tutusaus *et al.*, 2018). Nutrient addition is applied to maintain biomass in repeated-batch cultures, resulting in biomass gaining stability over time. In doing so, the strategy employed results in the renewal of partial biomass, which allows the extension of biofilm longevity and a more efficient lignin degradation process (Blánquez, Sarrà and Vicent, 2006). The biomass renovation technique extends the treatment process and hydraulic retention time (HRT) by overcoming the reduced enzymatic activity caused by the biomass age (Mir-Tutusaus *et al.*, 2017). The biofilm morphology allows the total biomass to be retained, resulting in renovating the whole biomass when fed with nutrients in the new treatment cycle. Pollutant removal is improved with increased HRT alongside the removal of accumulated toxic compounds as the spent media is removed with each cycle change (Mir-Tutusaus *et al.*, 2018). In summary, the maturation of biomass leads to a stable fungal treatment process, in turn maintaining enzymatic activity for a more extended period. Such stable methods favour biomass longevity and can further be tested in non-sterile wastewater treatment in natural conditions. To improve enzymatic production and maintain biomass stability, other groups have successfully tested this strategy in both sterile and non-sterile wastewater treatment processes (Badia-Fabregat *et al.*, 2016; Blánquez and Guieysse, 2008).

Adding phenolic compounds to wastewater treatment is reported to enhance the production of ligninolytic enzymes such as laccase and lignin peroxidase (Mishra *et al.*, 2017).

In the previous study, Chapter 4, the addition of phenolic lignin degradation products was reported to enhance the production of polyphenol oxidase (PPO) and versatile peroxidase (VPO), thus subsequently enhancing lignin degradation efficiency. Contrastingly, evidence states that extracellular ligninolytic enzyme secretion is affected only in nutrient-limiting conditions rather than in pollutants (Karapinar and Kargi, 2002). Taking both aspects into consideration - longevity of biofilm biomass and induction of ligninolytic enzyme activity - repeated-batch treatment is a promising solution.

Although a few fungi metabolise organic pollutants as a carbon source, the need for an additional assimilable carbon source has been reported by many authors (Cruz-Morató *et al.*, 2013; Badia-Fabregat *et al.*, 2015). For initial growth, *N. discreta* requires sucrose as a more readily available carbon (Pamidipati and Ahmed, 2017). Once the biofilms are formed, there seems to be a possibility that they can sustain growth and start metabolising lignin as the sole carbon source. The process economics can be improved by alternating wastewater media treatment cycles with and without sucrose addition.

It was hypothesised that repeated-batch treatment of industrial wastewater using mature biofilms would accelerate the fermentation rate for lignin and COD removal by eliminating the cellular lag phase involving the biofilm formation. Simultaneously, the study evaluated the effect of treating sucrose-sufficient and sucrose-deficient wastewater media for process efficiency. The two conditions differed in wastewater composition supplemented with sucrose in all cycles (Set A), and the other condition had an alternate period of no sucrose supplementation (Set B). The treatment process was operated in sterile-static conditions in a periodically repeated-batch reactor using lignin-rich wastewater as substrate. The reactor was operated at an HRT of 36 days. To evaluate the effect of process parameters with time, the

process period was divided into three cycles of treatment, allowing the adaptation of mature biofilms from lower concentrations of lignin to higher concentrations ranging from 500 mg L^{-1}, 1000 mg L^{-1} and 1500 mg L^{-1} of lignin and wastewater enriched with sucrose at 1000 mgL^{-1}. In a previous study, where *N. discreta* spores were inoculated in wastewater containing 1500 mg L^{-1} lignin, it showed slow growth with a lag of 6 days. In this study, the lag was overcome by inoculating spores in wastewater containing lower concentrations of lignin 500 mgL^{-1} and subsequently adapting the biofilms to higher concentrations. The biofilm process was tested for lignin and COD removal efficiency in wastewater.

5. 2 Materials and Method

5.2.1 Fermentation Setup

The entire repeated-batch treatment process is divided into three cycles of periodic batch treatments with a total hydraulic retention time of 36 days at pH 5. The experiments were set up in 250 ml of Erlenmeyer flasks, with a treatment volume of 100 ml, plugged with foam bungs and covered with aluminium foil. The biofilms were first grown in six reactors using an aseptic technique by inoculating 100 ml of wastewater media with *N. discreta* spores at 1 x 10^7 spores per ml. The reactors were incubated at 30^o C.

5.2.2 Wastewater Media Composition

Wastewater from the pulp and paper-making process was received from Mondi Frantschach, a paper-making company. The effluent was the filtrate from a bleaching plant collected during the pulp-brightening kraft process. The concentration of lignin in the wastewater was 1500 mg L^{-1}. The wastewater was diluted further to create three batches with lignin concentrations at 500 mg L^{-1}, 1000 mg L^{-1}, and the third batch was neat filtrate at 1500 mg L^{-}

[1]. All three wastewater cycles were supplemented with Vogel's media components (Section 2.3) with 1000 mg L^{-1} of sucrose as an additional carbon source (Table 5.1).

Cycle 1 was the growth phase, where the wastewater (500 mg L^{-1} lignin) was inoculated with spores. Cycle 2 of wastewater treatment began when the biofilms formed on the air-liquid interface from Cycle 1 (day 12) were transplanted on fresh wastewater media in new reactor flasks. The lignin concentration in Cycle 2 wastewater media was 1000 mg L^{-1} and was selectively supplemented with sucrose. The first set of triplicate reactors (Set A) was supplemented with sucrose (1000 mg L^{-1}), and the second set of triplicate reactors (Set B) was not supplemented with sucrose. The second cycle (Cycle 2) was carried out for 12 days, after which the spent wastewater media was withdrawn completely. To start the third wastewater treatment cycle, the mature biofilms were transferred again to fresh wastewater media containing 1500 mg L^{-1} lignin supplemented with sucrose (1000 mg L^{-1}) for another 12 days. The wastewater composition for Sets A and B was the same, with lignin concentration at 1500 mgL^{-1} and supplemented with sucrose at 1000 mgL^{-1}.

Table 5.1- Details of repeated-batch wastewater treatment showing the experiment cycles, sets, total HRT of the biofilms and wastewater media composition.

Cycle	Set	HRT (days)	Initial Lignin (mg L^{-1})	Initial Sucrose (mg L^{-1})
1	-	12	500	1000
2	A	24	1000	1000
2	B	24	1000	-
3	A	36	1500	1000
3	B	36	1500	1000

5.2.3 Inoculum

The inoculum for this study was prepared according to the method mentioned in Section 2.4. The *N. discreta* filaments were gently scraped from the PDA plate. The plate was flooded with Vogel's media, not supplemented with sucrose. The spore suspension was filtered through a sterile muslin cloth to remove the filaments. The spore count in the suspension was done on a haemocytometer using an optical microscope. The spores were adjusted to reach 1 X 10^7 spores per ml by adding Vogel's media to the suspension. The reactors containing the wastewater were inoculated with 1 ml of the spore suspension using aseptic techniques.

5.2.4 Lignin Estimation

In reactors where biofilms were transplanted, samples were drawn one hour after the transplant. The untreated and treated wastewater media samples were treated to remove the biomass according to the method detailed in Section 2.7. After the biomass removal, the lignin was precipitated and redissolved in DI water after drying. The samples were then injected in the HPLC column for estimation of lignin. The standard curve created using the precipitated lignin was used to calculate the residual lignin concentration. The lignin removal efficiency was calculated using the following equation:

$$Lignin\ Removal\ Efficiency\ (\%) = \frac{Lignin\ conc.(start) - Lignin\ conc.(end)}{Lignin\ conc.(start)} \times 100 \qquad (5.1)$$

5.2.5 Chemical Oxygen Demand

The chemical oxygen demand (COD) was measured using the Palintest COD/2000 Kits for samples collected before and after biofilm treatment. The COD removal efficiency was calculated using the following equation, where $COD_{(initial)}$ is the COD measured at the beginning of a treatment cycle and $COD_{(final)}$ is the COD measured at the end of the sample cycle:

$$COD\ \ Removal\ Efficiency(\%) = \frac{COD\ \ conc.(initial) - COD\ conc.(final)}{COD\ \ conc.(initial)} \times 100 \qquad (5.2)$$

5.2.6 Enzyme activity

The enzyme activity was determined for laccase, polyphenol oxidase and versatile peroxidase according to the protocol mentioned in Section 2.13. Laccase activity was measured using 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) as substrate in citrate buffer (Bourbonnais and Paice, 1990; Baldrian and Šnajdr, 2006). Dark green cations are produced because of the oxidation of ABTS by laccases, and the spectral time scan is measured spectrophotometrically at 420 nm wavelength. The enzyme activity (1 Unit) is quantified using the extinction coefficient (ε420) 36000 M^{-1} cm^{-1} (Wang *et al.*, 2016; Saito *et al.,* 2003). The polyphenol oxidase activity was measured using a pyrocatechol solution (0.2 M) prepared in citrate buffer (0.05 M) at pH 5. One unit of enzyme was defined as the amount of enzyme that forms 1 mol of product per minute. The oxidation of reactive black dye (RB5) in sodium tartrate buffer 50 mM at pH 3 measured VPO activity. One unit of enzyme is defined as the amount of enzyme that transforms 1mol of substrate consumed.

5.2.7 Biofilm Characterisation

The biofilms were harvested on day 36 from inoculation. The biofilms had multiple layers and were left to drain out excess water leaving on water-absorbent paper for 15 minutes. The fresh biofilms were used to measure the wet weight and WRV according to the protocol mentioned in Section 2.11. The known weight of the biofilm was used to extract the EPS according to the protocol mentioned in Section 2.12, and this EPS was further analysed to quantify the polysaccharides and proteins in the biofilm EPS. The biofilms were sprayed with isopropyl alcohol to arrest the cell growth and were stored at 4^{o} C for further analysis.

5.2.8 Microscopy

To get images of biofilms in native condition, without drying them, the imaging was done using a scanning electron microscope Thermofisher Quanta 650 ESEM with a Tungsten filament. The biofilms were imaged at the University of Nottingham (Nanoscale and Microscale Research Centre). Samples were mounted on a vertical stub held by wrapping an aluminium tape. The stub was fitted onto the Peltier stage cooled to 2^{o}C, and the chamber was pumped to 5.3 Torr following two purge cycles to flush with water vapour. The imaging mode was ESEM using the gaseous secondary electron detector (GSED) at 1000 x magnification.

5.3 Results and Discussion

5.3.1 Biofilm Growth

The first experiment (Cycle 1) was conducted to grow biofilms to fully cover the surface of wastewater media, showing strong and thick biofilms on day 12 from inoculation. The biofilms were seen to have covered the entire liquid surface on day five from inoculation. On day 12, after the biofilm transplant in sucrose-supplemented cultures (Set A) and sucrose-deficient cultures (Set B), the biofilms remained submerged under the wastewater until new cell growth started showing on the surface. In Set A, biofilms surfaced on day 2 after transplantation. In Set B, the biofilms showed a delay in fully emerging and covering the liquid surface on day 5. As the new top layer started to form, the biofilms began to float on the air-liquid interface, showing a distinct old biofilm layer under the new layer. On day 12, the biofilms in Set A cultures showed new layers formed on the surface. However, the cultures growing in Set B cultures showed poor growth and started disintegrating after day 9. The biofilms in the Set B reactors were weak and harvesting them for a transplant became challenging.

On day 12 of Cycle 2 (HRT - 24 days), the spent wastewater media was withdrawn and replaced with fresh wastewater media with 1500 mg L^{-1} of lignin supplemented with sucrose (1000 mg L^{-1}) in all the reactors. Similar to the previous Cycle, in Cycle 3, the biofilms first submerged and resurfaced as a new layer of cells formed on the entire surface. The biofilms were harvested and stored for further analysis on day 12 of Cycle 3 (HRT - 36 days) from inoculation.

5.3.2 Lignin Estimation

5.3.2.1 UV-Vis Spectroscopy

During the growth phase of the biofilms in Cycle 1, all reactors exhibited 42.3 +/- 1.22% lignin degradation (Figure 5.1). The Cycle 2 experiments started with 1000 mg L^{-1} lignin in untreated samples on day 0. The concentration of residual lignin was higher on day 3 of the treated compared to the untreated samples. This increase in lignin concentration can be explained by the residual lignin entrapped in the pores of the biofilms at the time of transfer, some of which may have dislodged and added to the culture medium in the new reactors. Therefore, the lignin removal efficiency was calculated from the third day of treatment for both Set A and Set B. After 12 days of treatment, the residual lignin concentration in Set A cultures, which were supplemented with sucrose, resulted in the lignin removal efficiency of 67.97 +/- 3.22 %. On the other hand, Set B cultures (sucrose deficient) had a higher residual lignin, resulting in 36.59 +/-2.5 % lignin removal. The lignin removal efficiency in the Cycle 2 experiment was significantly and negatively affected without sucrose ($p<0.05$). Also, there was a significant increase in lignin removal efficiency in Cycle 2 Set A compared to Cycle 1 treatment.

Cycle 3 wastewater treatment showed removal efficiencies of 46.92 +/- 0.67 % (Set A) and 32.56 +/- 1.37 % (Set B) at the end of the 12-day treatment (HRT 36 days), showing a significant difference between the two. During Cycle 2, the Set B biofilms started to deteriorate towards the end of the cycle, which affected the metabolic capabilities. Due to poor growth, the Set B biofilms showed lower lignin removal efficiencies. The Cycle 3 lignin removal efficiencies were significantly lower ($p<0.05$) than the Cycle 2 results. The trend, however, remained the same, with Set A being more efficient than Set B. This is because of the restricted diffusion of wastewater and mass transfer caused by forming multiple biofilms as new layers form on the top surface. In biofilms processes, the diffusion problems intensify as the biofilms become thicker and denser, resulting in varying pollutant removal rates across the biofilm as the mature biofilm layer becomes less active (Sperling and de Lemos Chernicharo, 2005; Matheus *et al.*, 2021).

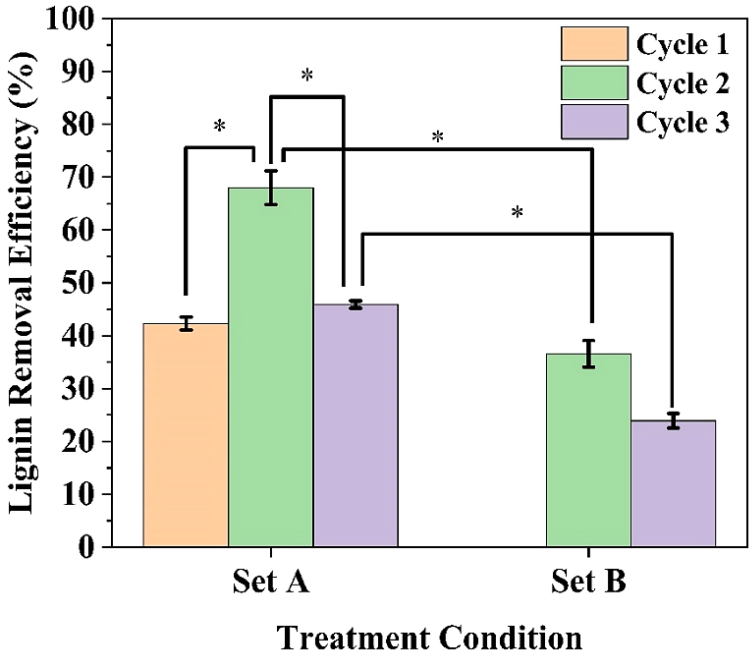


Figure 5.1 - Lignin removal efficiency (%) for Cycles 1, 2 and 3 wastewater selectively supplemented with sucrose (Set A and B). Significant difference is denoted by an asterisk (*) where $p<0.05$ as determined by the t

5.3.3.2 Reverse-Phase Liquid Chromatography

The chromatogram overlay of the Cycle 1 experiment is shown in Figure 5.2 a. The chromatograms compared are from untreated (day 0) and treated (day 12) wastewater media samples. The wastewater contained phenolic lignin-degraded products, as evidenced by the polar peak on the chromatogram at a retention time (RT) of 2.4 minutes. During the kraft process, the degradation of non-polar native lignin results in wastewater that is rich in phenolic lignin-degraded products. Figure 5.2 b is the enlarged polar peak area. The untreated wastewater samples show a significant peak area, represented as a black line. The treated samples (represented as a red line) taken on day 12 displayed a significantly smaller area ($p<0.05$) at RT 2.5 +/- 0.07 minutes.

The chromatograms obtained from Cycle 2 untreated samples formed the polar peak with a peak area of 1.83E+7 corresponding to 1000 mgL^{-1}. However, in the samples collected on day 3 from transplant (Set A and B), the peak area was more significant than the untreated samples. Figure 5.3 a is the chromatogram overlay of Cycle 2 Set A experiment samples from day 3 (black) and day 16 samples (red) initially supplemented with sucrose. Figure 5.3 b shows the enlarged chromatogram overlap for which the residual lignin concentrations were estimated to be 1898.58 mgL^{-1} and 648.63 mgL^{-1}. Like the previous results, the wastewater media samples showed major polar peaks formed for the lignin degradation products.

Similar to the results from the Set A sucrose-supplemented cultures, the Set B cultures (figure 5.4) the samples from day 3 of fungal treatment showed an increase in lignin concentration (1740.75 mg L^{-1}), which suggests that the biofilms, when transplanted from the previous lignin-rich cultures (500 mg L^{-1}), carried along lignin in the pores and when transplantation acclimatised, and the comparison was done from day 3 till the end of the treatment process. The residual lignin concentration on day 12, corresponding to the polar peak, was estimated to be 1103.85 mg L^{-1}.

The chromatogram overlaps in Figure 5.5 are from the samples from the Cycle 3 experiment with polar peak overlap of untreated (black) and treated cultures from day 36. On day 0, the residual lignin concentration in the Cycle 3 wastewater media, after a few hours of biofilm acclimatisation, was estimated to be 1913.06 mg L^{-1}. On day 12 of fungal biofilm treatment, the cultures supplemented with sucrose throughout (red line in figure 5.5) had lesser residual lignin concentration of 1032.41 mg L^{-1} in comparison to 1290.62 mg L^{-1} in the cultures subjected to intermittent no sucrose interval (green line in figure 5.5). This shows that the cultures continuously supplemented with sucrose showed more efficient lignin degradation. Though the cultures with intermittent no-sucrose periods showed smaller peak areas interpreted as more residual lignin, these can be considered more economical.

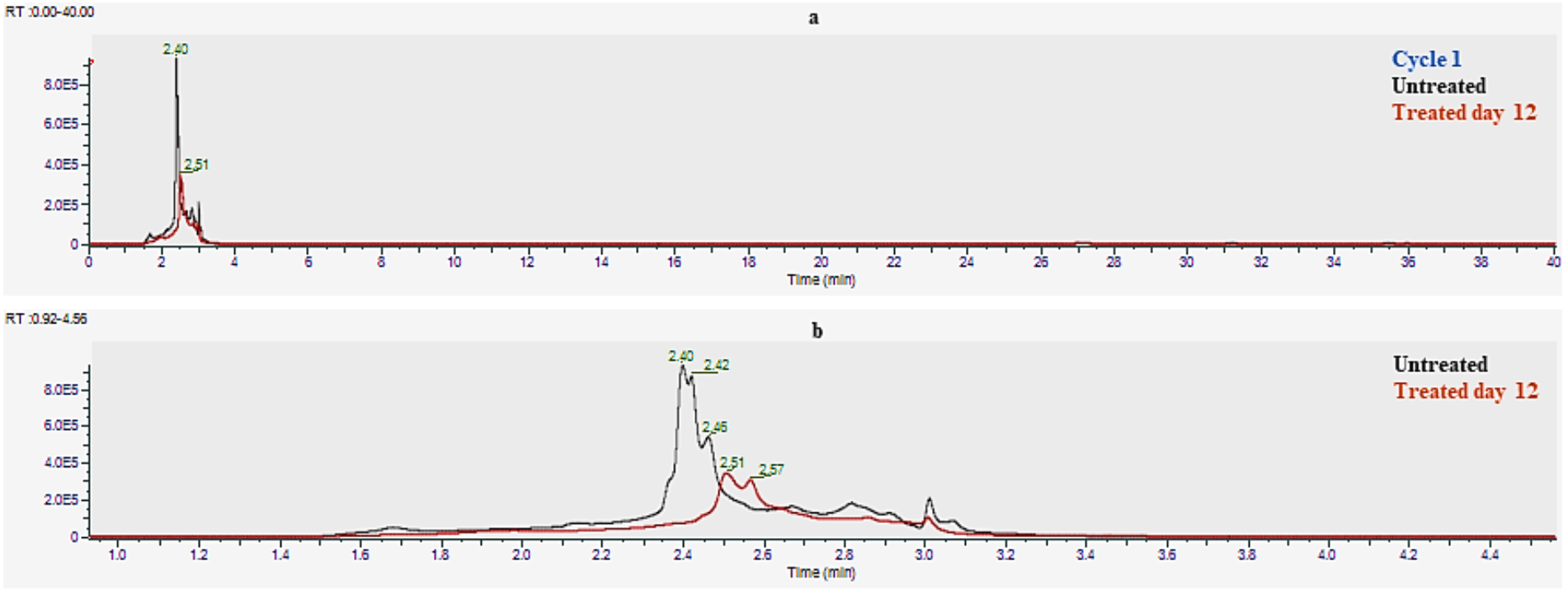


Figure 5.2 -Chromatogram overlay of spent wastewater media samples from cycle 1 wastewater treatment experiment comparing lignin content in untreated and treated samples (a) Complete chromatogram profile (b) Enlarged peak area

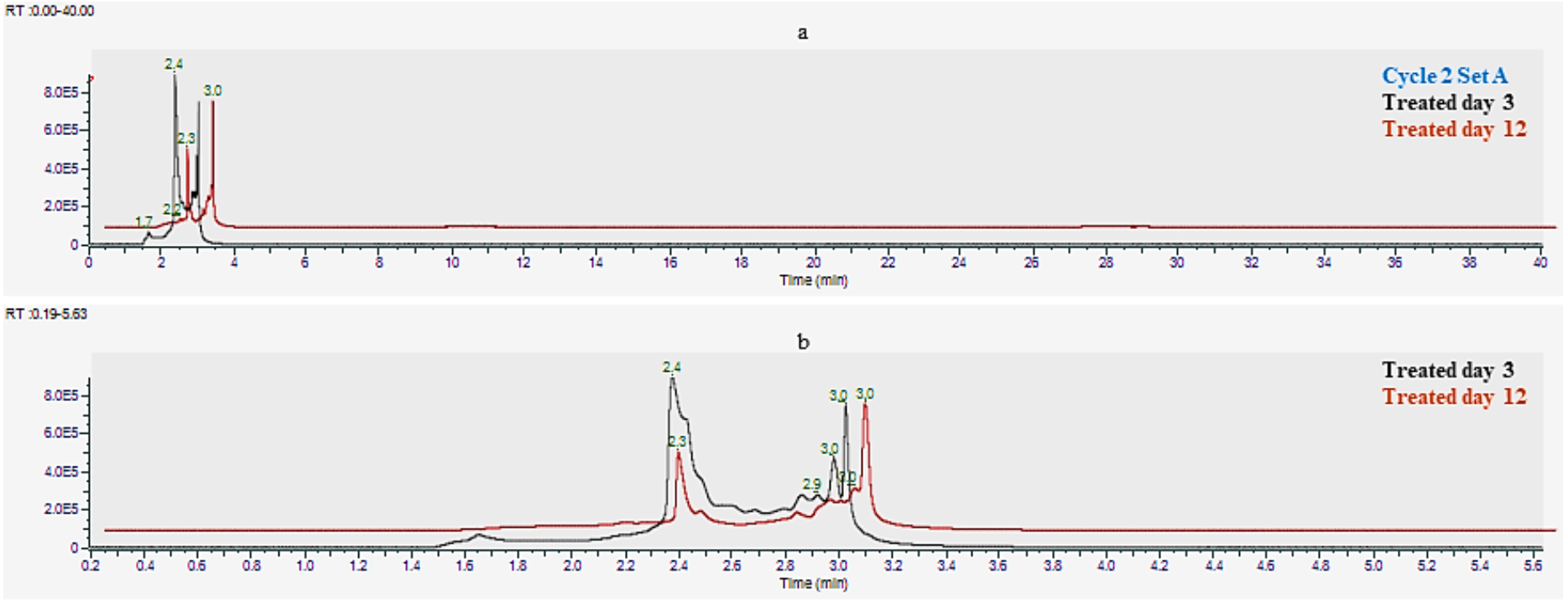


Figure 5.3-Chromatogram overlay of spent wastewater media samples from Cycle 2 (Set A) wastewater treatment experiment comparing lignin content in untreated and treated samples comparing day 3 treated samples (black) with day 16 treated samples (red) (a) Complete profile (b) Enlarged peak area.

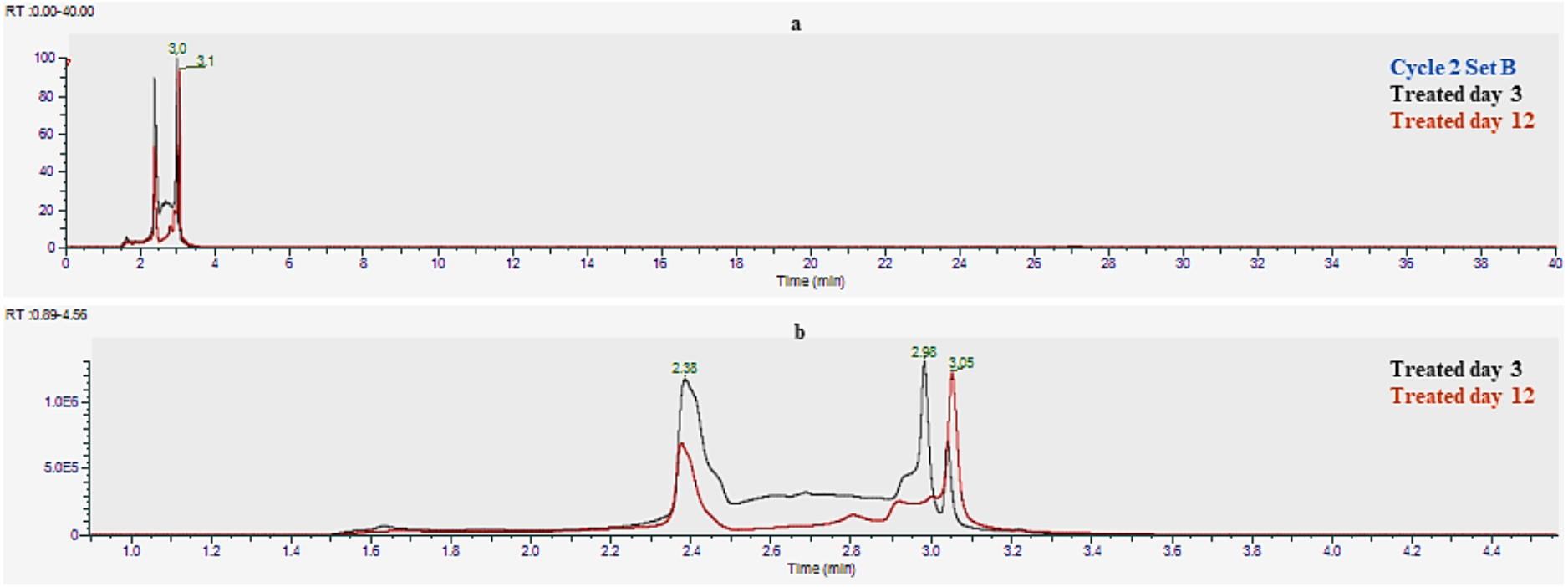


Figure 5.4 - Chromatogram overlay of spent wastewater media samples from Cycle 2 (Set B) wastewater treatment experiment comparing lignin content in untreated and treated samples comparing day 3 treated samples (black) with day 16 treated samples (red) (a) Completed profile (b) Enlarged peak area.

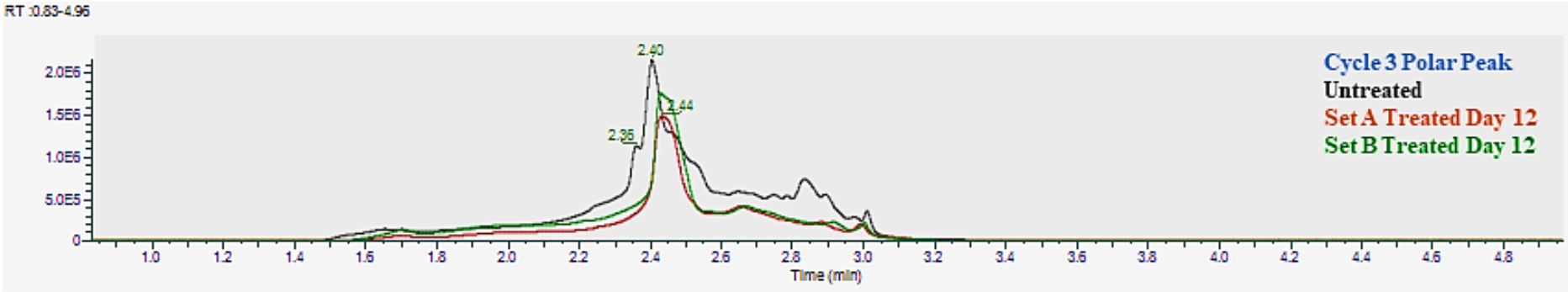


Figure 5.5 - Chromatogram overlap of Cycle 3 Set A and B spent wastewater media comparing lignin content in untreated samples (day 0) with fungal treated samples from day 12.

5.3.3 Chemical Oxygen Demand Removal Efficiency

During the first cycle of biofilm growth (Cycle 1), the removal of COD was 50 +/- 0.5 %, as shown in Figure 5.6. The Cycle 2 wastewater treatment resulted in a significant difference ($p<0.05$) in COD removal efficiencies between Set A and B, resulting in a higher COD removal efficiency of 78.36% in Set A compared to 40% in Set B on day 16 from the biofilm transplant. A strong correlation was found between the removal efficiencies of lignin and COD (R-0.88; $p < 0.05$) in Cycle 2 wastewater treatment. The presence of sucrose significantly increases the efficiency of COD removal in cultures. However, in the sucrose-supplemented cultures, lignin removal efficiencies were high too. This indicates that the removal of lignin is the primary factor in achieving higher COD removal, while the presence of sucrose plays a secondary role. The COD removal efficiency was more elevated in Cycle 2 with sucrose supplementation compared to Cycle 1. The COD removal efficiency trend differed in Cycle 3 wastewater treatment. Set B had a higher efficiency of 59.24 +/- 2.3 %, while Set A had a lower efficiency of 47 +/- 0.5 %. This trend in COD removal is inversely related to lignin removal efficiency during Cycle 3 wastewater treatment. This suggests that in Cycle 3 Set B cultures, biofilms consumed sucrose preferentially for growth instead of lignin while recovering from poor growth.

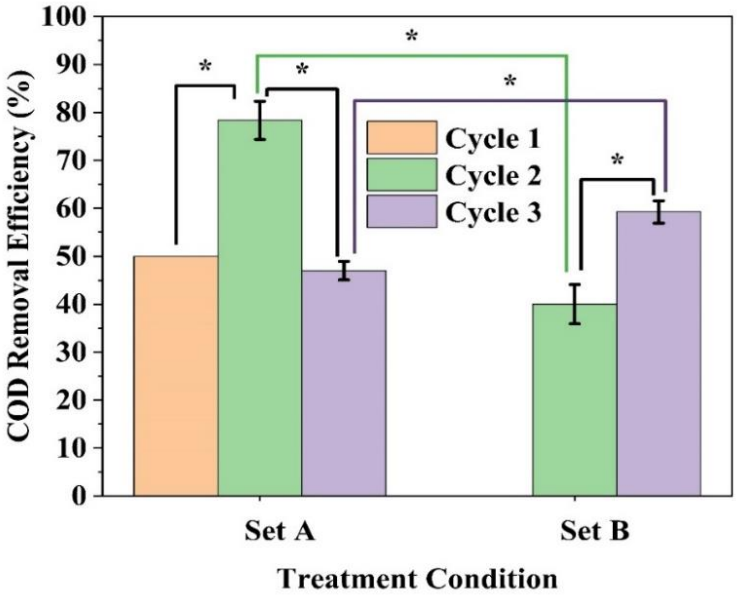


Figure 5.6 - COD Removal efficiency (%) for Cycles 1, 2 and 3 wastewater selectively supplemented with sucrose (Set A and B). Significant difference is denoted by an asterisk (*) where $p<0.05$ as determined by the one-way ANOVA and tukey test. Significant difference is denoted by an asterisk (*) where $p<0.05$.

5.3.4 Activity of Lignin-Degrading Enzymes

5.3.4.1 Laccase Activity

Enzyme activity for laccase, PPO and VPO was monitored over 36 days from inoculation. It has become a common notion that most fungi produce laccase because it has been demonstrated in several fungi (Brijwani, Rigdon and Vadlani, 2010); however, for *N. discreta,* laccase activity was not seen in any of the previous studies. Initially, during the biofilm growth phase (Cycle 1), no laccase activity was seen in any of the reactors but was noted from the beginning of Cycle 2, as shown in Figure 5.7. The addition of xenobiotic compounds, such as lignin, xylidine and veratryl alcohol, induces lacca(Pazarlıoğlu, Sariişik and Telefoncu, 2005)(Pazarlıoğlu, Sariişik and Telefoncu, 2005). The appearance of laccase activity in Cycle 2 batch treatment was due to the introduction of untreated lignin in fresh wastewater. In another study, the addition of copper sulphate in the sucrose-grown *Pleurotus sajor-caju* was also reported to play a role in the induction of laccase (Bettin *et al.*, 2014). We

had already investigated the effect of copper sulphate in the optimisation study and had found $CuSO_4$ had an impact on PPO and VPO activity during the growth phase when supplemented in the wastewater media initially at the time of inoculation. However, adding $CuSO_4$ in fresh wastewater media on day 13 must have induced laccase. It can be inferred that adding new wastewater containing lignin and copper sulphate induced laccase in *N. discreta* biofilms, resulting in this study's first recorded laccase activity. This also suggests that laccase in *N. discreta* is not a growth-associated enzyme but is induced in suitable environmental conditions.

During the second cycle of wastewater treatment (Cycle 2), laccase activity was noted from day 7 onwards but at a minimal level in both conditions (0.0028 UL^{-1} and 0.003 UL^{-1}). On day 9, the laccase activity was significantly high ($p<0.05$) in Set A cultures supplemented with sucrose, showing an 82-fold increase compared with Set B cultures, showing only a 1.8-fold increase. The addition of sucrose, in Set A cultures, led to increased laccase titers, which is consistent with a previous study where the induction of consecutive laccases in the ascomycetes *Botryosphaeria* sp was observed after the addition of various carbohydrates as the sole carbon source (Alves da Cunha *et al.,* 2003). Henceforth, the laccase activity lowered for both the experiment sets, which was because of the consumption of total sucrose and the formation of lignin degradation intermediate products that are previously reported as inhibitors of laccase enzyme (Pamidipati and Ahmed, 2020). Cycle 3 laccase activity was noted from day 5 onwards, with a significant ($p < 0.05$) increase at the end of the process on day 12. In Cycle 3 treatment, both the Sets were supplemented with sucrose- Set A cultures had a 5.16-fold increase compared to Set B, which showed a higher 13.72-fold increase.

From these results, it can be explained that the presence of sucrose played an essential role in increasing the laccase activity, resulting in higher laccase activity for sucrose-supplemented cultures. The production of fungal laccase is reported to be affected negatively by excess

sucrose by another author, as it allows the constitutive production of enzymes but represses the induction (Brijwani, Rigdon and Vadlani, 2010). Sucrose-starved biofilms showed a rapid increase in laccase activity when supplemented with sucrose in the last cycle. However, it was noted that the dry biomass of biofilms grown in sucrose-supplemented batches throughout was more (0.91 gg^{-1}) than the cultures with intermittent no sucrose (0.27 gg^{-1}). It was observed that laccase activity per unit of dry biomass was higher in cultures exposed to irregular periods without sucrose supplementation. Details of biomass wet and dry weight are explained in section 5.3.6.1 below. However, the lignin degradation during the Cycle 3 experiment was at the lowest in all the treatment cycles, with 18.67% for Set A) and 22.92% for Set B. Hence, it can be noted that laccase did not have a vital role in lignin degradation.

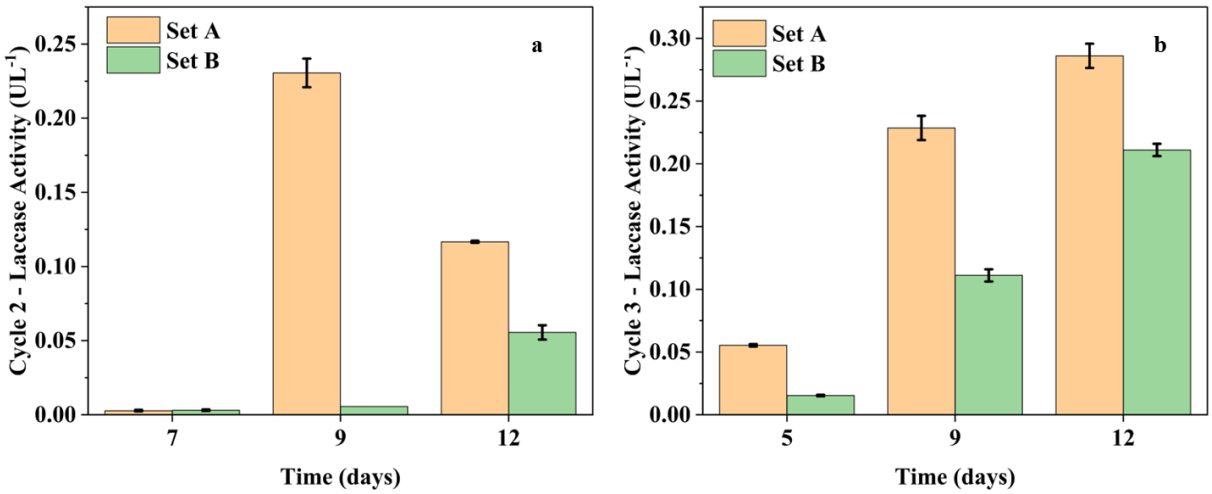


Figure 5.7- Laccase Activity (UL^{-1}) for cultures supplemented (Set A) and deficient of sucrose in Cycle 2 (Set B) (a) Cycle 2 batch treatment with selective supplementation of sucrose in wastewater medium (b) Cycle 3 of wastewater treatment supplemented with sucrose in all cycles (Set A) and subjected to intermittent no sucrose supplementation (Set B).

5.3.4.2 Polyphenol Oxidase Activity

Polyphenol Oxidase Activity (Figure 5.8) was noted from the Cycle 1 experiment onwards, showing a 5.59-fold increase compared to the initial level. The Cycle 2 experiments had a linear increasing trend and showed more activity for sucrose-supplemented cultures (Figure 5.8 b). PPO activity was observed in both sets on the biofilm transplant's third day.

The cultures in Set A, supplemented with sucrose, had a 2.6-fold increase in PPO activity compared to a 6.25-fold increase in Set B cultures, which were sucrose deficient. The PPO activity per unit biomass was calculated to be higher for Set B. However, due to the slow metabolism of lignin in the absence of sucrose, the enzyme produced per unit spent media would have resulted in lower activity. Another author has reported that sucrose does not affect PPO activity; however, adding a higher level of sucrose (10%) resulted in the inactivation of PPO (Haddouche, Phalak and Tikekar, 2015).

The lignin degradation efficiencies in Cycle 2 treatment showed a positive correlation with the PPO activity (R-0.83; $p < 0.05$), suggesting PPO to be the most important for lignin degradation by *N. discreta* biofilms. Cycle 3 PPO activity (Figure 5.8 c) was noted from the day of biofilm transplantation as the biofilms were actively producing the enzyme, which could have diffused into the wastewater media after transplant. The PPO activity in Set A cultures had a 13.02-fold increase. However, Set B cultures showed a consistent PPO activity with only a 2.49-fold increase. In Set B, the PPO activity ($p < 0.05$) remained significantly low throughout Cycle 3 compared to Cycle 2. The lignin removal efficiency in Set B also showed the same trend, with significantly lower lignin removal noted for Cycle 3. This could be due to enzyme inactivation caused by the formation of in-process enzyme inhibitors (Aitken, Venkatadri and Irvine, 1989). In a study by Dosoretz et al., a decline in lignin peroxidase activity in *Phanerophyte crysosporium-treated* cultures correlated with extracellular protease appearance in glucose-limited cultures (Dosoretz *et al.,* 1990) which could be another reason for low PPO activity in Set B cultures.

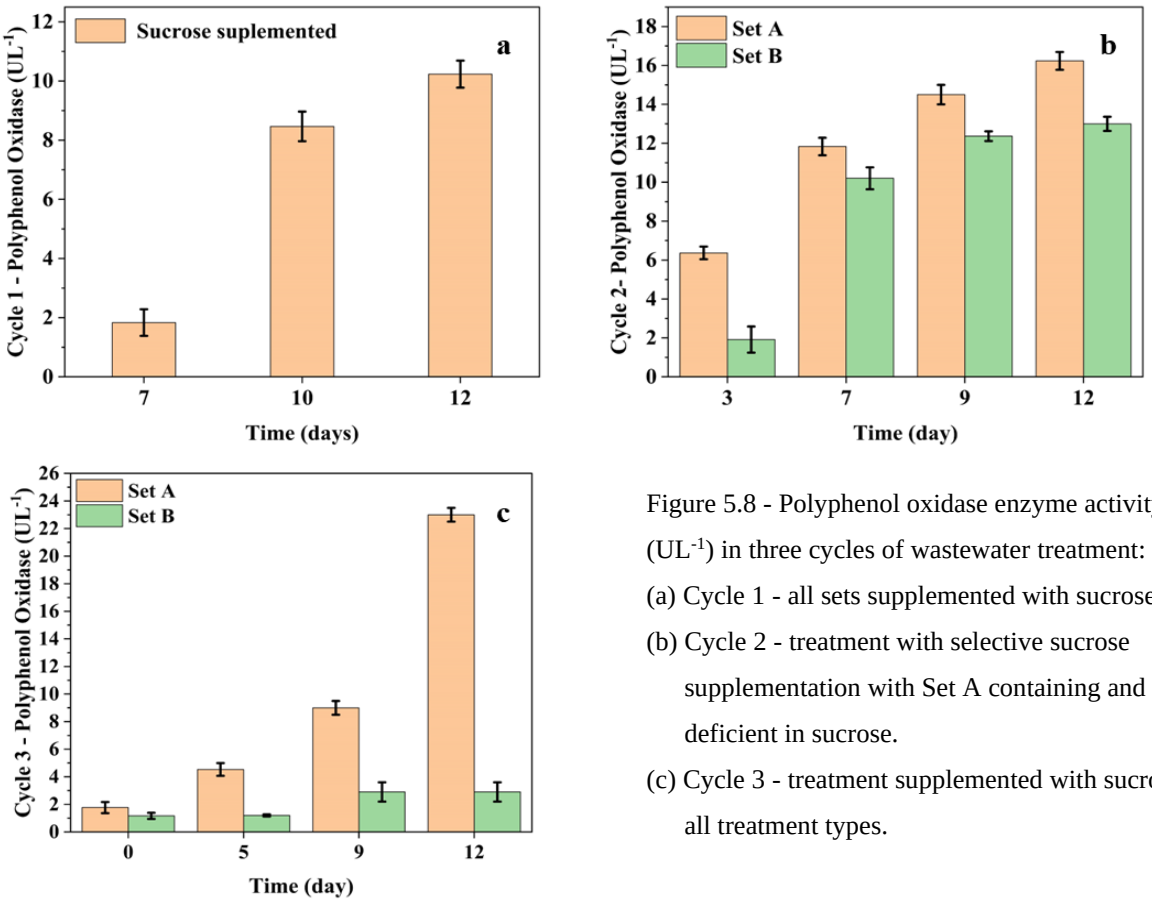


Figure 5.8 - Polyphenol oxidase enzyme activity (UL^{-1}) in three cycles of wastewater treatment:
(a) Cycle 1 - all sets supplemented with sucrose
(b) Cycle 2 - treatment with selective sucrose supplementation with Set A containing and Set B deficient in sucrose.
(c) Cycle 3 - treatment supplemented with sucrose in all treatment types.

5.3.4.3 Versatile Peroxidase Activity

As shown in Figure 5.9 a, the versatile peroxidase activity during Cycle 1 showed a 3.3-fold increase. During Cycle 2 (Figure 5.9 b), the VPO activity seen after the biofilm transplant in sucrose-supplemented cultures shows a nonlinear trend with the decline in activity from day 3 till day 9; however, the activity increased on day 12, showing a 2-fold increase. This is because the biofilms adjusted to the culture conditions and increased activity in nutrient-limiting states. The Set B cultures that were sucrose deficient showed consistent VPO activity throughout the process with a 1.6-fold increase - a contrasting trend for the Cycle 3 experiment. In Cycle 3, though Set A showed higher activity than Set B, there was an overall decrease in VPO activity, with a 2.8-fold and 2.5-fold reduction in Set A and Set B, respectively. Since the biofilms were actively producing the enzymes at the beginning of

Cycle 3, the reasons that could have lowered the activity could be the formation of intermediate products (Pamidipati and Ahmed, 2020), which inhibited the enzyme activity or due to the accumulation of toxins as the biofilms matured.

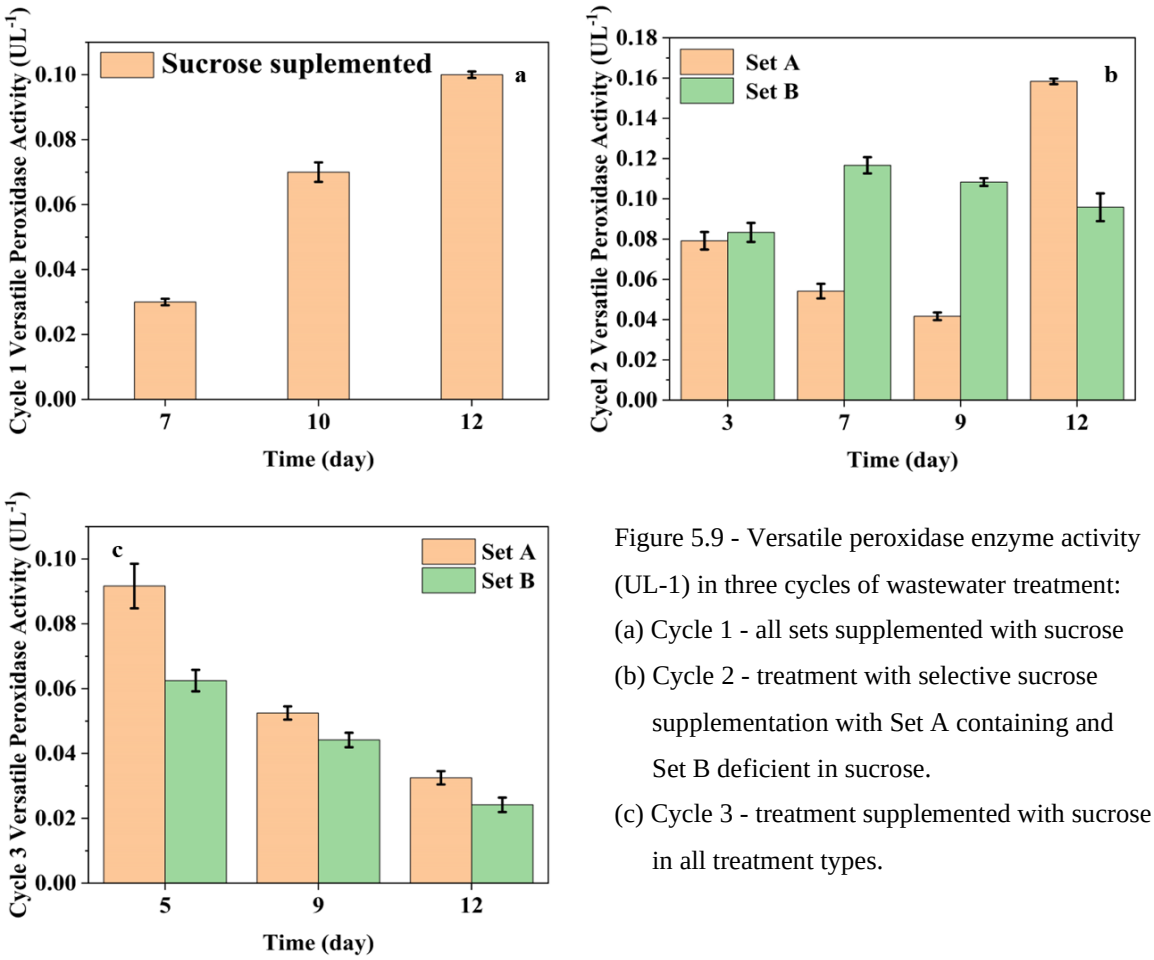


Figure 5.9 - Versatile peroxidase enzyme activity (UL-1) in three cycles of wastewater treatment:
(a) Cycle 1 - all sets supplemented with sucrose
(b) Cycle 2 - treatment with selective sucrose supplementation with Set A containing and Set B deficient in sucrose.
(c) Cycle 3 - treatment supplemented with sucrose in all treatment types.

5.3.5 Biofilm Characterisation

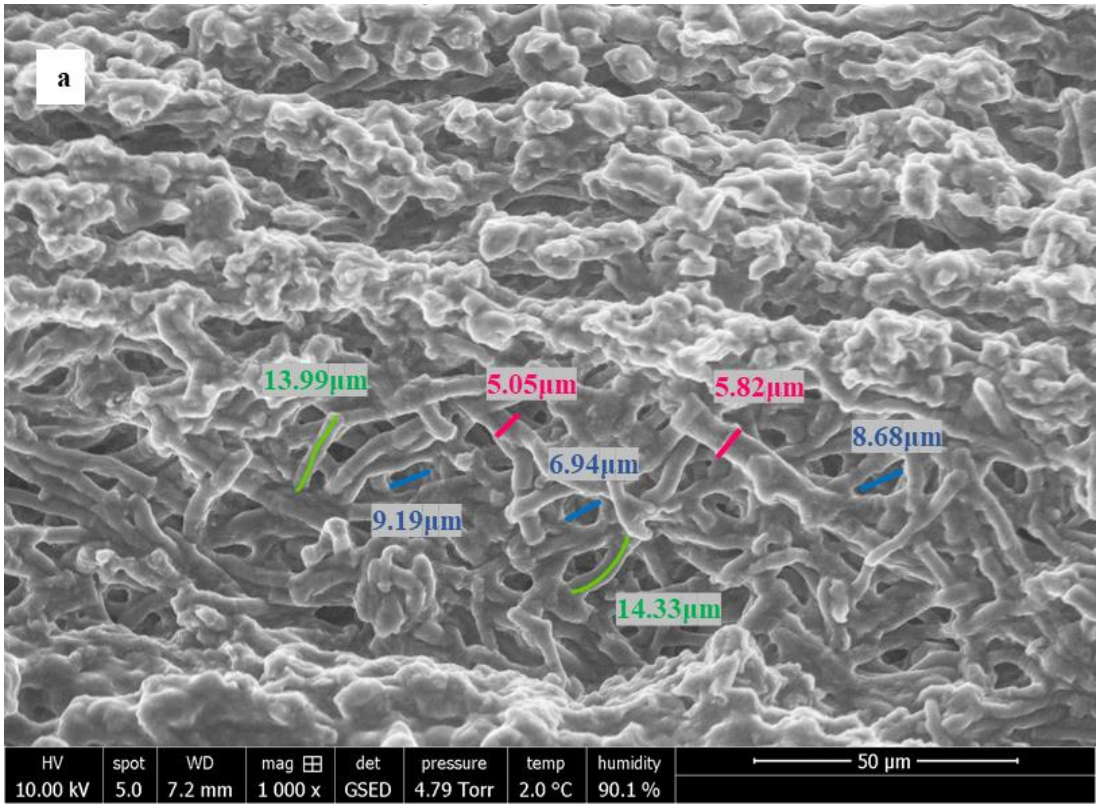
5.3.5.1 Biofilm Properties and Extracellular Matrix Components

The biofilms were harvested on day 36 from inoculation. The biofilm dry biomass and water retention value were analysed and quantified, as shown in Figure 5.10. It was observed that the biofilms harvested from the Set A treatment were heavier, thicker and stronger than the Set B biofilms. Additionally, the dry biomass in Set A biofilms was 3.3 times higher than in Set B. Sucrose is metabolised faster than lignin due to its simple structure. The ease of sucrose degradability also leads to cell growth, which explains the greater biomass in Set A

biofilms. Previous studies on *N. discreta* biofilm show that carbon sources influence biofilm properties such as porosity, water retention value and extracellular matrix (Ahmed, Narayanan and Veni, 2020; Tabraiz *et al.,* 2022). As sucrose-deficient conditions are created in Cycle 2 for the Set B treatment, the metabolic energy is diverted into carbohydrate production rather than cell growth (Rughoonundun, Mohee and Holtzapple, 2012). The EPS extracted from the biofilms was analysed chemically to quantify the polysaccharide and protein concentrations. The concentration of polysaccharides per unit dry biomass in Set A was significantly lower ($p < 0.05$), which was 1.6-fold lower than for Set B. The sucrose-deficient conditions created in the Cycle 2 Set B experiment resulted in nutrient-limiting conditions, which led to the secretion of secondary metabolites as EPS (Tabraiz *et al.,* 2022). Biofilm EPS containing more polysaccharide content absorbs more water, resulting in higher WRV (Rughoonundun, Mohee and Holtzapple, 2012). The WRV of Set A biofilms was 92.09 %. In comparison, Set B biofilms had 96.38 %, which supports the theory about polysaccharides being hydrophilic and adsorb water to the biofilms, making them more fluid like.

5.3.5.2 Biofilm Microstructure

The microstructures of the biofilms can be observed in Figure 5.11 through microscopic images. The microstructural features such as pore diameter, filament length, and thickness were distinguishable in the images analysed using ImageJ software. The biofilms in Set A (Figure 5.11a) exhibit thicker, denser, and globular filaments with shorter lengths and smaller pore areas. On the other hand, Set B biofilms showed long, slender filaments and significantly larger ($p<0.05$) pore areas. Unlike bacteria, fungal cells are immotile, and filament growth is stimulated in nutrient-limiting conditions, resulting in longer filaments. It has been established that long filaments also contribute to larger pore areas (Aravinda Narayanan and Ahmed, 2019).



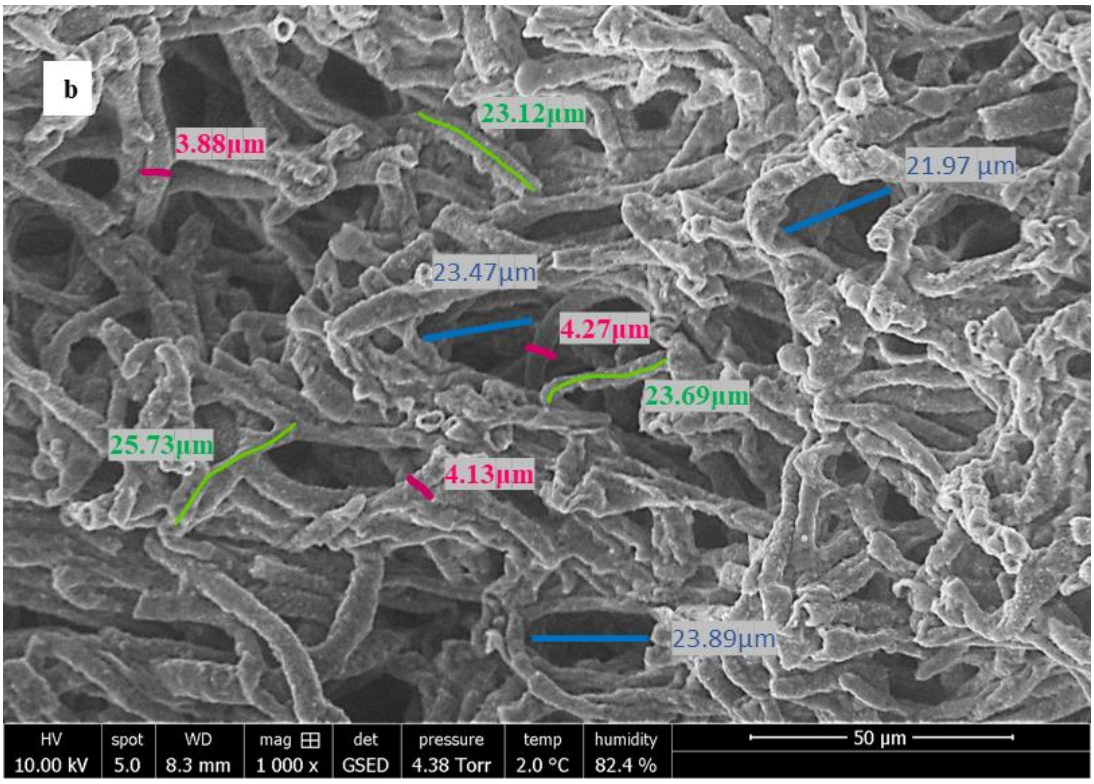


Figure 5.11– Scanning electron microscopy images of biofilms in native condition are presented. The images depict the pore diameter in blue, filament length in green and filament thickness in pink for biofilms harvested from Set A (a) and Set B (b) reactors after the third Cycle of wastewater treatment. The images are analysed using ImageJ application.

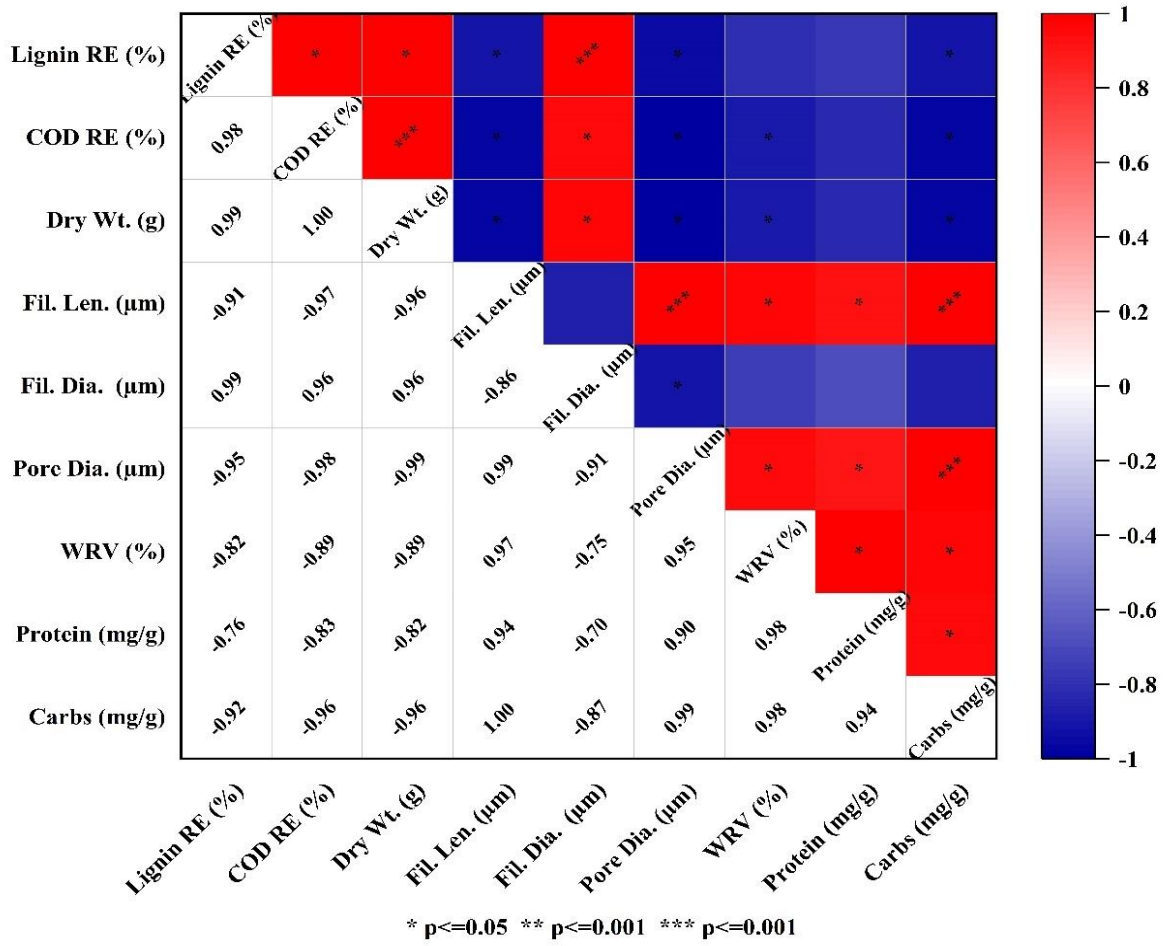


Figure 5.12 - Pearson's correlation plot showing the correlations between lignin removal efficiency (Lignin RE, %). COD removal efficiency (COD RE, %), filament length (Fil. Len., µm), filament diameter (Fil. Dia., µm), pore diameter (Pore Dia., µm), water retention value (WRV, %), proteins (Protein, mg/g) and polysaccharides (Carbs, mg/g). The asterisks in the upper triangle signify the variables to have a significant correlation and the colours denote the positive (red) and negative (blue) correlation. A statistically significant difference represented by asterisk *=($p<0.05$), ** = ($p<0.01$), *** = ($p<0.005$). The Pearson's coefficient values (R) are presented as the labels in the lower triangle.

5.3.6 Correlations Between Process Efficiencies and Biofilm Properties

This section presents the correlation between the biofilm properties and the lignin and COD removal efficiencies with the biofilm characteristics such as filament length, diameter, and total pore diameter. The water retention value and the biofilm ECM's protein content correlate. In Pearson's Correlation plot Figure 5.12, the lignin and COD removal efficiencies

for Cycle 2 strongly correlated (R - 0.98, $p < 0.05$). The lignin and COD removal efficiency also positively correlated with the biofilm dry biomass and filament thickness, which was because the Set A cultures, which were supplemented with sucrose, encouraged cell growth and showed significantly higher lignin removal due to more biofilm biomass present in the cultures. An inverse correlation with filament length was noted as the cultures deficient in sucrose had longer filaments due to the formation of nutrient-limiting conditions. The filaments grow longer to reach out to nutrients. In Set B, the nutrient conditions forced filaments to grow longer, and the pollutant removal efficiencies were lower. Filament length strongly correlated with pore diameter, water retention value and the ECM protein content (Ahmed, Narayanan and Veni, 2020).

5.4 Conclusion

This study evaluated the extended HRT of *N. discreta* biofilms to note its effect on lignin and COD removal efficiency. Laccase activity was seen for the first time in *N. discreta* biofilms when fresh wastewater containing lignin was added to the cultures. Laccase production could have been induced by lignin in new wastewater, which has been reported as ligninolytic enzyme inducers. The trend in lignin removal efficiency and COD correlates (0.98, $p<0.05$) with the polyphenol oxidase enzyme activity. The chromatograms obtained from wastewater samples treated with biofilms grown in cultures continuously supplemented with sucrose in all batches showed new peaks drifting towards the polar side of the chromatograms, suggesting continuous degradation. Longer HRT (36 days) resulted in a more stable process, which showed a 12.5-fold increase in PPO and a 44-fold increase in Laccase activity. The process resulted in significantly high ($p<0.05$) PPO enzyme activity compared to the initial cycles along with activation of Laccase. These promising results can be applied to scale up in non-sterile wastewater treatment conditions. The significantly lower lignin and COD removal efficiencies noted in Cycle 3 were due to the formation of multiple layers of biofilms, which

restricted the diffusion of wastewater and mass transfer. It is important to note that the initial lignin concentration at the beginning of Cycle 2 was higher than the previous cycle. This can be interpreted as sucrose helping the cultures increase biomass. Still, the influential role was the addition of lignin and longer HRT that induced more ligninolytic enzymes and resulted in more lignin and COD degradation.

---

6. Evaluation of a Scale-Up Process for the Treatment of Lignin-Rich Wastewater by *Neurospora discreta* Biofilms in a Repeated-Batch System

6.1 Introduction

Large-scale wastewater treatment is explored in systems for practical significance and requires detailed study before its transfer to an industrial scale. Up-scaling the process in non-sterile conditions is critical to getting a more realistic picture and technological advancements of a biological process (Ryan, Leukes and Burton, 2005). Before implementing the treatment process at an industrial level, it is necessary to develop fungal biofilm reactors that can be operated under industrial conditions using natural wastewater. The wastewater is autoclaved at lab-scale, and an aseptic technique is used; however, at an industrial level, the conditions are the opposite (Blánquez, Sarrà and Vicent, 2008). Since sterilisation is an energy-intensive and time-consuming process, it is not feasible to autoclave large volumes of wastewater**.** A lack of suitable and cost-effective methods hinders the large-scale application of fungal biofilm treatment.

Many of the extracellular ligninolytic enzyme-producing higher fungi, such as *Phanerochaete chrysosporium* (Kiran *et al.*, 2019), *Neurospora crassa* (Luke and Burton, 2001), *Pleurotus* species (Ragunathan and Swaminathan, 2004), have been explored for the treatment of lignin-rich wastewater at lab scale. However, use of fungi in large-scale wastewater treatment is complex and has not been explored yet (Ehaliotis *et al.*, 1999). One of the challenges for a biological process in non-sterilized conditions is to adapt the cells to outcompete other microbial contaminants. Additionally, bacterial communities present in the non-sterilized wastewater may change the metabolism and fungal growth (Svobodová and Novotný, 2018). Although fungal cells and biofilms are resilient to pollution and bacterial contamination, they are impacted by changes in cellular metabolism, resulting in low pollutant removal efficiencies (Edwards and Kjellerup, 2013).

Under non-sterilised conditions, a stable wastewater treatment process requires high biomass concentration (Nicolella, Van Loosdrecht and Heijnen, 2000). For the maintenance of steady fungal biofilm growth, nutrient feeding is essential. The concentration of the biofilm biomass is dependent on the concentration of the substrate in the wastewater. Once the high biomass concentration is reached, it can be maintained for reuse instead of re-initiating the process from inoculation. Since the biofilm-based wastewater treatment has two distinct phases - biofilm and culture medium - the biofilm biomass can be easily separated from the treated wastewater medium. The biofilm biomass can be retained in the bioreactor and fed repeatedly with a fresh, nutrient-rich wastewater medium for further treatment while treating bulk volumes of wastewater.

Another limitation of the fungal biological treatment processes is that the biomass and metabolic activity depend on incubation temperature (Akpor, Adelani-Akande and Aderiye, 2013). Process economics are improved if the wastewater treatment is at room temperature. *N. discreta* is believed to have greater genetic diversity among all the known *Neurospora* species (Dettman, Jacobson and Taylor, 2006). The fungus was found under the bark of trees damaged by wood fire, showing that it can withstand extreme temperatures and readily adapt to environmental changes (Romero-Olivares, Taylor and Treseder, 2015). One of the studies reported that *N. discreta* strains tested grew faster at warmer temperatures than colder ones (Romero-Olivares, Taylor and Treseder, 2015). However, low temperatures are said to lower the organic pollutant removal efficiencies than nutrient removal efficiency (Sankaran *et al.*, 2010). The studies reported in the previous chapters were conducted at 30^{o} C. Testing the fungal biofilm treatment at room temperature is essential to improve process economics.

Several large-scale biofilm-based biotechnology processes using artificially immobilised cells have been reported for wastewater treatment (Wu, Xiao and Yu, 2005; Moga *et al.*, 2019). However, the naturally formed biofilms have not been reported for treatment in repeated-

batch systems for large-scale wastewater systems till now. The potential of utilising the fungal biofilms formed by *N. discreta* on a large scale must be explored.

Repeated fed-batch wastewater treatment was tested previously. The method reuses biofilms retained in the reactor for multiple cycles. The fresh wastewater rich in nutrients will maintain the stable fungal biomass and simultaneous removal of lignin and COD. The seeding medium is also known to influence biofilm growth and efficiency. It was hypothesised that the repeated-batch treatment would maintain a high biomass, increasing extracellular enzyme production in non-sterilised wastewater treatment. *Neurospora discreta* biofilms were evaluated for lignin and COD removal and stable biomass maintenance using non-sterilized wastewater as a growth medium at room temperature. In the previous study, the static-repeated-batch wastewater treatment in sterile conditions at 30^{o} C resulted in an efficient, stable process with an extended 36 days of biofilm biomass retention at a flask scale. The process was upscaled in a tray reactor in a non-sterile ambient (22^{o} C) environment. The scale-up process under non-sterilised conditions was investigated by varying the wastewater media composition in the three treatment cycles.

6.2 Materials and Methods

6.2.1 Fermentation Setup in Tray Reactors

In this study, bench-top tray bioreactors with large surface areas were chosen (Figure 6.1). The reactors were set up in polypropylene trays (Thermo Scientific™ Catalog number: 6910-0618). The dimensions of the tray were 46 x 15 x 6.7 cm (length x width x height). Two tray reactors were set up; the total volume of the wastewater treated in the tray bioreactor was 800 cm^3 (ml) each. The surface area of the biofilms formed on the liquid-air interface was 108 cm^2. The control reactors were set in 250 ml Erlenmeyer flasks with a media volume of 100 cm^3 (ml) for each experiment set. The tray reactors were covered with loosely fit

polypropylene tray lids, and the Erlenmeyer flasks were plugged with foam bungs covered with aluminium foil. The reactors were not incubated and were left at room temperature (22^{o} C) on a clean laboratory bench top in a static state.

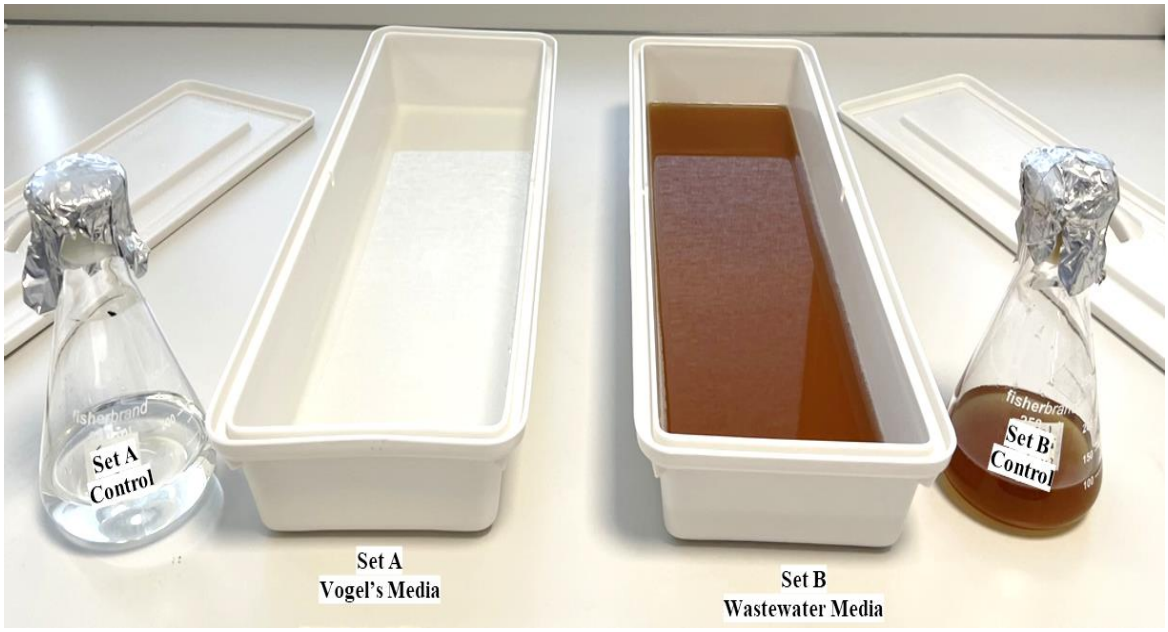


Figure 6.1 - Fermentation setup for repeated batch treatment of wastewater. Erlenmeyer flasks were set as controls for each experiment set. Biofilm treatment was investigated by comparing the initial growth media, Vogels media (Set A) and wastewater media (Set B) by inoculating *N. discreta* spores.

6.2.2 Wastewater Media

Wastewater provided by a paper-making company, Mondi Frantschach, was used for the study reported here (Section 2.4). The initial concentration of lignin was 1500 mgL^{-1}. To test the biofilm treatment as a repeated-batch process, three wastewater media batches were prepared by diluting and supplementing the industrial wastewater with Vogel's media components and sucrose (Table 6.1). The wastewater media used throughout the experiments was not sterilised. The first cycle (Cycle 1) of the experiment was conducted to grow the biofilms in different growth media. In the first set of reactors, Set A (flask-100 ml and tray reactors- 800 ml), the biofilms were grown by inoculating Vogel's media with *N. discreta* spores. In Set B (flask-100 ml and tray-800 ml reactors), the biofilms were grown by

inoculating industrial wastewater containing 500 mgL^{-1} of lignin, supplemented with Vogel's media components and 1000 mgL^{-1} of sucrose (Section 2.3).

For Cycle 2 of wastewater treatment, the treated wastewater was drawn out from under the fully-formed biofilms 12 days after inoculation and industrial wastewater containing 1000 mgL^{-1} lignin and 1000 mgL^{-1} sucrose and supplemented with components of Vogel's media (section 2.2) was added. The with the same composition was added to all the reactors containing the full-grown biofilms. On day 12 of treatment, the treated wastewater media was drawn out. For the third cycle (Cycle 3) of wastewater treatment, the lignin concentration in wastewater was 1500 $mgL^{-1,}$ supplemented with 1000 mgL^{-1} of sucrose and Vogel's media component, as mentioned in section 2.2. The reactors were sampled regularly during all the repeated-batch treatments.

Table 6.1 - Experiment design with concentrations of lignin and sucrose in wastewater media used in Cycle 1, 2 and 3 and in Sets A and B of experiments.

Cycle	**Set**	**HRT (days)**	**Initial Lignin (mgL^{-1})**	**Initial Sucrose (mgL^{-1})**
1	**A**	12	-	1000
1	**B**	12	500	1000
2	**A/B**	12	1000	1000
3	**A/B**	12	1500	1000

6.2.3 Inoculation

The spore suspension was prepared on the fifth day of streaking on the PDA plate using the aseptic technique (Section 2.5). The spores were collected in full minimal media and filtered through a muslin cloth to obtain a spore suspension of 10^7 spores ml^{-1}.

6.2.4 Lignin Estimation

The biofilm-treated wastewater samples were first centrifuged after raising the pH to 10.5, followed by centrifuging at 5000 RCF for 30 mins (Section 2.9). The supernatant was

collected and acidified to precipitate the lignin. The biomass formed a pellet and was discarded. The supernatant containing all the lignin was acidified with conc. H_2SO_4 to drop pH below 2 and the lignin as precipitated and left to dry. The completely dried lignin was redissolved in DI water and was used for HPLC analysis on a C-18 column using a water-acetonitrile system using RP-HPLC. The lignin estimation for all the batches of wastewater treatment was measured using the HPLC method mentioned in section 2.9.2. The lignin estimation was done before and after fungal treatment, and the lignin removal efficiency was calculated according to equation (6.1) by comparing the lignin concentration estimated using the chromatogram peak formed by samples. For data representation, the HPLC chromatograms were overlapped using Freestyle software.

$$Lignin\ Removal\ Efficiency(\%) = \frac{Lignin\ conc.(start) - Lignin\ conc.(end)}{Lignin\ conc.(start)} \times 100 \qquad (6.1)$$

6.2.5 Chemical Oxygen Demand

The chemical oxygen demand (COD) was measured according to the protocol mentioned in Section 2.10. The COD measured at the beginning of the individual batch experiment was compared with the COD measured at the end of the treatment to calculate the COD removal efficiency (equation 6.2).

$$COD\ Removal\ Efficiency\ (\%) = \frac{COD\ (start) - COD(end)}{COD\ (start)} \times 100 \qquad (6.2)$$

6.2.6 Enzyme Activity

The enzyme activity was measured according to the protocol mentioned in Section 2.14. Laccase activity was measured using ABTS as substrate in citrate buffer (Bourbonnais and Paice, 1990; Baldrian and Šnajdr, 2006).

ABTS oxidation by laccases leads to the formation of dark green cations. The spectral time scan is measured at 420 nm. The enzyme activity (1 Unit) is quantified using the extinction coefficient (ε420) 36000 M^{-1} cm^{-1} (Wang *et al.,* 2016; Saito *et al.,* 2003). The polyphenol oxidase activity was measured using a pyrocatechol solution (0.2 M) prepared in citrate buffer (0.05 M) at pH 5. One unit of enzyme was defined as the amount of enzyme that forms 1 mol of product per minute. The oxidation of reactive black dye (RB5) in sodium tartrate buffer 50 mM at pH 3 measured Versatile peroxidase activity. One unit of enzyme is defined as the amount of enzyme that transforms 1mol of substrate consumed.

6.2.7 Biofilm Characterisation

The biofilms were harvested on day 36 from inoculation and were first left on water-absorbing paper to drain excess water for 10 minutes. The known weight of biofilm samples was used to characterise biofilm properties using physio-chemically methods, as mentioned in Section 2.11. The biofilms were characterised for water retention value, dry weight per unit biomass, EPS polysaccharides and EPS proteins. The SEM microscope used was a Hitachi TM4000plus microscope, and the biofilms were imaged with the settings as voltage 15kV, magnification-X1.50 k, and Backscattered electron (BSE) mode.

6.3 Results and Discussion

6.3.1 Biofilm Growth

During Cycle 1, the biofilms started to form on the air-liquid interface after spore inoculation from the second day onwards. The biofilms continued to thicken by forming a new layer. However, the biofilms formed on Vogel's media (Set A) were thin, slimy, and fragile compared to the sturdy and thick biofilms formed on lignin-rich wastewater (Set B). This was due to the presence of hydrophobic suspended lignin particles in unfiltered wastewater that

act as carriers and provide extra support to the fungal spores. Studies show that thicker fungal biofilms are quicker on carriers in wastewater treatment processes (Zhang *et al.*, 2022). After the fresh media was fed to begin Cycle 2 of wastewater treatment, the Set A biofilms also started forming new layers and picked up growth. Set B became even thicker by forming new layers. Additionally, at the end of Cycle 3, the Set B biofilm in the tray reactor showed the formation of abundant spores on the surface (Figure 6.2). For *N. discreta* growth, previous studies report a positive correlation between biomass and spore production, which can explain the formation of abundant spores in the thicker biofilms in Set B (Romero-Olivares, Taylor and Treseder, 2015).

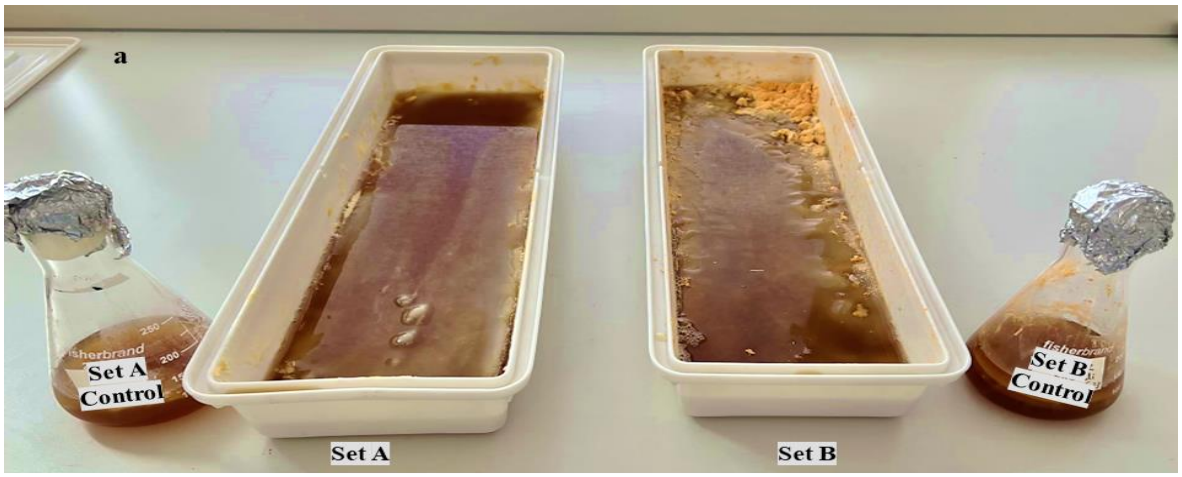


Figure 6.2 - Image of reactor set up showing biofilms at the beginning of Cycle 3 of wastewater treatment showing Sets A and B showing biofilms on the surface with abundant spores on the surface of biofilms in Set B reactors.

6.3.2 Lignin Estimation

6.3.2.1 UV-Vis Spectroscopy

The lignin removal efficiency (%) was calculated by comparing the untreated and treated samples, shown in Figure 6.3. For the Cycle 1 experiment- during the biofilm growth phase, the tray reactors showed much lower lignin removal (12.47 %) as the biofilms were seen much later (day 7) in comparison to flask reactors that showed better growth and degradation

(37.10 %). The delayed growth and lower lignin degradation efficiency can be attributed to the lower temperatures. In the previous studies, the wastewater treatment experiments were carried out at 30^{o} C in an incubator and showed a lignin removal efficiency of 42.3 +/- 1.22 % (Section 5.3.2). Low temperatures of 22^{o} C may have caused the lignin removal efficiency to be lowered by 5.2% in flask reactors. This result is in accordance with a study on *Neurospora intermedia*, which shows that high temperature had the highest positive impact in maximum colour removal at 30^{o} C in mill effluent (Kaushik and Thakur, 2009).

For the second round of treatment (Cycle 2), the Set B tray reactor achieved the maximum lignin removal efficiency of 63.39 %, followed by the Set A tray reactor, which showed 34.90 % lignin removal (Figure 6.3 a). A higher lignin removal efficiency was observed for Set B compared to Set A in flask reactors (53.80 %, vs 45.54 %, respectively) (Figure 6.3 a). The biofilms formed in Set A tray reactors at the end of Cycle 1 were poorly formed and showed void no growth area at the centre of the tray due to poor growth in Vogel's medium. During Cycle 2, the biomass in the Set A tray was lesser than in the Set B tray, which resulted in lower lignin removal efficiency in the Set A tray. Overall, in Cycle 2, lignin removal efficiency was higher for biofilm treatments in wastewater throughout (Set B), with tray reactors being the most efficient. Cycle 3 treatment showed lower lignin removal efficiencies (Figure 6.3 b) in all the reactors, following the same trend between Set A and B as in Cycle 2. During Cycle 3, the efficiencies of lignin degradation were lower than in Cycle 2. The Set B tray reactor showed a maximum lignin removal efficiency of 45.46 % compared to 37.56 % for the flask treatment. The Set B tray rectors showed a significantly higher ($p < 0.05$) degradation than the Set A tray. In a study conducted by Rajwar et al. (2017), it was found that fungal treatment of pulp and paper mill effluent resulted in significant lignin removal. The study showed that the second cycle had higher lignin removal rates than the first and third cycles, which had slower removal rates (Kaushik and Thakur, 2009).

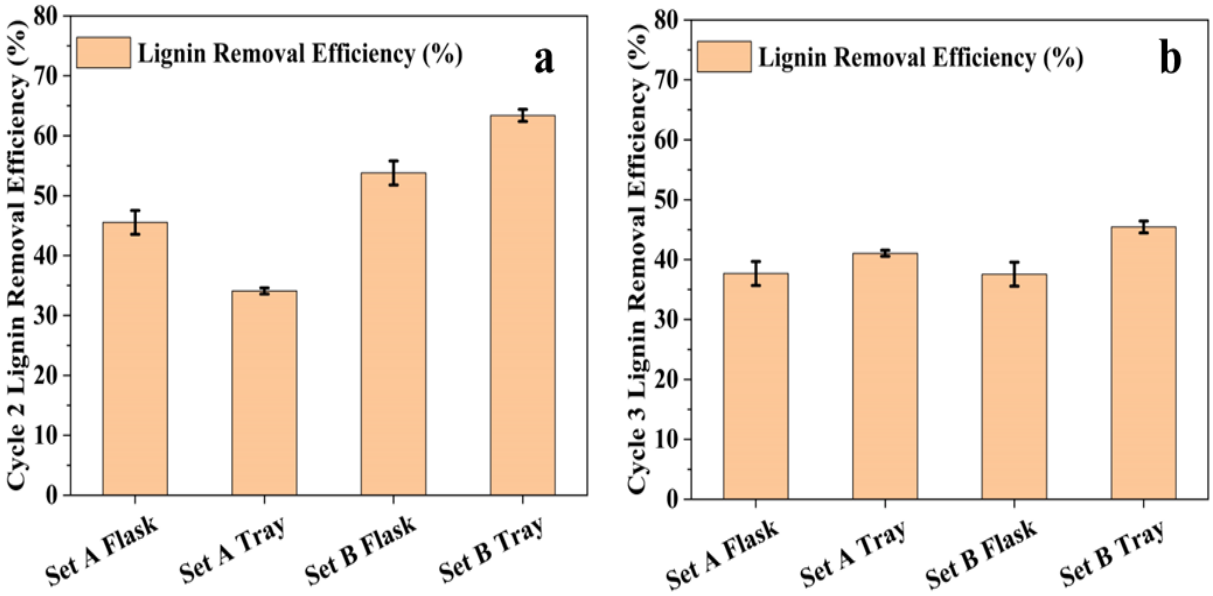


Figure 6.3 - Lignin removal efficiency (%) for biofilm treatment for comparing the treatment by biofilms grown in Vogel's media (Set A) and industrial wastewater (Set B) for: (a) Cycle 2 (b) Cycle 3

6.3.2.2 Reverse-Phase Liquid Chromatography

For the first wastewater treatment cycle, the lignin estimation was done for the experiment set containing lignin-rich industrial wastewater (Set B). Figure 6.4 a shows the chromatogram overlap of treated samples from day 12 from the flask and tray reactors compared to the untreated samples. The chromatograms showed a prominent polar peak at 2.4 minutes for all the samples, and no non-polar peak was seen (Figure 6.4 a). Compared to the kraft lignin standard (Section 2.9.2), no nonpolar peak was noted for any treatment cycles. Figure 6.4 b. shows the enlarged polar peak area of the chromatogram overlap. During the growth phase of biofilms (Cycle 1), the biofilms formed in flasks exhibited smaller peak areas compared to the larger peak areas seen for the trays. This indicates more significant lignin degradation in the flasks than in the tray. This could be because, in the tray, biofilms took longer to cover the liquid surface and were strong enough to maintain their integrity on day 7. Comparatively, flask reactors showed fully-formed biofilms earlier on day 5. This confirms that the growth rate of fungi is known to be affected by the temperature as it affects the microorganism's metabolism. In the case of *N. discreta*, the biomass is reported to be

affected by an increase in temperature, and so is the metabolic rate, showing a low growth rate at lower temperatures (Romero-Olivares, Taylor and Treseder, 2015). Figure 6.5a shows the chromatogram profile overlay for the second wastewater treatment cycle (Cycle 2) for Set A and B experiments. The significant peaks seen were polar but were split to form two peaks at RT 2.4 and 3.0 minutes. There were no nonpolar peaks seen. Figure 6.5 b shows the enlarged peak area comparing chromatograms of the spent wastewater from the tray and flask reactors. The total peak area for the untreated sample was 1.95E+07. The peak areas for Set A and Set B trays showed smaller peak areas for Set B tray (1.29E+07 and 7.19E+06 respectively). The chromatogram overlay (Figure 6.6 a) showing the enlarged polar peak area for Set A exhibits a larger peak area in the flask than the tray. On comparing the peak areas, it was confirmed that the lignin degradation was higher in the tray reactors, which could have been due to larger surface areas in trays enabling more biofilm and wastewater contact area, resulting in better lignin degradation. The chromatogram overlay showing the enlarged polar peak area for Set B (Figure 6.6 b) again exhibits a smaller peak area for the tray reactor than the flask. These results confirm the UV-spectrometry results where the Tray reactors showed better lignin removal.

The chromatogram overlay (Figure 6.7) for the third wastewater treatment cycle (Cycle 3) exhibits the lignin content for untreated and treated spent media samples with an initial lignin concentration of 1500 mgL^{-1}. The only distinct, prominent peak for all the samples was polar formed at RT 2.3 minutes for untreated samples and at RT 2.4 minutes for all the biofilm-

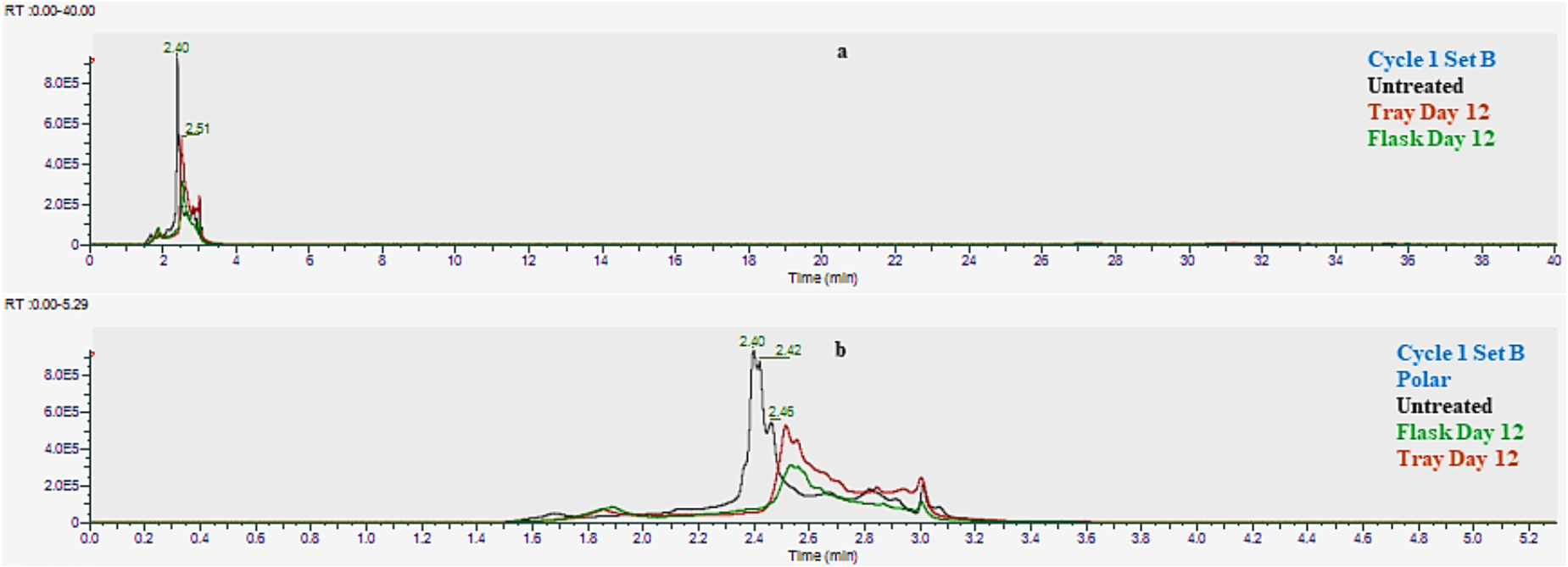


Figure 6.4 - Chromatogram overlay of spent wastewater media samples from Cycle 1 Set B of wastewater treatment experiment comparing lignin content in untreated and treated samples showing: (a) formation of major polar peak only from untreated and treated samples (b) enlarged peak area.

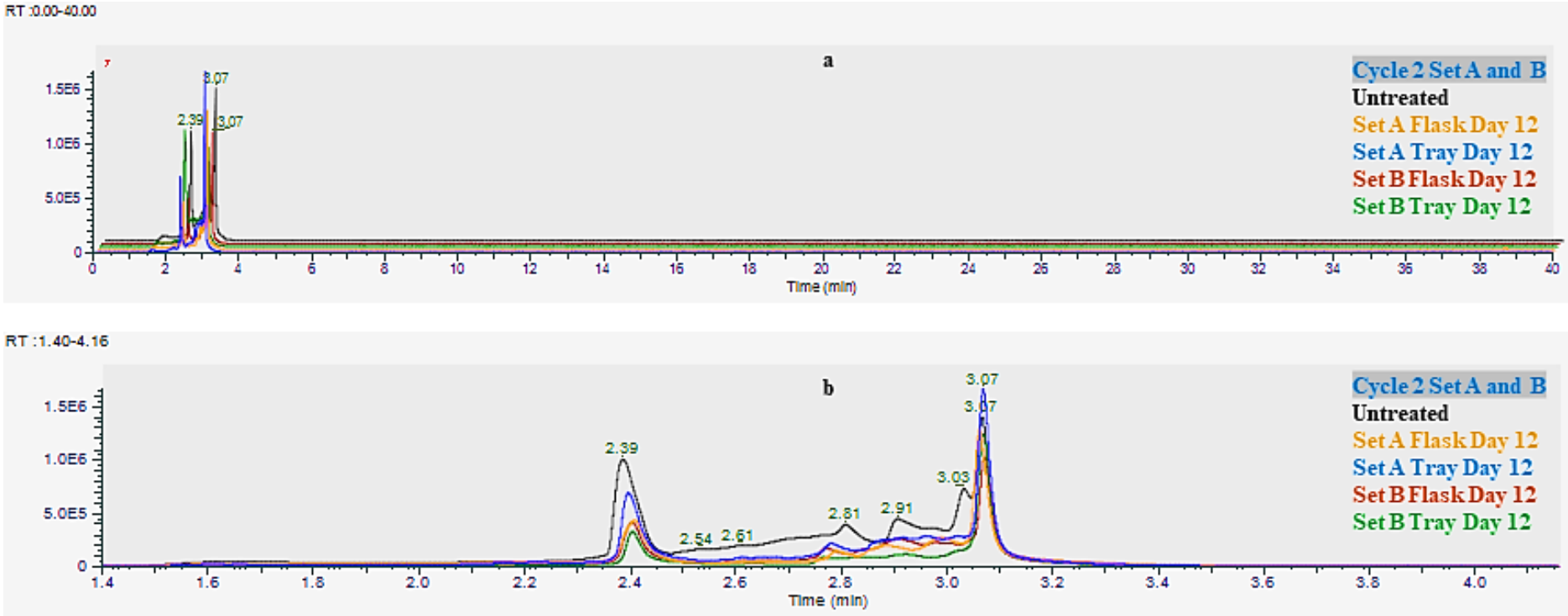


Figure 6.5 - Chromatogram overlay of spent wastewater media samples from Cycle 2 of wastewater treatment experiment comparing lignin content in untreated and treated samples showing (a) Complete profile showing a polar peak only (b) Enlarged peak area

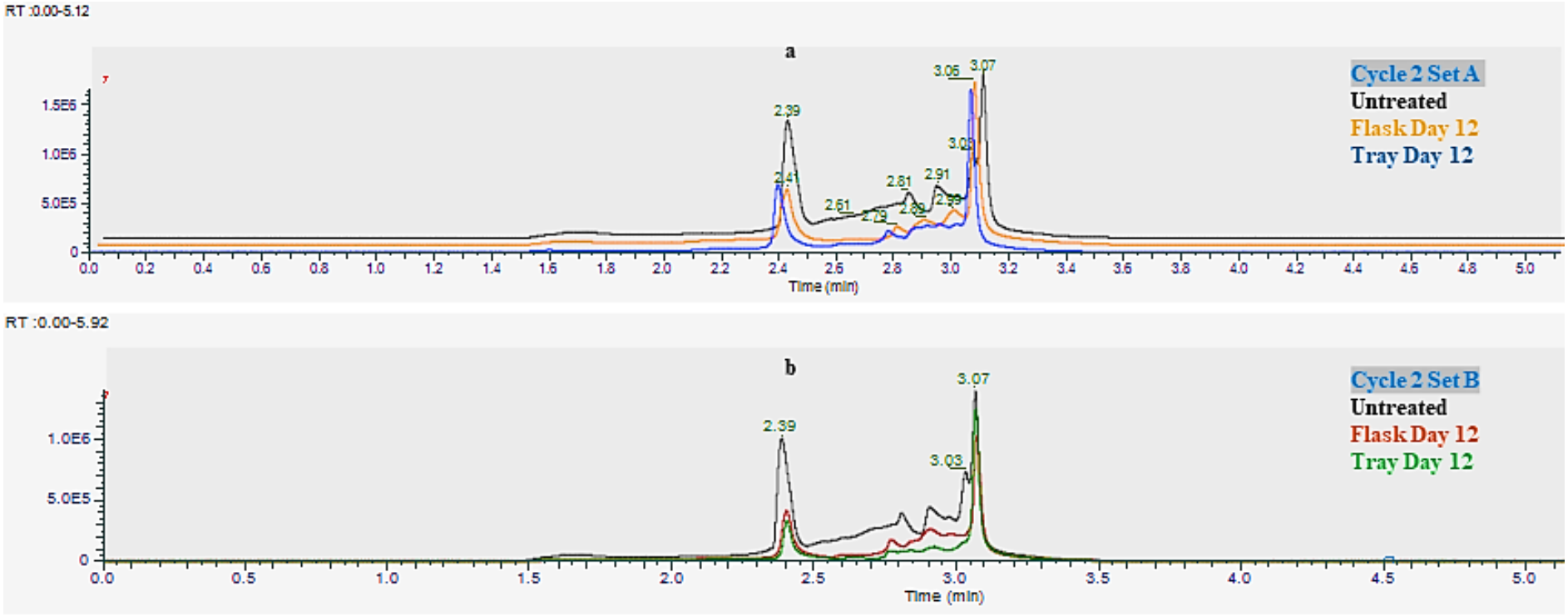


Figure 6.6 - Chromatogram overlay of spent wastewater media samples from Cycle 2 of wastewater treatment experiment comparing lignin content in untreated and treated samples showing: (a) Set A chromatograms comparing the sample form tray and flask reactors (b) Set B chromatograms comparing the sample form tray and flask

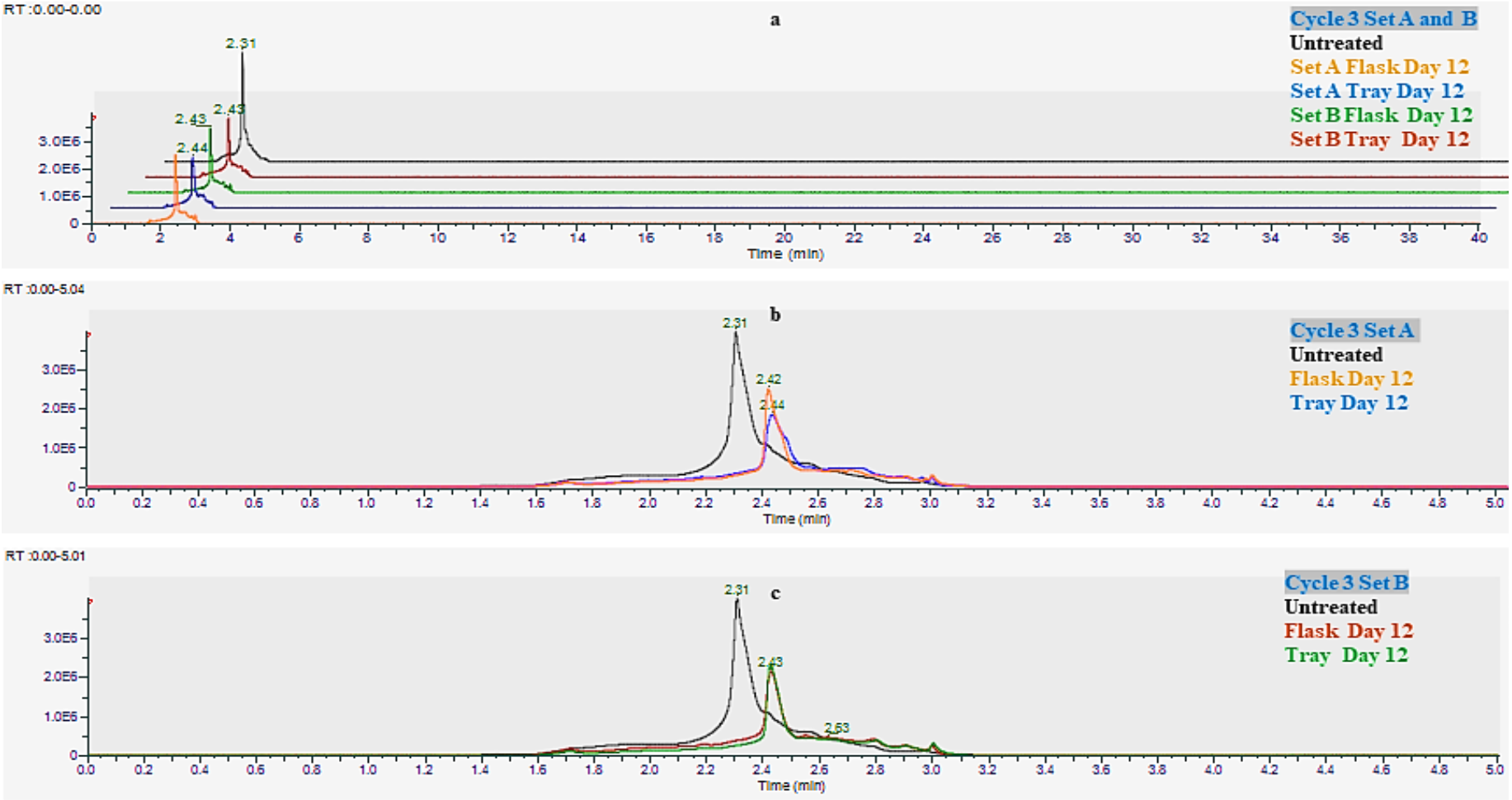


Figure 6.7 Chromatogram overlay of spent wastewater media samples from Cycle 3 of wastewater treatment experiment comparing lignin content in untreated and treated samples showing: (a) Complete chromatogram profile (b) Set A chromatograms comparing the sample from tray and flask reactors (b) Set B chromatograms comparing the sample from tray and flask reactors.

treated samples. Figure 6.7 (b) shows a chromatogram overlay of spent media for Set A, where biofilms were grown initially on Vogel's media. The polar peak area shows a taller and larger peak area for the flask reactor and a smaller peak area for the tray reactor. The peak area integrated for the untreated samples was 4.92E + 07. The peak area for the Set A tray was higher than Set B (Figure 6.7 c), showing the maximum lignin removal in the Set B tray. The HPLC results confirm that of all three cycles, the most efficient results were seen for Cycle 2.

6.3.4 Chemical Oxygen Demand Removal Efficiency

The COD measured for the three wastewater batches on day 0 were 2950.99 mgL^{-1}, 5256.21 mgL^{-1} and 7730.14 mgL^{-1}. Figure 6.8 shows the COD removal efficiency (%) for all the treatment cycles. During the biofilm growth phase after inoculation (Cycle 1), the COD removal efficiency in Vogel's media (Set A) was negligible at 10.53 % and 1.75% in the flask and tray, respectively. Less COD removal was noted since there was delayed, and poor growth of biofilms formed on Vogel's media at room temperature. The lignin removal efficiency (%) for these two reactors was negligible, which can be explained by saying that the sugar was consumed mainly during the growth and formation of biofilms, and lignin metabolism slowed down primarily due to lower environmental temperatures. On the other hand, in Set B, the biofilms formed on lignin-rich wastewater media showed a COD removal of 50% and 57.69% in the flask and tray, respectively. The biofilms in these reactors were sturdy and covered the entire surface of the reactors, which was the reason behind efficient COD and lignin removal in these reactors.

In Cycle 2, Set B showed maximum COD removal for tray reactors (51.26%) and for the flask reactor 45.35 %. The Set A cultures resulted in lower COD removal efficiencies, with 40.49% in the flask and 32% in the tray. The poor growth of Set A Cycle 1 hampered lignin removal due to

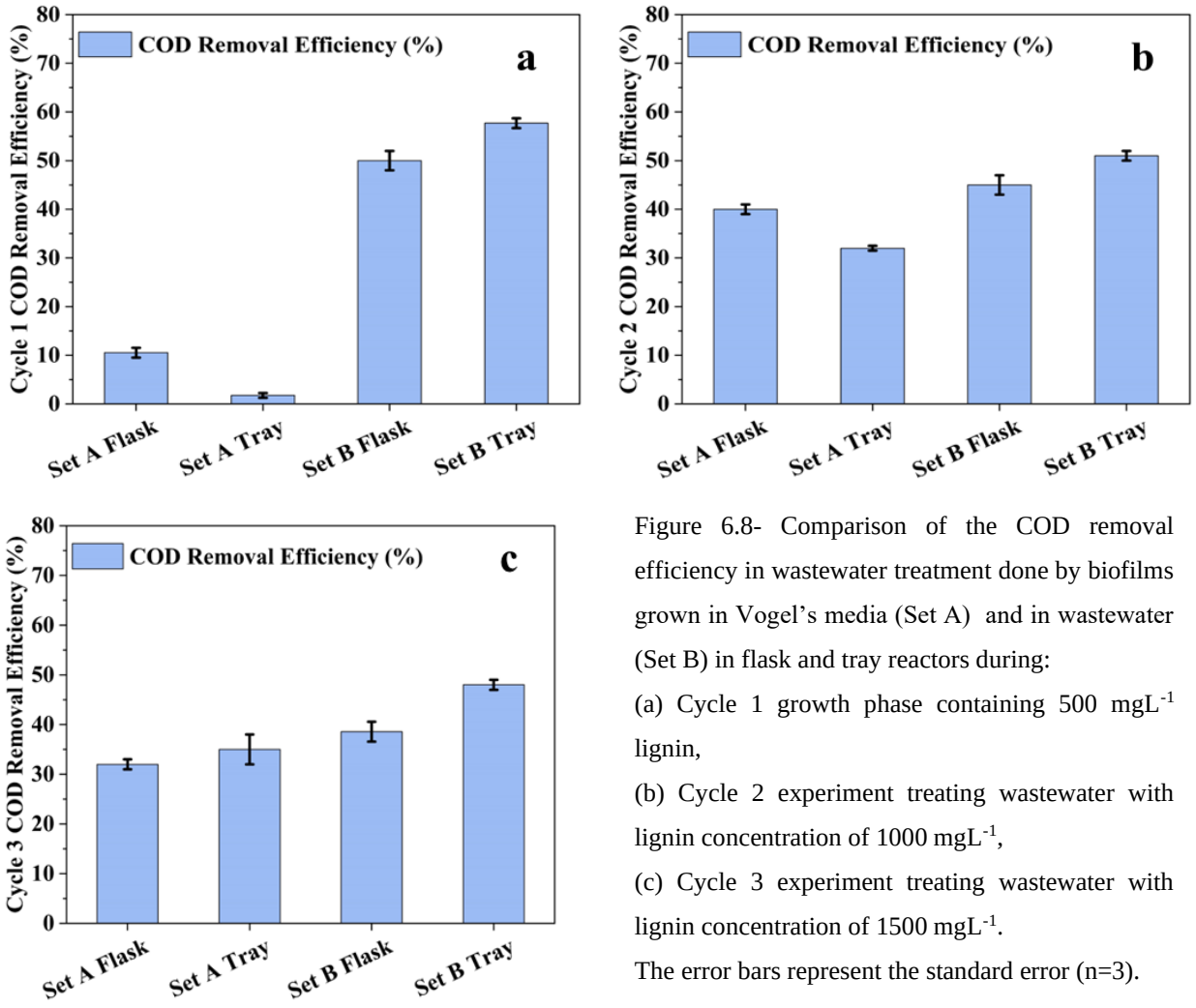


Figure 6.8- Comparison of the COD removal efficiency in wastewater treatment done by biofilms grown in Vogel's media (Set A) and in wastewater (Set B) in flask and tray reactors during:

(a) Cycle 1 growth phase containing 500 mgL^{-1} lignin,

(b) Cycle 2 experiment treating wastewater with lignin concentration of 1000 mgL^{-1},

(c) Cycle 3 experiment treating wastewater with lignin concentration of 1500 mgL^{-1}.

The error bars represent the standard error (n=3).

the reported dependence of the process efficiency on cell density in repeated-batch treatments (Rajwar, Paliwal and Rai, 2017). In the present study, the initial growth medium of Set B, which contained lignin-rich wastewater, was beneficial for biofilm growth. The overall COD removal efficiencies declined in Cycle 3 compared to Cycle 2 for all reactors. In Cycle 3 Set A, the tray reactor showed 35 % compared to 32% in the flask reactor. Set B maximum COD removal efficiency was 48% for the tray reactors, while the flask showed 38%. The trend in COD removal correlates with the lignin removal efficiency. The trend in COD removal correlates with the lignin removal efficiency (R - 0.92, $p < 0.05$).

6.3.5 Activity of Lignin-Degrading Enzymes

6.3.5.1 Laccase Activity

During the biofilm growth phase, Cycle 1, no laccase activity was noted for the first 12 days from inoculation (Figure 6.9). On day 12, the fresh batch of lignin-rich media at 1000 mgL^{-1} was added to all the reactors. In the second cycle (Cycle 2), laccase activity was noted from day 3 in both Sets A and B but was much lower than the results from Cycle 2. This was because of the formation of lignin degradation intermediates that inhibit laccase activity in *N. discreta* (Pamidipati and Ahmed, 2020). On day 7, the activity increased and showed in both sets. However, on day 9, the laccase activity in Set A tray showed significantly high activity (1.38 UL^{-1}) compared to all the other treatments. Set B reactors showed significantly high laccase activity of 4.17 UL^{-1} on day 14.

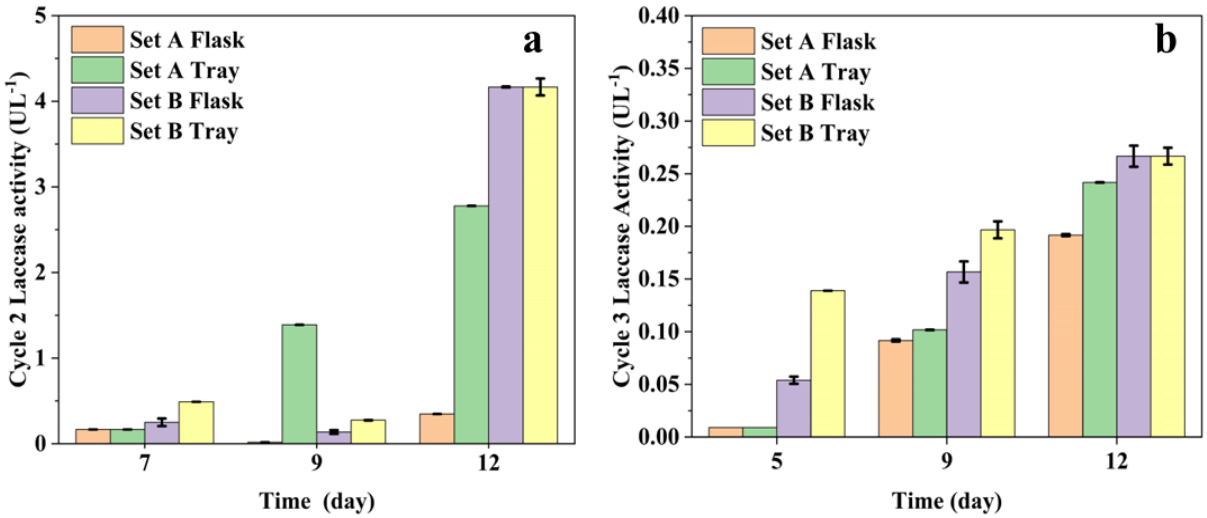


Figure 6.9- Laccase activity in cultures treated with biofilms grown on Vogel's media and on industrial wastewater (a) Cycle 2 experiments treating industrial wastewater containing 1000 mgL^{-1} lignin (b) Cycle 3 experiment treating industrial wastewater containing 1500 mgL^{-1} lignin. The error bars represent the standard error (n=3). The asterisk (*) indicates there is a statistically significant difference ($p<0.05$) between the two

The in-process lignin intermediates play a role in the inhibition of laccase activity. This enzyme is also reported to be induced in other species, *Neurospora crassa* and white-rot fungi, by the addition of copper sulphate acting as the inducer (Pamidipati and Ahmed, 2020; Levin, Forchiassin and Ramos, 2002; Schilling *et al.*, 1992). It can be inferred that laccase is not growth-associated and was induced by adding fresh wastewater in Cycle 2. Laccase activity was noted from the second batch of treatment when the biofilm biomass had matured.

6.3.5.2 Polyphenol Oxidase Activity

Polyphenol oxidase activity was noted in all three cycles of wastewater treatment (Figure 6.10). The first wastewater treatment cycle (Cycle 1) showed PPO activity from day 7 of biofilm growth in all the reactors. On day 12, the maximum PPO activity showed a 122-fold increase (24 UL^{-1}) in Set B tray reactors and a 115-fold increase in the control. The Set A tray and control reactors showed an 11.2-fold and 6.7-fold lower PPO activity than Set B. Figure 6.10 b shows the PPO activity in Cycle 2 of wastewater treatment, which was noted from day 3 onwards. The maximum PPO activity was reported on day 12 of Cycle 2 treatment, with a maximum activity of 28.3 UL^{-1} for the tray reactor. The PPO activity for the flask reactors was lower than for the tray reactor for wastewater-grown biofilms. Though the overall PPO activity was much lower in Set A, the flask reactors showed higher activity than the tray reactors, which showed the lowest 1.8-fold lower PPO activity than Set B tray. This could be because the biofilms grown in Vogel's media in Cycle 1 were still recuperating from poor growth. Figure 6.10 (c) shows the PPO enzyme activity for Cycle 3 of the experiment, where the enzyme activity noted on day zero could have been due to enzymes from biofilm EPS diffusing into the wastewater.

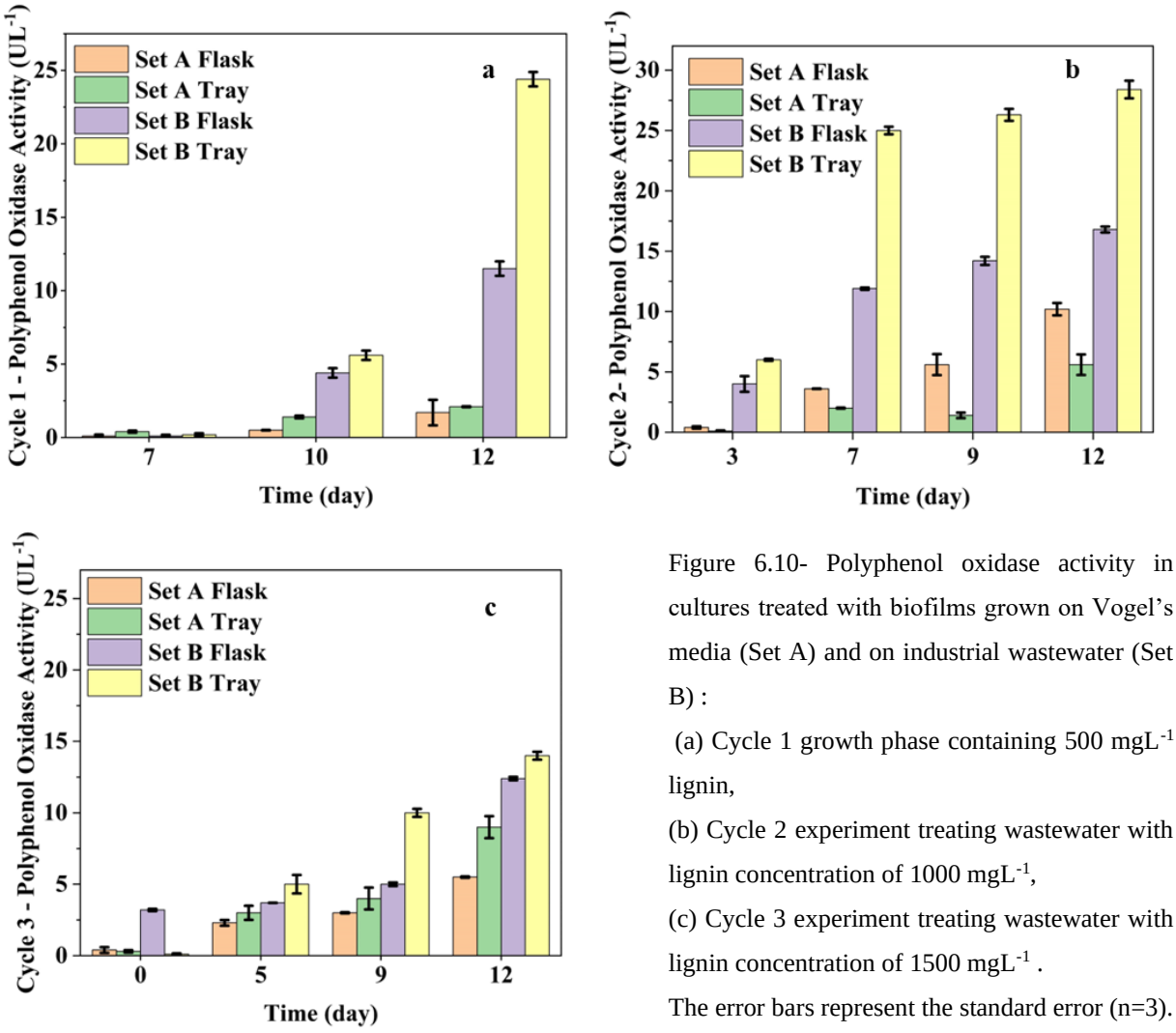


Figure 6.10- Polyphenol oxidase activity in cultures treated with biofilms grown on Vogel's media (Set A) and on industrial wastewater (Set B) :

(a) Cycle 1 growth phase containing 500 mgL^{-1} lignin,

(b) Cycle 2 experiment treating wastewater with lignin concentration of 1000 mgL^{-1},

(c) Cycle 3 experiment treating wastewater with lignin concentration of 1500 mgL^{-1} .

The error bars represent the standard error (n=3).

Though, for the rest of the Cycle 3 experiment, the PPO activity was noted to increase continuously till day 13, it was much lower compared to the set 2 experiments. This could justify the low lignin removal efficiency (%) and COD in the Cycle 3 experiments, reported in the previous sections. It can also be inferred that the biofilms took longer to acclimatise to higher lignin concentration in the Cycle 3 experiments.

6.5.3 Versatile Peroxidase Activity

The VPO activity for the three cycles of experiments is shown in Figure 6.11. During Cycle 1, VPO activity was noted in both the absence (Set A) and presence (Set B) of lignin from day 7 onwards. The Set B tray and flask reactors showed higher VPO activity, increasing by 3.1-fold and 3-fold, respectively. The Set A activity was lower than Set B and demonstrated a 14-fold and 12-fol increase on day 12 from day 7. This confirms the presence of lignin in the treated media is essential for VPO enzyme induction, which results in higher enzyme activity. Figure 6.11 (b) shows the activity for Cycle 2, where the VPO activity was noted from day 3 onwards, with the highest activity for Set B tray and control reactors. The Set B reactors showed lower VPO activity from day 7 until it again increased on day 14, showing a 2.8-fold and 2.7-fold increase from day 7. This was supported by the fact that the biofilm in the Set B reactors stayed submerged under the liquid surface after the media change, and the lack of oxygen resulted in a decline in VPO activity till a new layer on the surface was seen on day 9. The biofilm growth phase in Set B Vogel's media resulted in weak biofilms that picked up growth and looked healthier from the Cycle 2 experiment onwards. As a result, lower VPO activity was noted The addition of sucrose led to increased laccase titers, which is consistent with a previous study where the induction of consecutive laccases in the ascomycetes *Botryosphaeria* sp was observed after the addition of various carbohydrates as the sole carbon source an opposite trend was noted.

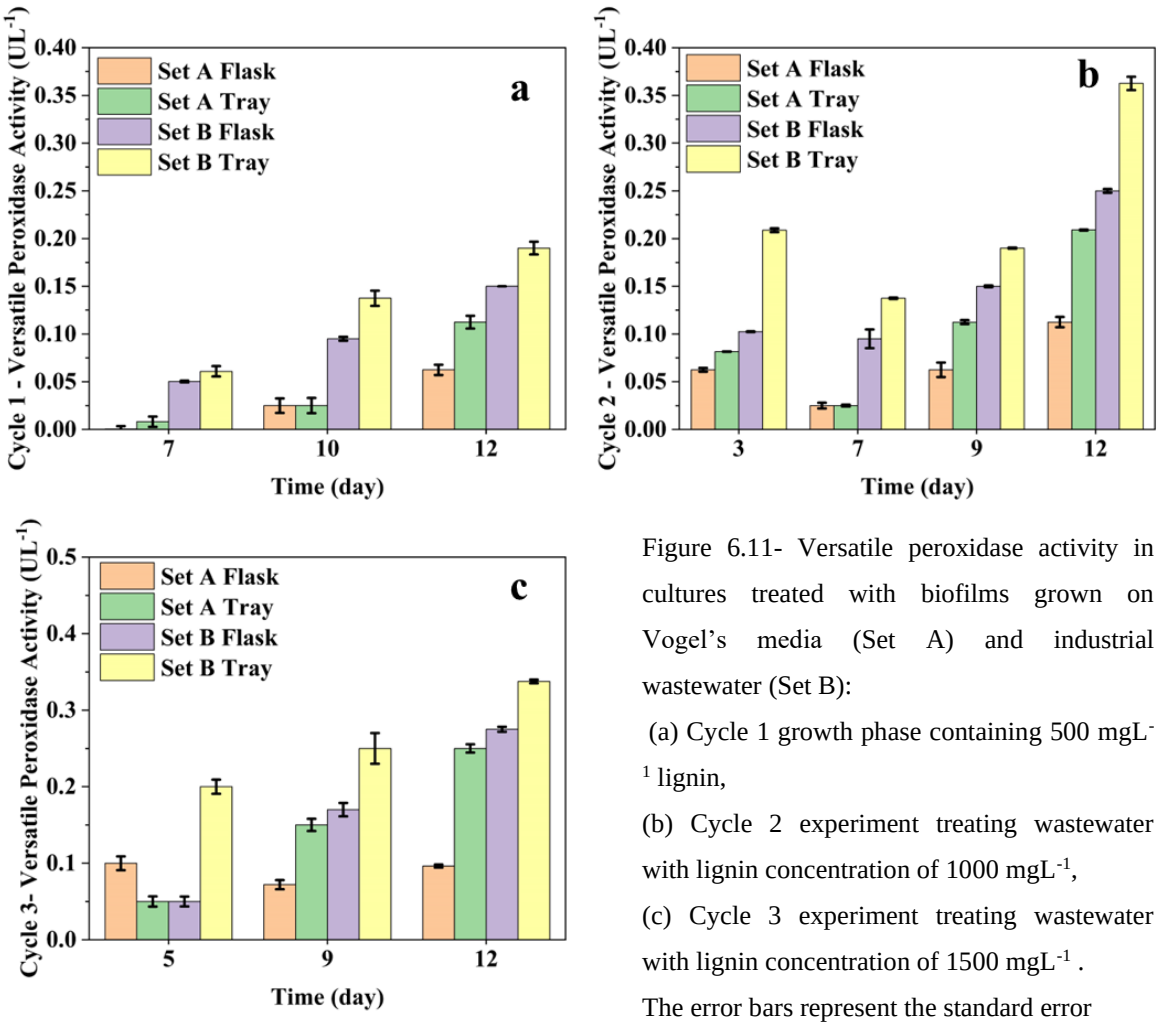


Figure 6.11- Versatile peroxidase activity in cultures treated with biofilms grown on Vogel's media (Set A) and industrial wastewater (Set B):

(a) Cycle 1 growth phase containing 500 mgL^{-1} lignin,

(b) Cycle 2 experiment treating wastewater with lignin concentration of 1000 mgL^{-1},

(c) Cycle 3 experiment treating wastewater with lignin concentration of 1500 mgL^{-1} .

The error bars represent the standard error (n=3).

6.3.6 Biofilm Composition - Extracellular Matrix Components

6.3.4.1 Biofilm Properties

The harvested biofilms were analysed (Figure 6.12) to quantify the properties such as biofilm dry weight per unit, biofilm wet weight, and water retention value (%). The EPS extracted from the biofilms was analysed chemically to quantify the polysaccharide concentration per unit biomass (mgg^{-1}) and the protein concentration per unit biomass (mgg^{-1}). The biofilms grown in flasks were less watery than the tray bioreactors. The Set B wastewater-grown biofilms had the

maximum WRV values of 98.76% for the tray reactors, followed by the flask-grown biofilms (96.25%). Set A biofilms had higher water content for tray reactors (92.98%) than the flask-grown biofilms (90.91%). On quantification of the dry weight per unit biomass, it was noted it inversely correlated with the WRV, confirming that the biofilms heavier in Tray (Set B) had more water content. Due to enhanced lignin degradation in the Set B reactors, limiting conditions were created, which resulted in the biofilms producing more extracellular matrix rich in polysaccharides and proteins (Ahmed, Narayanan and Veni, 2020).

The polysaccharide concentration in the EPS can explain the WRV trend as polysaccharides are hydrophilic molecules, and biofilms with higher polysaccharide content had higher WRV (Rughoonundun, Mohee and Holtzapple, 2012). Therefore, the wastewater-grown biofilms with the highest polysaccharide content had the highest WRV and lowest number of cells indicated by the dry weight per unit biomass. Similarly, the protein content is also the highest for the wastewater-grown biofilms treating the wastewater in a tray reactor. A strong positive correlation exists between the lignin and COD removal efficiencies with the WRV, polysaccharides and protein content. (R-0.92, $p < 0.05$). This explains that the culture where the biofilms were grown in wastewater directly was the most efficient in lignin and COD removal compared to those grown without lignin. Though biofilm processes have been studied to develop wastewater treatment processes, the studies on biofilm composition are limited to bacterial biofilms only (Lazarova and Manem, 1995). Fungal biofilms and their composition in wastewater treatment have not been studied till now in this context.

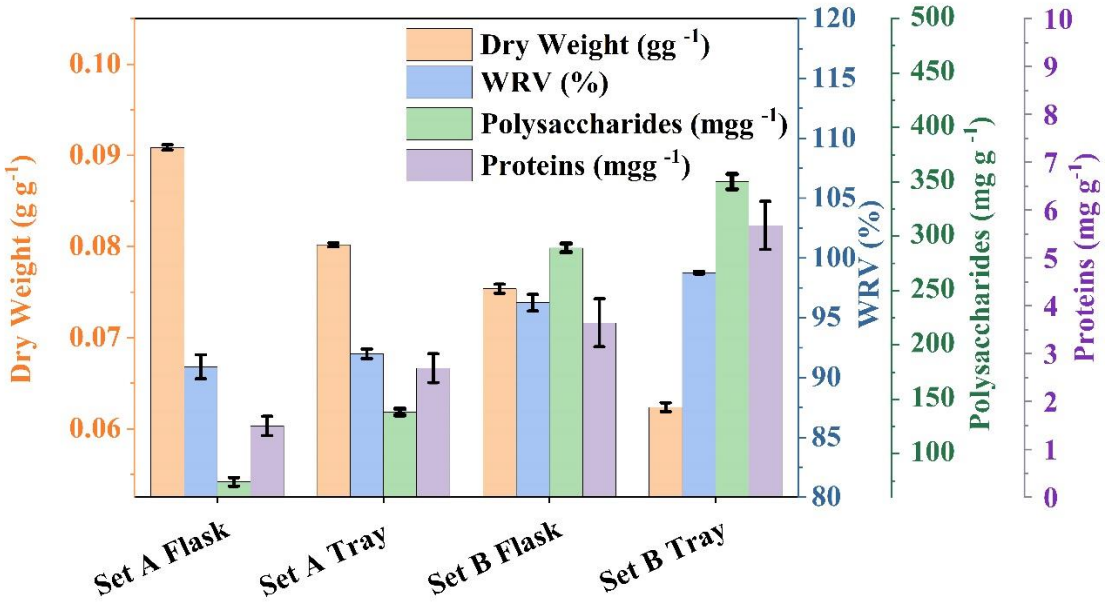


Figure 6.12 - Biofilm properties showing dry biomass per unit biofilm weight as dry weight (g g^{-1}), water retention value (%), polysaccharides per unit biomass (mg g^{-1}), and protein per unit biomass (mg g^{-1}). The error bars represent the standard error (n= 3).

6.3.4.2 Biofilm Microstructure

The diffusion of the substrate through the biofilms largely depends on the porosity of the biofilm, substrate concentration and mass transfer at the biofilm-liquid interface (Heijnen *et al.*, 1989). The biofilm microstructure is reported to be influenced by nutrient conditions (Sankaran *et al.*, 2010; Ahmed, Narayanan and Veni, 2020). Figure 6.13 shows the scanning electron microscopic images of the biofilms harvested on day 39 from inoculation. The SEM images show the biofilms grown on industrial wastewater to be more porous with longer filaments and greater pore diameter. The biofilms grown by inoculating spores in Vogel's media (Figure 6.13 a and b) visually looked compact in structure in comparison to more porous biofilms formed by inoculating wastewater media rich in lignin (Figure 6.13 c and d). The flask reactors (Figure 6.13 a), with biofilms grown on Vogel's media, show abundant spore forming on the biofilms, which

is an indication that the biofilms were still increasing as these biofilms had poor growth during the initial growth phase (Cycle 1). Since the wastewater-grown biofilm treatment proved to be more efficient, it seems crucial to focus on the microstructure of these biofilms first (Figure 6.14). The average pore diameter and total pores area are the highest for the biofilms grown in wastewater medium. These biofilms also had thinner and longer filaments.

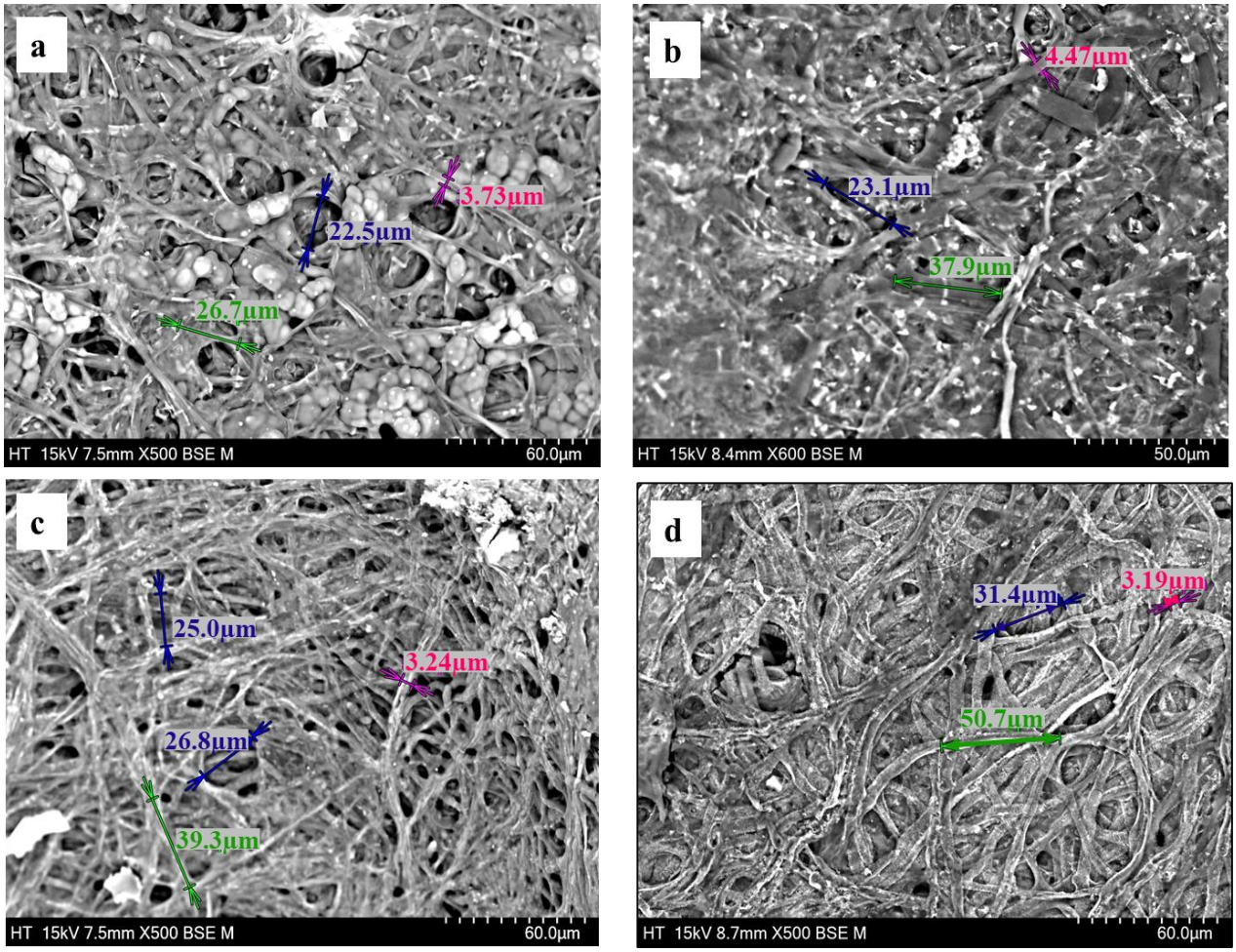


Figure 6.13 - Scanning electron microscopy image (500 X magnification) of biofilms growth from spore inoculation in treatment media composition: (a) media grown biofilms (100 ml), (b) media grown biofilms (800 ml) (c) lignin-rich wastewater grown biofilm (100 ml) and (d) lignin-rich wastewater grown biofilm (800 ml). Microstructure measurements include pore diameter (blue), filament length (green) and filament thickness (pink).

This is an attribute of biofilms growing in nutrient-limiting conditions; here, the degradation of lignin was reported to be maximum; hence, the filaments grew longer to reach the limiting carbon source-lignin in this case (Aravinda Narayanan and Ahmed, 2019). In particular, the biofilms grown in a tray reactor (800 ml) were the most porous, measuring an average of 8000 pixels of total ariel pore area. This was followed by the flask reactors biofilms grown on wastewater media with an average of 7500 pixels of ariel pore area. The biofilms grown on flask reactors had an average pore diameter of 22.5 µm, filament length of 26.7 µm and filament thickness of 3.73 µm. In comparison to the flasks, the tray reactors showed larger pore diameter (23.1 µm), longer filaments (37.9 µm) and thinner filaments (3.47 µm).

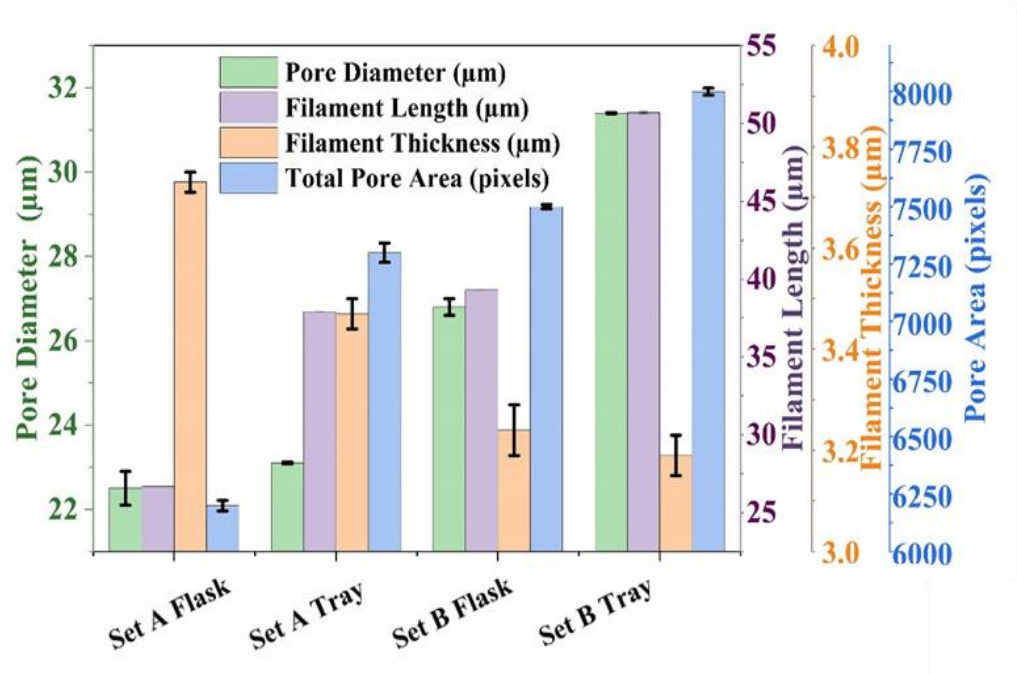


Figure 6.14 - Biofilm microstructure analysis showing average pore diameter, filament length, filament thickness and total ariel pore area (pixels) for biofilms grown by inoculating spores in Vogel's media and in wastewater rich in lignin for treatment volumes of 100 ml and 800 ml in flask and tray reactors. The error bars represent the standard error (n=3).

6.3.7 Correlations Between Process Efficiencies and Biofilm Properties

The process efficiencies for Cycle 2 treatment were further analysed to investigate the correlations with biofilm properties. The lignin and COD removal efficiencies showed a strong positive correlation (R - 0.99 , $p < 0.05$). The enzyme activity for day 12 of treatment of PPO also showed a strong positive correlation with lignin and COD removal efficiencies. However, the VPO activity did not show any correlation. This was because the optimal temperature for VPO stability is reported to be between 25 to 70^{o} C (Knop *et al.,* 2016) and the low temperature tested in the current study must have negatively affected the enzyme activity. This result is confirmed by looking at the VPO activity results shown in Section 6.3.5. The relationship between WRV, polysaccharides and proteins follow the same trends as were noted in the previous studies, showing a strong positive correlation among themselves; however, there was no correlation noted with the process efficiencies.

6.4 Discussion

Lower temperatures reduced the process efficiency. The initial growth media drastically affected the biofilm formation and growth, at least during the first cycle. The presence of lignin in the wastewater media did have a strong positive effect on biofilm formation, enzyme production and process efficiency. The second cycle of wastewater treatment did give promising results, indicating the process can be reproducible at this scale and up to the second cycle of wastewater treatment. The low enzyme activity noted towards the end of the second cycle and the significant reduction during the third cycle indicates the possibility of contamination. This could be due to microbial competitive inhibition, or the formation of lignin degradation intermediated, which inhibits enzyme production. Since there were no visible signs of contamination till the end of the third cycle, the formation of new linin degradation products may likely have inhibited enzyme

production. Another reason for poor process efficiency in the third cycle of wastewater treatment could be the presence of mature biofilms. The biofilms consume their biomass; however, it is inefficient if the process suffers from slow nutrient removal rates. It is hence proposed that the old biofilms are removed. Also, biofilm processes suffer from mass transfer and diffusion limitations due to forming a gradient in the mature and new biomass.

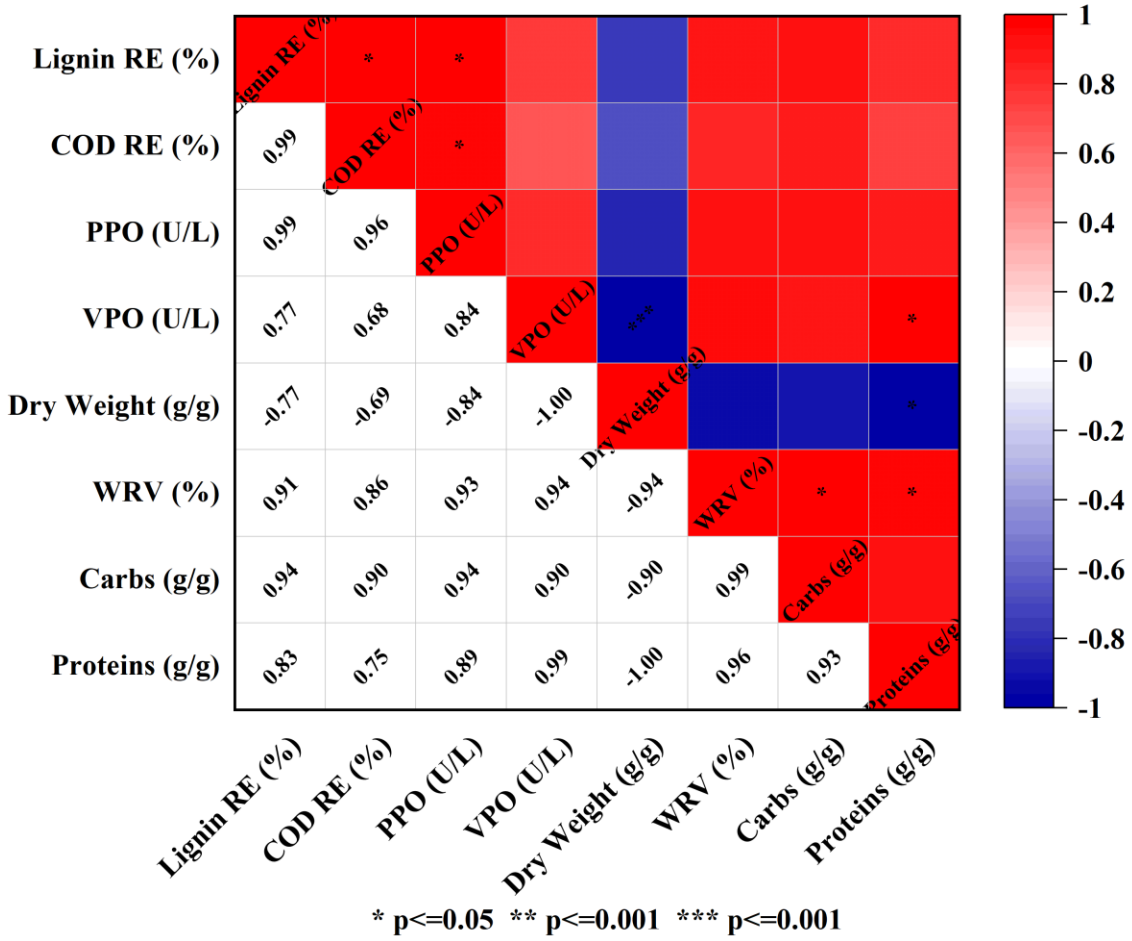


Figure 6.14 - Pearson's correlation plot showing the correlations between lignin removal efficiency (Lignin RE, %). COD removal efficiency (COD RE, %), polyphenol oxidase activity (PPO, UL-1), versatile peroxidase activity (VPO, UL-1), polysaccharides (Carbs, mgg-1), proteins (mgg-1), water retention value (WRV, %), dry biomass (g). The asterisks in the upper triangle signify the variables to have a significant correlation and the colours denote the positive (red) and negative (blue) correlation. A statistically significant difference represented by asterisk *=($p<0.05$), ** = ($p<0.01$), *** = ($p<0.005$). The Pearson's coefficient values (R) are presented as the labels in the lower triangle.

The mature biofilms begin to disintegrate and add organic matter into the wastewater media, and instead of consuming the pollutant, it begins to consume its biomass; however, if the process suffers from slow nutrient removal rates, the process is inefficient. In the biofilms formed by *N. discreta*, like any other biofilm, the wastewater with dissolved lignin degradation products is transferred through the porous water channels formed in the biofilms due to the criss-cross meshwork of fungal filaments (Flemming and Wingender, 2010). This wastewater transfer through the biofilm creates a carbon source gradient across the biofilm layers, and lignin is consumed during this diffusion process (Nicolella, Van Loosdrecht and Heijnen, 2000). The established carbon source diffusion gradient results in a growth rate gradient within the biofilms, forming a multi-layer biofilm structure (May *et al.,* 2019). The slower-growing mature biofilms are found at the bottom, with new biofilms forming on the surface (Heijnen *et al.,* 1989). Ideally, the formation of multilayers leads to a larger surface area beneficial for enhanced biofilm and wastewater interaction, leading to a more efficient wastewater treatment process. However, the gradient formation across the layers slows the process down. The mature biofilms are not metabolising efficiently and block the active diffusion of biofilm metabolites to the wastewater media from the top layers. Removing mature biomass during wastewater cycle change can result in a robust process.

6.5 Conclusion

The scale-up process in non-sterile and ambient temperatures resulted in a stable and effective method for repeated-batch treatment of wastewater with effective lignin and COD removal efficiency after the biofilms were fully grown. The process resulted in the easy adaptation of biofilms to a fresh batch of wastewater containing a higher concentration of lignin and COD. The media composition used for the biofilm growth affected the biofilm properties and, hence, the

lignin removal efficiency. The results from this study are expected to form a base for further research and exploration of this biofilm-based wastewater treatment process at full scale.

---

7. Conclusion and Future Work

7.1 Thesis Highlights

This thesis describes the development of an efficient biological process for the treatment of lignin-rich wastewater. This work aimed to develop the lignin degradation process in synthetic and industrial wastewater using novel fungal biofilms formed by the fungus *Neurospora discreta*. The significance of this work includes the crucial contributions made towards the development and optimisation of a scalable and sustainable process for the treatment of wastewater generated from pulp and paper mills.

This work achieved its objectives as follows:

1. A statistical approach was applied to identify the optimal process parameters using Taguchi Design of Experiments on synthetic wastewater media. The parameters investigated were pH, copper sulphate and trace elements at three levels.

   Lignin degradation efficiencies and enzyme activities of polyphenol oxidase and versatile peroxidase were evaluated as process responses. Results indicated that pH significantly impacted lignin degradation (p-value $\leq$ 0.002), followed by copper sulphate (p-value $\leq$ 0.005). Furthermore, pH 5 was identified as the optimal condition, resulting in significant lignin degradation and enhanced enzymatic activities. The highest lignin degradation (70%) and enzyme activities were achieved at pH 5 in a 14-day process. Increasing copper sulphate concentrations resulted in decreasing lignin removal efficiencies and enzyme activities of PPO. This study demonstrated the relationship between biofilm properties, such as porosity, water retention value, polysaccharide content, and lignin removal efficiency. Furthermore, versatile peroxidase, a ligninolytic enzyme, was reported in *Neurospora* sp. for the first time in this study.

2. The addition of natural lignin degradation intermediates was evaluated as inducers for ligninolytic enzymes produced by *N. discreta* (Babič and Pavko, 2012). This study assessed the effect of the addition of fungal-treated spent media collected from different time points to the biofilm treatment process. The results showed significantly increased ($p<0.003$) lignin and COD removal efficiencies in all the fed cultures. The cultures fed spent media from day 10 mainly exhibited exceptionally high lignin and COD removal efficiencies, increased by 15.88% and 19.2% (p-value 0.001) compared to the unfed cultures. The spent media fed in these cultures was analysed to identify the lignin degradation products showing the presence of Sinapic acid, Resveratrol and Nordihydroguaiaretic acid, which can potentially be classified as natural inducers in wastewater treatment processes using fungi belonging to the Ascomycota group. The novelty of using lignin degradation products in spent media was explored for the first time.

3. A repeated-batch process for wastewater treatment was evaluated for its effect on the fungal biofilm system efficiencies. The wastewater treated in three cycles by transferring the mature biofilms showed better results in the second cycle of the treatment. Also, it was seen that the biofilms were actively metabolising lignin even when the wastewater was initially deprived of sucrose, which is needed for initial growth as an easily assimilable carbon source. Laccase enzyme activity was seen for the first time and was concluded to have been induced by the addition of fresh wastewater rich in lignin and copper sulphate. The study resulted in a stable process with enhanced production of enzymes and treatment of new wastewater cycles with

the retained biomass. This study opens new facets for the procedure, showing the potential of being applied as a stable continuous process for wastewater treatment.

4. Novel bioreactor systems were evaluated using polystyrene trays to serve as biofilm reactors for a scale-up process for the first time. The biofilm scale-up process investigated resulted in a stable continuous operation with no contamination and was successfully executed for 36 days, treating three cycles of wastewater. The lignin and COD removal in the second cycle exhibited 63.39 % and 51% lignin and COD removal efficiencies. Laccase activity was induced from the second cycle in addition to the VPO, and PPO enzyme activity was significantly high in Cycle 2 of treatment. The scale-up process at ambient temperatures is a promising and economical wastewater system for industrial scale. Results from this study are expected to form a base for further development and scale-up of a biofilm-based wastewater treatment process at full scale.

7.2 Future Work

The thesis outlines a biofilm-based approach to effectively degrade lignin in industrial wastewater from pulp and paper mills that use the kraft process. Results from this study can be used to scale up the process to a pilot study, furthering it to an industrial-level treatment process.

1. An in-depth study to identify and quantify intermediate lignin degradation products formed during fungal biofilm treatment of wastewater can lead to the discovery of new products that are potentially value-added (Chen *et al.*, 2012). The fungal biofilm method for lignin degradation can be developed to ensure the accumulation of these new products rather than complete degradation. Processing phenolic monomers from lignin biodegradation yields valuable secondary metabolites, potentially discovering new

metabolites (Chandra and Bharagava, 2013). When used to induce useful enzymes, these degradation products can have another significant industrial value.

2. To understand the *N. discreta* biofilm degradation mechanism, understanding enzyme expression at the molecular level will open more avenues for process improvement (Asemoloye *et al.,* 2021). Bioaugmentation for enzyme production can be further improved by genetically modifying the organism by targeting the enzyme expression at the gene level.

3. A detailed survey of optimising process parameters to enhance enzyme activity will result in designing a process to treat wastewater at a large scale (Hubbe *et al.,* 2016). Pilot scale studies can be carried out to verify the laboratory scale studies. During the scale-up process, lignin and COD removal efficiencies were lower at room temperature compared to the studies conducted at 30^{o} C in sterile conditions. The approach holds the potential to be further optimised for enhanced enzyme production. Agitation as another process parameter that may improve the process efficiency by resolving problems such as biofilm fouling and pore-clogging in the continuous treatment process (Zheng *et al.,* 2018).

4. When industrial lignin is used in a growth medium for microorganisms, obtaining the same enzyme induction results remains challenging (Iram, Berenjian and Demirci, 2021). In addition, the precise microenvironment of lignin-based culture media, crucial during lignin degradation, remains poorly understood (Amaral *et al.,* 2012). Further research is

necessary to determine if the degree of lignin degradation during pulping procedures impacts the activation of ligninolytic enzymes.

5. The repeated-batch treatment process is a more efficient and potentially economical alternative to the other continuous methods, such as the sequence-batch technique (Yesilada *et al.*, 2010)ost analysis of the process is essential for evaluating process feasibility and improvisation.

7.3 Concluding Remarks and Practical Consideration

In conclusion, the fungal biofilm-based wastewater treatment process efficiently removes lignin and COD in batch and repeated-batch conditions. The biofilms studied can be viewed as living membranes that can be modified to change their microstructure by adjusting the culture conditions. To use these systems at an industrial scale, they must be evaluated further for robustness, sensitivity, and accuracy to ensure reliable and repeatable results. This wastewater treatment process is significant as these biofilms are biodegradable, making them a sustainable and eco-friendly alternative for wastewater treatment.

8 Publications and Other Research Activities Arising from This Thesis

8.1 Publications

Tabraiz, S., Aiswarya, N.M., Taneja, H., Narayanan, R.A. and Ahmed, A., 2022. Biofilm-based simultaneous nitrification, denitrification, and phosphorous uptake in wastewater by *N. discreta*. Journal of Environmental Management, 324, p.116363.

8.2 Manuscripts in Preparation

- Optimisation of a novel biofilm-based process for treating lignin-rich wastewater by *Neurospora discreta*- Taneja, H. and Ahmed, A.

8.3 Presentations

- 2023 - Poster presentation at the EBNet ECR conference at Hariott-Watt University, Edinburgh, Scotland
- 2023 - Abstract Acceptance at 4th International Conference for Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability
- 2022 - Poster Presentation at a conference organised by the postgraduate research forum organised by Canterbury Christ Church University
- 13 Dec 2021- Poster presentation at the conference organised by the Ecology Research Group on Valorisation and Sustainable Land Management, Canterbury Christ Church University
- 22 June 2021- Poster presentation in the Postgraduate Forum in the School of Life Sciences, Canterbury Christ Church University
- 06 Dec 2021- Talk on Current Issues in Science presented my research in the event organised for undergraduate students on Current Issues in Science.

9 References

Abramoff, M.D., Magalhães, P.J. and Ram, S.J. (2004) 'Image processing with ImageJ', *Biophotonics International,* 11(7), pp. 36.

Adler, E. (1977) 'Lignin chemistry—past, present and future', *Wood Science and Technology*, 11(3), pp. 169-218.

Ahmad, M., Taylor, C.R., Pink, D., Burton, K., Eastwood, D., Bending, G.D. and Bugg, T.D. (2010) 'Development of novel assays for lignin degradation: comparative analysis of bacterial and fungal lignin degraders', *Molecular Biosystems,* 6(5), pp. 815-821.

Ahmed, A., Narayanan, R.A. and Veni, A.R. (2020) 'Influence of carbon source complexity on porosity, water retention and extracellular matrix composition of Neurospora discreta biofilms', *Journal of Applied Microbiology*, 128, pp. 1099-1108. doi: -10.1111/jam.14539 https://onlinelibrary.wiley.com/doi/abs/10.1111/jam.14539.

Aitken, M.D., Venkatadri, R. and Irvine, R.L. (1989) 'Oxidation of phenolic pollutants by a lignin degrading enzyme from the white-rot fungus Phanerochaete chrysosporium', *Water Research,* 23(4), pp. 443-450.

Akolekar, D.B., Bhargava, S.K., Shirgoankar, I. and Prasad, J. (2002) 'Catalytic wet oxidation: an environmental solution for organic pollutant removal from paper and pulp industrial waste liquor', *Applied Catalysis A: General*, 236(1-2), pp. 255-262.

Akpor, O.B., Adelani-Akande, T.A. and Aderiye, B.I. (2013) 'The effect of temperature on nutrient removal from wastewater by selected fungal species', *International Journal of Current Microbiology and Applied Sciences,* 2(9), pp. 328-340.

Almonti, D., Baiocco, G. and Ucciardello, N. (2021) 'Pulp and paper characterization by means of artificial neural networks for effluent solid waste minimization—A case study', *Journal of Process Control,* 105, pp. 283-291.

Al-Rasheed, R.A. (2005) *Water treatment by heterogeneous photocatalysis an overview 4th SWCC Acquired experience symposium.* , Saudi Arabia: Jeddah.Citeseer, pp. 14.

Alves da Cunha, M.A., Barbosa, A.M., Giese, E.C. and Dekker, R.F. (2003) 'The effect of carbohydrate carbon sources on the production of constitutive and inducible laccases by Botryosphaeria sp.', *Journal of Basic Microbiology,* 43(5), pp. 385-392.

Amaral, J.S., Sepúlveda, M., Cateto, C.A., Fernandes, I.P., Rodrigues, A.E., Belgacem, M.N. and Barreiro, M.F. (2012) 'Fungal degradation of lignin-based rigid polyurethane foams', *Polymer Degradation and Stability,* 97(10), pp. 2069-2076.

Aravinda Narayanan, R. and Ahmed, A. (2019) 'Arrested fungal biofilms as low-modulus structural bio-composites: Water holds the key', *The European Physical Journal E,* 42(10), pp. 1-8.

Arora, D.K. (2003) *Fungal biotechnology in agricultural, food, and environmental applications* Boca Raton: CRC Press, pp. 11-21 .

Ascacio-Valdés, J.A., Buenrostro, J.J., De la Cruz, R., Sepúlveda, L., Aguilera, A.F., Prado, A., Contreras, J.C., Rodríguez, R. and Aguilar, C.N. (2014) 'Fungal biodegradation of pomegranate ellagitannins', *Journal of Basic Microbiology,* 54(1), pp. 28-34. doi: 10.1002/jobm.201200278 https://api.istex.fr/ark:/67375/WNG-2SGXM5BM-5/fulltext.pdf.

Asemoloye, M.D., Marchisio, M.A., Gupta, V.K. and Pecoraro, L. (2021) 'Genome-based engineering of ligninolytic enzymes in fungi', *Microbial Cell Factories*, 20, pp. 1-18.

Asgher, M., Bhatti, H.N., Ashraf, M. and Legge, R.L. (2008) 'Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system', *Biodegradation,* 19, pp. 771-783.

Asif, M.B., Hai, F.I., Singh, L., Price, W.E. and Nghiem, L.D. (2017) 'Degradation of pharmaceuticals and personal care products by white-rot fungi—a critical review', *Current Pollution Reports*, 3, pp. 88-103.

Asina, F., Brzonova, I., Kozliak, E., Kubátová, A. and Ji, Y. (2017) 'Microbial treatment of industrial lignin: Successes, problems and challenges', *Renewable and Sustainable Energy Reviews,* 77, pp. 1179-1205.

Asina, F., Brzonova, I., Voeller, K., Kozliak, E., Kubátová, A., Yao, B. and Ji, Y. (2016) 'Biodegradation of lignin by fungi, bacteria and laccases', *Bioresource Technology,* 220, pp. 414-424.

Babič, J. and Pavko, A. (2012) 'Enhanced enzyme production with the pelleted form of D. squalens in laboratory bioreactors using added natural lignin inducer', *Journal of Industrial Microbiology and Biotechnology*, 39(3), pp. 449-457.

Badia-Fabregat, M., Lucas, D., Gros, M., Rodríguez-Mozaz, S., Barceló, D., Caminal, G. and Vicent, T. (2015) 'Identification of some factors affecting pharmaceutical active compounds (PhACs) removal in real wastewater. Case study of fungal treatment of reverse osmosis concentrate', *Journal of Hazardous Materials,* 283, pp. 663-671.

Badia-Fabregat, M., Lucas, D., Pereira, M.A., Alves, M., Pennanen, T., Fritze, H., Rodríguez-Mozaz, S., Barceló, D., Vicent, T. and Caminal, G. (2016) 'Continuous fungal treatment of non-sterile veterinary hospital effluent: pharmaceuticals removal and microbial community assessment', *Applied Microbiology and Biotechnology*, 100, pp. 2401-2415.

Bajpai, P. and Bajpai, P. (2015) 'Basic overview of pulp and paper manufacturing process', *Green Chemistry and Sustainability in Pulp and Paper Industry*, , pp. 11-39.

Bajpai, P. and Kondo, R. (2012) *Biotechnology for environmental protection in the pulp and paper industry* Springer Science & Business Media.

Bajwa, D.S., Pourhashem, G., Ullah, A.H. and Bajwa, S.G. (2019) 'A concise review of current lignin production, applications, products and their environmental impact', *Industrial Crops and Products*, 139, pp. 111526.

Balcıoğlu, I.A., Tarlan, E., Kıvılcımdan, C. and Saçan, M.T. (2007) 'Merits of ozonation and catalytic ozonation pre-treatment in the algal treatment of pulp and paper mill effluents', *Journal of Environmental Management,* 85(4), pp. 918-926.

Baldrian, P. (2006) 'Fungal laccases – occurrence and properties', *FEMS Microbiology Reviews,* 30(2), pp. 215-242. doi: 10.1111/j.1574-4976.2005.00010.x .

Baldrian, P. and Šnajdr, J. (2006) 'Production of ligninolytic enzymes by litter-decomposing fungi and their ability to decolorize synthetic dyes', *Enzyme and Microbial Technology,* 39(5), pp. 1023-1029.

Barapatre, A. and Jha, H. (2017) 'Degradation of alkali lignin by two ascomycetes and free radical scavenging activity of the products', *Biocatalysis and Biotransformation,* 35(4), pp. 269-286.

Barapatre, A. and Jha, H. (2016) 'Decolourization and biological treatment of pulp and paper mill effluent by lignin-degrading fungus Aspergillus flavus strain F10', *International Journal of Current Microbiology and Applied Science,* 5(5), pp. 19-32.

Basha, K.M., Rajendran, A. and Thangavelu, V. (2010) 'Recent advances in the biodegradation of phenol: a review', *Asian Journal of Experimental Biological Sciences,* 1(2), pp. 219-234.

Bayat, Z., Hassanshahian, M. and Cappello, S. (2015) 'Immobilization of microbes for bioremediation of crude oil polluted environments: a mini review', *The Open Microbiology Journal,* 9, pp. 48.

Bending, G.D. and Read, D.J. (1997) 'Lignin and soluble phenolic degradation by ectomycorrhizal and ericoid mycorrhizal fungi', *Mycological Research,* 101(11), pp. 1348-1354. doi: 10.1017/S0953756297004140 https://dx.doi.org/10.1017/S0953756297004140.

Berka, R.M., Schneider, P., Golightly, E.J., Brown, S.H., Madden, M., Brown, K.M., Halkier, T., Mondorf, K. and Xu, F. (1997) 'Characterization of the gene encoding an extracellular laccase of Myceliophthora thermophila and analysis of the recombinant enzyme expressed in Aspergillus oryzae', *Applied and Environmental Microbiology,* 63(8), pp. 3151-3157.

Bettin, F., Montanari, Q., Calloni, R., Gaio, T.A., Silveira, M.M. and Dillon, A. (2014) 'Additive effects of CuSO4 and aromatic compounds on laccase production by Pleurotus sajor-caju PS-2001 using sucrose as a carbon source', *Brazilian Journal of Chemical Engineering,* 31, pp. 335-346.

Bhoria, P., Singh, G., Sharma, J.R. and Hoondal, G.S. (2012) 'Biobleaching of wheat straw-rich-soda pulp by the application of alkalophilic and thermophilic mannanase from Streptomyces sp. PG-08-3', *African Journal of Biotechnology,* 11(22), pp. 6111-6116.

Biermann, C.J. (1997) *Handbook of pulping and papermaking*. 2. ed., [Nachdr.] edn. San Diego [u.a.]: Acad. Press.

Blanco, A., Hermosilla, D. and Negro, C. (2016) *Water reuse within the paper industry* Switzerland: Springer, pp. 213-237 .

Blánquez, P. and Guieysse, B. (2008) 'Continuous biodegradation of 17β-estradiol and 17α-ethynylestradiol by Trametes versicolor', *Journal of Hazardous Materials,* 150(2), pp. 459-462.

Blánquez, P., Sarrà, M. and Vicent, M.T. (2006) 'Study of the cellular retention time and the partial biomass renovation in a fungal decolourisation continuous process', *Water Research,* 40(8), pp. 1650-1656.

Blánquez, P., Sarrà, M. and Vicent, T. (2008) 'Development of a continuous process to adapt the textile wastewater treatment by fungi to industrial conditions', *Process Biochemistry,* 43(1), pp. 1-7.

Bourbonnais, R., Leech, D. and Paice, M.G. (1998) 'Electrochemical analysis of the interactions of laccase mediators with lignin model compounds', *Biochimica et Biophysica Acta (BBA)-General Subjects,* 1379, pp. 381-390. doi: 10.1016/S0304-4165(97)00117-7 .

Bourbonnais, R. and Paice, M.G. (1990) 'Oxidation of non-phenolic substrates: an expanded role for laccase in lignin biodegradation', *FEBS Letters,* 267(1), pp. 99-102.

Brijwani, K., Rigdon, A. and Vadlani, P.V. (2010) 'Fungal Laccases: Production, Function, and Applications in Food Processing', *Enzyme Research,* 2010, pp. 149748-10. doi: 10.4061/2010/149748 https://www.airitilibrary.com/Article/Detail/P20151207001-201012-201706260023-201706260023-20-29.

Bruce, A. (1998) *Forest products biotechnology*. 1st edn. London [u.a.]: Taylor & Francis.

Bruijnincx, P.C., Rinaldi, R. and Weckhuysen, B.M. (2015) 'Unlocking the potential of a sleeping giant: lignins as sustainable raw materials for renewable fuels, chemicals and materials', *Green Chemistry,* 17(11), pp. 4860-4861.

Buchholz, K., Kasche, V. and Bornscheuer, U.T. (2012) *Biocatalysts and enzyme technology*. 2., edn. Weinheim: Wiley-Blackwell.

Bugg, T.D., Ahmad, M., Hardiman, E.M. and Rahmanpour, R. (2011) 'Pathways for degradation of lignin in bacteria and fungi', *Natural Product Reports,* 28(12), pp. 1883-1896.

Burton, S.G. (1994) 'Biocatalysis with polyphenol oxidase: a review', *Catalysis Today,* 22(3), pp. 459-487.

Buswell, J.A., Odier, E. and Kirk, T.K. (1987) 'Lignin biodegradation', *Critical Reviews in Biotechnology,* 6(1), pp. 1-60.

Buyukkamaci, N. and Koken, E. (2010) 'Economic evaluation of alternative wastewater treatment plant options for pulp and paper industry', *Science of the Total Environment,* 408(24), pp. 6070-6078.

Cabrera, M.N. (2017) 'Pulp mill wastewater: characteristics and treatment', *Biological Wastewater Treatment and Resource Recovery,* 2, pp. 119-139.

Calvo-Flores, F.G. and Dobado, J.A. (2010) 'Lignin as renewable raw material', *ChemSusChem,* 3(11), pp. 1227-1235.

Camarero, S., Cañas, A.I., Nousiainen, P., Record, E., Lomascolo, A., MartÍnez, M.J. and MartÍnez, ÁT. (2008) 'p-Hydroxycinnamic acids as natural mediators for laccase oxidation of recalcitrant compounds', *Environmental Science & Technology,* 42(17), pp. 6703-6709.

Cañas, A.I. and Camarero, S. (2010) 'Laccases and their natural mediators: biotechnological tools for sustainable eco-friendly processes', *Biotechnology Advances,* 28(6), pp. 694-705.

Cancel, A.M., Orth, A.B. and Tien, M. (1993) 'Lignin and veratryl alcohol are not inducers of the ligninolytic system of Phanerochaete chrysosporium', *Applied and Environmental Microbiology,* 59(9), pp. 2909-2913.

Candeias, L.P., Folkes, L.K., Dennis, M.F., Patel, K.B., Everett, S.A., Stratford, M.R. and Wardman, P. (1994) 'Free-radical intermediates and stable products in the oxidation of indole-3-acetic acid', *The Journal of Physical Chemistry,* 98(40), pp. 10131-10137.

Chakar, F.S. and Ragauskas, A.J. (2004) 'Review of current and future softwood kraft lignin process chemistry', *Industrial Crops and Products,* 20(2), pp. 131-141.

Chan, Y.J., Chong, M.F., Law, C.L. and Hassell, D.G. (2009) 'A review on anaerobic–aerobic treatment of industrial and municipal wastewater', *Chemical Engineering Journal,* 155(1-2), pp. 1-18.

Chandra, R. and Bharagava, R.N. (2013) 'Bacterial degradation of synthetic and kraft lignin by axenic and mixed culture and their metabolic products', *Journal of Environmental Biology,* 34(6), pp. 991.

Chandra, R., Sharma, P., Yadav, S. and Tripathi, S. (2018) 'Biodegradation of endocrine-disrupting chemicals and residual organic pollutants of pulp and paper mill effluent by biostimulation', *Frontiers in Microbiology,* 9, pp. 960.

Chandra, R. and Singh, R. (2012) 'Decolourisation and detoxification of rayon grade pulp paper mill effluent by mixed bacterial culture isolated from pulp paper mill effluent polluted site', *Biochemical Engineering Journal*, 61, pp. 49-58.

Chauhan, P.S. (2020) 'Role of various bacterial enzymes in complete depolymerization of lignin: A review', *Biocatalysis and Agricultural Biotechnology*, 23, pp. 101498.

Chen, Y., Chai, L., Tang, C., Yang, Z., Zheng, Y., Shi, Y. and Zhang, H. (2012) 'Kraft lignin biodegradation by Novosphingobium sp. B-7 and analysis of the degradation process', *Bioresource Technology*, 123, pp. 682-685.

Cheng, Q.Z., Wang, J.X., McNeel, J.F. and Jacobson, P.M. (2010a) 'Water retention value measurements of cellulosic materials using a centrifuge technique.', *BioResources*, 5(3), pp. 1945-1954.

Cheng, Q., Wang, J., McNeel, J. and Jacobson, P. (2010b) 'Water retention value measurements of cellulosic materials using a centrifuge technique', *BioResources*, 5(3), pp. 1945-1954.

Chenthamarakshan, A., Parambayil, N., Miziriya, N., Soumya, P.S., Lakshmi, M.S.K., Ramgopal, A., Dileep, A. and Nambisan, P. (2017) 'Optimization of laccase production from Marasmiellus palmivorus LA1 by Taguchi method of Design of experiments', *BMC Biotechnology*, 17(1), pp. 12. doi: 10.1186/s12896-017-0333-x .

Cho, M., Moinuddin, S.G., Helms, G.L., Hishiyama, S., Eichinger, D., Davin, L.B. and Lewis, N.G. (2003) '( )-Larreatricin hydroxylase, an enantio-specific polyphenol oxidase from the

creosote bush (Larrea tridentata)', *Proceedings of the National Academy of Sciences*, 100(19), pp. 10641-10646.

Chopra, A.K. and Singh, P.P. (2012) 'Removal of color, COD and lignin from pulp and paper mill effluent by Phanerochaete chrysosporium and Aspergillus fumigatus', *Journal of Chemical and Pharmaceutical Research*, 4(10), pp. 4522-4532.

Choudhury, S., Sahoo, N., Manthan, M. and Rohella, R.S. (1998) 'Fungal treatment of pulp and paper mill effluents for pollution control', *Journal of Industrial Pollution Control*, 14, pp. 1-13.

Christopher, L.P., Yao, B. and Ji, Y. (2014) 'Lignin biodegradation with laccase-mediator systems', *Frontiers in Energy Research*, 2, pp. 12.

Christov, L. and Van Driessel, B. (2003) 'Waste Water Bioremediation in the Pulp and Paper Industry', *Indian Journal of Biotechnology*, 2(3), pp. 444-450.

Chu, R., Li, S., Zhu, L., Yin, Z., Hu, D., Liu, C. and Mo, F. (2021) 'A review on co-cultivation of microalgae with filamentous fungi: Efficient harvesting, wastewater treatment and biofuel production', *Renewable and Sustainable Energy Reviews*, 139, pp. 110689.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. (1995) 'Microbial biofilms', *Annual Review of Microbiology*, 49(1), pp. 711-745.

Crestini, C., Melone, F., Sette, M. and Saladino, R. (2011) 'Milled wood lignin: a linear oligomer', *Biomacromolecules*, 12(11), pp. 3928-3935.

Cruz-Morató, C., Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., Marco-Urrea, E., Vicent, T. and Sarrà, M. (2013) 'Degradation of pharmaceuticals in non-sterile urban wastewater by Trametes versicolor in a fluidized bed bioreactor', *Water Research,* 47(14), pp. 5200-5210.

D'annibale, A., Celletti, D., Felici, M., Di Mattia, E. and Giovannozzi-Sermanni, G. (1996) 'Substrate specificity of laccase from Lentinus edodes', *Acta Biotechnologica,* 16(4), pp. 257-270.

Dashtban, M., Schraft, H., Syed, T.A. and Qin, W. (2010) 'Fungal biodegradation and enzymatic modification of lignin', *International Journal of Biochemistry and Molecular Biology,* 1(1), pp. 36.

Demain, A.L. (1998) 'Induction of microbial secondary metabolism', *International Microbiology,* 1(4), pp. 259-264.

Dettman, J.R., Jacobson, D.J. and Taylor, J.W. (2006) 'Multilocus sequence data reveal extensive phylogenetic species diversity within the Neurospora discreta complex', *Mycologia,* 98(3), pp. 436-446.

Dhagat, S. and Jujjavarapu, S.E. (2022) 'Utility of lignin-modifying enzymes: a green technology for organic compound mycodegradation', *Journal of Chemical Technology & Biotechnology,* 97(2), pp. 343-358.

Dionisi, D., Majone, M., Tandoi, V. and Beccari, M. (2001) 'Sequencing batch reactor: Influence of periodic operation on performance of activated sludges in biological wastewater treatment', *Industrial & Engineering Chemistry Research,* 40(23), pp. 5110-5119.

Dogaris, I., Mamma, D. and Kekos, D. (2013) 'Biotechnological production of ethanol from renewable resources by Neurospora crassa: an alternative to conventional yeast fermentations?', *Applied Microbiology and Biotechnology,* 97(4), pp. 1457-1473.

Doherty, W.O., Mousavioun, P. and Fellows, C.M. (2011) 'Value-adding to cellulosic ethanol: Lignin polymers', *Industrial Crops and Products,* 33(2), pp. 259-276.

Dosoretz, C.G., Dass, S.B., Reddy, C.A. and Grethlein, H.E. (1990) 'Protease-mediated degradation of lignin peroxidase in liquid cultures of Phanerochaete chrysosporium', *Applied and Environmental Microbiology,* 56(11), pp. 3429-3434.

Duval, A. and Lawoko, M. (2014) 'A review on lignin-based polymeric, micro-and nano-structured materials', *Reactive and Functional Polymers,* 85, pp. 78-96.

Edwards, S.J. and Kjellerup, B.V. (2013) 'Applications of biofilms in bioremediation and biotransformation of persistent organic pollutants, pharmaceuticals/personal care products, and heavy metals', *Applied Microbiology and Biotechnology,* 97(23), pp. 9909-9921.

Eggert, C., Temp, U. and Eriksson, K. (1996) 'The ligninolytic system of the white rot fungus Pycnoporus cinnabarinus: purification and characterization of the laccase', *Applied and Environmental Microbiology,* 62(4), pp. 1151-1158.

Ehaliotis, C., Papadopoulou, K., Kotsou, M., Mari, I. and Balis, C. (1999) 'Adaptation and population dynamics of Azotobacter vinelandii during aerobic biological treatment of olive-mill wastewater', *FEMS Microbiology Ecology,* 30(4), pp. 301-311.

Ekstrand, E., Larsson, M., Truong, X., Cardell, L., Borgström, Y., Björn, A., Ejlertsson, J., Svensson, B.H., Nilsson, F. and Karlsson, A. (2013) 'Methane potentials of the Swedish pulp and paper industry–A screening of wastewater effluents', *Applied Energy*, 112, pp. 507-517.

El-Bestawy, E., El-Sokkary, I., Hussein, H. and Keela, A.F.A. (2008) 'Pollution control in pulp and paper industrial effluents using integrated chemical–biological treatment sequences', *Journal of Industrial Microbiology and Biotechnology*, 35(11), pp. 1517-1529.

Elisashvili, V., Asatiani, M.D. and Kachlishvili, E. (2020) 'Revealing the features of the oxidative enzyme production by white-rot Basidiomycetes during fermentation of plant raw materials', *Microbial Enzymes and Biotechniques: Interdisciplinary Perspectives*, , pp. 107-130.

Elisashvili, V., Kachlishvili, E., Khardziani, T. and Agathos, S.N. (2010) 'Effect of aromatic compounds on the production of laccase and manganese peroxidase by white-rot basidiomycetes', *Journal of Industrial Microbiology and Biotechnology*, 37(10), pp. 1091-1096.

Eriksson, K.E. and Kolar, M.C. (1985) 'Microbial degradation of chlorolignins', *Environmental Science & Technology*, 19(11), pp. 1086-1089.

Eriksson, K.L., Blanchette, R.A., Ander, P., Eriksson, K.L., Blanchette, R.A. and Ander, P. (1990) 'Biodegradation of lignin', *Microbial and Enzymatic Degradation of Wood and Wood Components*, , pp. 225-333.

Fabbri, F., Bischof, S., Mayr, S., Gritsch, S., Jimenez Bartolome, M., Schwaiger, N., Guebitz, G.M. and Weiss, R. (2023) 'The Biomodified Lignin Platform: A Review', *Polymers*, 15(7), pp. 1694.

Federation, W.E. and Aph Association (2005) 'Standard methods for the examination of water and wastewater', *American Public Health Association (APHA): Washington, DC, USA*, 21.

Ferreira, J.A., Varjani, S. and Taherzadeh, M.J. (2020) 'A critical review on the ubiquitous role of filamentous fungi in pollution mitigation', *Current Pollution Reports*, 6, pp. 295-309.

Flemming, H. and Wingender, J. (2010) 'The biofilm matrix', *Nature Reviews Microbiology*, 8(9), pp. 623-633.

Flemming, H., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A. and Kjelleberg, S. (2016) 'Biofilms: an emergent form of bacterial life', *Nature Reviews Microbiology*, 14(9), pp. 563-575.

Froehner, S.C. and Eriksson, K. (1974) 'Induction of Neurospora crassa laccase with protein synthesis inhibitors', *Journal of Bacteriology*, 120(1), pp. 450-457.

Fulekar, M.H., Pathak, B., Fulekar, J. and Godambe, T. (2012) 'Bioremediation of organic pollutants using Phanerochaete chrysosporium'*Fungi as Bioremediators* Springer, pp. 135-157.

Gao, H., Wang, Y., Zhang, W., Wang, W. and Mu, Z. (2011) 'Isolation, identification and application in lignin degradation of an ascomycete GHJ-4', *African Journal of Biotechnology*, 10(20), pp. 4166-4174.

Garg, A., Mishra, I.M. and Chand, S. (2007) 'Catalytic wet oxidation of the pretreated synthetic pulp and paper mill effluent under moderate conditions', *Chemosphere (Oxford)*, 66(9), pp. 1799-1805. doi: 10.1016/j.chemosphere.2006.07.038 https://dx.doi.org/10.1016/j.chemosphere.2006.07.038.

Garg, S.K. and Modi, D.R. (1999) 'Decolorization of Pulp-Paper Mill Effluents by White-Rot Fungi', *Critical Reviews in Biotechnology,* 19, pp. 85-112. doi: 10.1080/0738-859991229206 https://www.tandfonline.com/doi/abs/10.1080/0738-859991229206.

Gellerstedt, G. and Lindfors, E. (1984) 'Structural changes in lignin during kraft pulping', *Holzforschung,* , pp. 151-158.

Gianfreda, L., Xu, F. and Bollag, J. (1999) 'Laccases: a useful group of oxidoreductive enzymes', *Bioremediation Journal,* 3(1), pp. 1-26.

Gierer, J. (1980) 'Chemical aspects of kraft pulping', *Wood Science and Technology,* 14(4), pp. 241-266.

Gold, M.H. and Alic, M. (1993) 'Molecular biology of the lignin-degrading basidiomycete Phanerochaete chrysosporium', *Microbiological Reviews,* 57(3), pp. 605-622.

Gomes, I.B., Simões, M. and Simões, L.C. (2020) 'Copper surfaces in biofilm control', *Nanomaterials,* 10, pp. 2491.

Gonçalves, C., Rodriguez-Jasso, R.M., Gomes, N., Teixeira, J.A. and Belo, I. (2010) 'Adaptation of dinitrosalicylic acid method to microtiter plates', *Analytical Methods,* 2, pp. 2046. doi: 10.1039/C0AY00525H .

González, L.F., Sarria, V. and Sánchez, O.F. (2010) 'Degradation of chlorophenols by sequential biological-advanced oxidative process using Trametes pubescens and TiO2/UV', *Bioresource Technology,* 101(10), pp. 3493-3499.

Govender, S., Pillay, V.L. and Odhav, B. (2010) 'Nutrient manipulation as a basis for enzyme production in a gradostat bioreactor', *Enzyme and Microbial Technology,* 46(7), pp. 603-609.

Govumoni, S.P., Jahnavi, G., Sravanthi, K., Haragopal, V., Venkateshwar, S. and Rao, L.V. (2015) 'Extracellular lignocellulolytic enzymes by Phanerochaete chrysosporium (MTCC 787) under solid-state fermentation of agro wastes.', *International Journal of Current Microbiology and Applied Sciences,* 4(10), pp. 700-710.

Gupta, G.K., Liu, H. and Shukla, P. (2019) 'Pulp and paper industry–based pollutants, their health hazards and environmental risks', *Current Opinion in Environmental Science & Health,* 12, pp. 48-56.

Gupte, A., Gupte, S. and Patel, H. (2007) 'Ligninolytic enzyme production under solid-state fermentation by white rot fungi', *Journal of Scientific and Industrial Research,* 66, pp. 611-614.

Habets, L. and Driessen, W. (2007) 'Anaerobic treatment of pulp and paper mill effluents–status quo and new developments', *Water Science and Technology,* 55(6), pp. 223-230.

Haddouche, L., Phalak, A. and Tikekar, R.V. (2015) 'Inactivation of polyphenol oxidase using 254 nm ultraviolet light in a model system', *LWT-Food Science and Technology,* 62(1), pp. 97-103.

Hagelqvist, A. (2013) 'Batchwise mesophilic anaerobic co-digestion of secondary sludge from pulp and paper industry and municipal sewage sludge', *Waste Management,* 33(4), pp. 820-824.

Haile, A., Gelebo, G.G., Tesfaye, T., Mengie, W., Mebrate, M.A., Abuhay, A. and Limeneh, D.Y. (2021) 'Pulp and paper mill wastes: utilizations and prospects for high value-added biomaterials', *Bioresources and Bioprocessing,* 8, pp. 1-22.

Hammel, K.E., Kapich, A.N., Jensen Jr, K.A. and Ryan, Z.C. (2002) 'Reactive oxygen species as agents of wood decay by fungi', *Enzyme and Microbial Technology,* 30(4), pp. 445-453.

Haq, I., Mazumder, P. and Kalamdhad, A.S. (2020) 'Recent advances in removal of lignin from paper industry wastewater and its industrial applications–A review', *Bioresource Technology,* 312, pp. 123636.

Hatakka, A. and Hammel, K.E. (2011) 'Fungal biodegradation of lignocelluloses'*Industrial applications* Springer, pp. 319-340.

Hatfield, R. and Fukushima, R.S. (2005) 'Can lignin be accurately measured?', *Crop Science,* 45(3), pp. 832-839.

He, L., Huang, H., Zhang, Z. and Lei, Z. (2015) 'A review of hydrothermal pretreatment of lignocellulosic biomass for enhanced biogas production', *Current Organic Chemistry,* 19(5), pp. 437-446.

Heijnen, J.J., Mulder, A., Enger, W. and Hoeks, F. (1989) 'Review on the application of anaerobic fluidized bed reactors in waste-water treatment', *The Chemical Engineering Journal,* 41(3), pp. B37-B50.

Heinzkill, M., Bech, L., Halkier, T., Schneider, P. and Anke, T. (1998) 'Characterization of laccases and peroxidases from wood-rotting fungi (family Coprinaceae)', *Applied and Environmental Microbiology,* 64, pp. 1601-1606. doi: 10.1128/aem.64.5.1601-1606.1998 .

Hermosilla, D., Merayo, N., Gascó, A. and Blanco, Á (2015) 'The application of advanced oxidation technologies to the treatment of effluents from the pulp and paper industry: a review', *Environmental Science and Pollution Research,* 22, pp. 168-191.

Hernandez, M., Rodriguez, J., Perez, M.I., Ball, A.S. and Arias, M.E. (1997) '13 C NMR cross polarization and magic angle spinning (CPMAS) and gas chromatography/mass spectrometry analysis of the products from a soda pulp mill effluent decolourised by two Streptomyces strains', *Applied Microbiology and Biotechnology,* 47, pp. 272-278.

Higuchi, T. (2012) *Biochemistry and molecular biology of wood* Springer Science & Business Media.

Hooda, R., Bhardwaj, N.K. and Singh, P. (2015) 'Screening and identification of ligninolytic bacteria for the treatment of pulp and paper mill effluent', *Water, Air, & Soil Pollution,* 226(9), pp. 305.

Huang, L. and Wang, M.J. (1995) 'Image thresholding by minimizing the measures of fuzziness', *Pattern Recognition,* 28, pp. 41-51. doi: 10.1016/0031-3203(94)E0043-K https://dx.doi.org/10.1016/0031-3203(94)E0043-K.

Hubbe, M.A., Metts, J.R., Hermosilla, D., Blanco, M.A., Yerushalmi, L., Haghighat, F., Lindholm-Lehto, P., Khodaparast, Z., Kamali, M. and Elliott, A. (2016) 'Wastewater treatment

and reclamation: a review of pulp and paper industry practices and opportunities', *BioResources*, 11(3), pp. 7953-8091.

Hutchins, F.E. (1901) *Toxicity of pulp and paper mill effluent. A literature review*, pp. 4-32 .

Iram, A., Berenjian, A. and Demirci, A. (2021) 'A review on the utilization of lignin as a fermentation substrate to produce lignin-modifying enzymes and other value-added products', *Molecules*, 26(10), pp. 2960.

Janshekar, H., Brown, C. and Fiechter, A. (1981) 'Determination of biodegraded lignin by ultraviolet spectrophotometry', *Analytica Chimica Acta*, 130(1), pp. 81-91.

Janusz, G., Pawlik, A., Sulej, J., Świderska-Burek, U., Jarosz-Wilkołazka, A. and Paszczyński, A. (2017) 'Lignin degradation: microorganisms, enzymes involved, genomes analysis and evolution', *FEMS Microbiology Reviews*, 41(6), pp. 941-962.

Jordan, A. (1993) 'Integrated pollution control and the evolving style and structure of environmental regulation in the UK', *Environmental Politics*, 2(3), pp. 405-427.

Kamali, M. and Khodaparast, Z. (2015) 'Review on recent developments on pulp and paper mill wastewater treatment', *Ecotoxicology and Environmental Safety*, 114, pp. 326-342.

Karapinar, K.I. and Kargi, F. (2002) 'Biological decolorization of textile dyestuff containing wastewater by Coriolus versicolor in a rotating biological contactor', *Enzyme and Microbial Technology*, 30(2), pp. 195-199.

Karunarathna, M.S. and Smith, R.C. (2020) 'Valorization of lignin as a sustainable component of structural materials and composites: Advances from 2011 to 2019', *Sustainability,* 12(2), pp. 734.

Katiyar, P., Srivastava, S.K. and Kushwaha, D. (2020) 'Bioethanol extraction and its production from agricultural residues for sustainable development', *Waste to Energy: Prospects and Applications,* , pp. 143-163.

Kaushik, G. and Thakur, I.S. (2009) 'Isolation of fungi and optimization of process parameters for decolorization of distillery mill effluent', *World Journal of Microbiology and Biotechnology,* 25, pp. 955-964.

Keyser, P., Kirk, T.K. and Zeikus, J. (1978) 'Ligninolytic enzyme system of Phanaerochaete chrysosporium: synthesized in the absence of lignin in response to nitrogen starvation', *Journal of Bacteriology,* 135(3), pp. 790-797.

Khan, M.U. and Ahring, B.K. (2019) 'Lignin degradation under anaerobic digestion: Influence of lignin modifications-A review', *Biomass and Bioenergy,* 128, pp. 105325.

Khiyami, M.A., Pometto, A.L. and Kennedy, W.J. (2006) 'Ligninolytic enzyme production by Phanerochaete chrysosporium in plastic composite support biofilm stirred tank bioreactors', *Journal of Agricultural and Food Chemistry,* 54(5), pp. 1693-1698.

Kiran, S., Huma, T., Jalal, F., Farooq, T., Hameed, A., Gulzar, T., Bashir, A., Rahmat, M., Rahmat, R. and Rafique, M.A. (2019) 'Lignin degrading system of Phanerochaete chrysosporium and its exploitation for degradation of synthetic dyes wastewater', *Polish Journal of Environmental Studies,* 28(3), pp. 1749-1757.

Kirk, T.K. and Farrell, R.L. (1987) 'Enzymatic" combustion": the microbial degradation of lignin', *Annual Reviews in Microbiology*, 41(1), pp. 465-501.

Kishi, K., Wariishi, H., Marquez, L., Dunford, H.B. and Gold, M.H. (1994) 'Mechanism of manganese peroxidase compound II reduction. Effect of organic acid chelators and pH', *Biochemistry*, 33(29), pp. 8694-8701.

Kishimoto, N., Nakagawa, T., Okada, H. and Mizutani, H. (2010) 'Treatment of paper and pulp mill wastewater by ozonation combined with electrolysis', *Journal of Water and Environment Technology*, 8(2), pp. 99-109.

Knop, D., Levinson, D., Makovitzki, A., Agami, A., Lerer, E., Mimran, A., Yarden, O. and Hadar, Y. (2016) 'Limits of versatility of versatile peroxidase', *Applied and Environmental Microbiology*, 82(14), pp. 4070-4080.

Knop, D., Yarden, O. and Hadar, Y. (2015) 'The ligninolytic peroxidases in the genus Pleurotus: divergence in activities, expression, and potential applications', *Applied Microbiology and Biotechnology*, 99, pp. 1025-1038.

Kong, W., Fu, X., Wang, L., Alhujaily, A., Zhang, J., Ma, F., Zhang, X. and Yu, H. (2017) 'A novel and efficient fungal delignification strategy based on versatile peroxidase for lignocellulose bioconversion', *Biotechnology for Biofuels*, 10(1), pp. 1-15.

Kringstad, K.P. and Lindström, K. (1984) 'Spent liquors from pulp bleaching', *Environmental Science & Technology*, 18(8), pp. 236A-248A.

Labois, C., Stempien, E., Schneider, J., Schaeffer-Reiss, C., Bertsch, C., Goddard, M. and Chong, J. (2021) 'Comparative study of secreted proteins, enzymatic activities of wood degradation and stilbene metabolization in grapevine botryosphaeria dieback fungi', *Journal of Fungi,* 7(7), pp. 568.

Lacki, K. and Duvnjak, Z. (1998) 'Transformation of 3, 5-dimethoxy, 4-hydroxy cinnamic acid by polyphenol oxidase from the fungus Trametes versicolor: Product elucidation studies', *Biotechnology and Bioengineering,* 57(6), pp. 694-703.

Lacki, K. and Duvnjak, Z. (1996) 'Modeling the enzymatic transformation of 3, 5-dimethoxy, 4-hydroxy cinnamic acid by polyphenoloxidase from the white-rot fungus Trametes versicolor', *Biotechnology and Bioengineering,* 51(3), pp. 249-259.

Lawoko, M., Henriksson, G. and Gellerstedt, G. (2005) 'Structural differences between the lignin− carbohydrate complexes present in wood and in chemical pulps', *Biomacromolecules,* 6(6), pp. 3467-3473.

Lazarova, V. and Manem, J. (1995) 'Biofilm characterization and activity analysis in water and wastewater treatment', *Water Research,* 29(10), pp. 2227-2245.

Lee, R.A., Bédard, C., Berberi, V., Beauchet, R. and Lavoie, J. (2013) 'UV–Vis as quantification tool for solubilized lignin following a single-shot steam process', *Bioresource Technology,* 144, pp. 658-663.

Lerch, K. (1983) 'Neurospora tyrosinase: structural, spectroscopic and catalytic properties', *Molecular and Cellular Biochemistry,* 52, pp. 125-138.

Levin, L., Forchiassin, F. and Ramos, A.M. (2002) 'Copper induction of lignin-modifying enzymes in the white-rot fungus Trametes trogii', *Mycologia,* 94(3), pp. 377-383.

Li, F., Ma, F., Zhao, H., Zhang, S., Wang, L., Zhang, X. and Yu, H. (2019) 'A lytic polysaccharide monooxygenase from a white-rot fungus drives the degradation of lignin by a versatile peroxidase', *Applied and Environmental Microbiology,* 85(9), pp. 2803.

Liu, H. and Fang, H.H. (2002) 'Extraction of extracellular polymeric substances (EPS) of sludges', *Journal of Biotechnology,* 95(3), pp. 249-256.

Liu, S., Mishra, G., Amidon, T.E. and Gratien, K. (2009) 'Effect of hot-water extraction of woodchips on the kraft pulping of eucalyptus woodchips', *Journal of Biobased Materials and Bioenergy,* 3(4), pp. 363-372.

López, A.M.Q., Silva, A.L.d.S. and Santos, E.C.L.d. (2017) 'The fungal ability for biobleaching/biopulping/bioremediation of lignin-like compounds of agro-industrial raw material', *Química Nova,* 40, pp. 916-931.

Luke, A.K. and Burton, S.G. (2001) 'A novel application for Neurospora crassa: progress from batch culture to a membrane bioreactor for the bioremediation of phenols', *Enzyme and Microbial Technology,* 29(6-7), pp. 348-356.

Ma, F., Xiong, Z., Zheng, Y., Yu, X. and Zhang, X. (2008) 'Repeated batch process for biological treatment of black liquor using brown-rot basidiomycete Fomitopsis sp. IMER2', *World Journal of Microbiology and Biotechnology,* 24, pp. 2627-2632.

Madadi, M. and Abbas, A. (2017) 'Lignin degradation by fungal pretreatment: a review', *Journal of Plant Pathology and Microbiology,* 8(398), pp. 2.

Mahdinia, E. and Demirci, A. (2020) '4 Biofilms in Fermentation', *Microbial Biofilms: Properties and Applications in the Environment, Agriculture, and Medicine,* , pp. 73.

Malaviya, P. and Rathore, V.S. (2007) 'Bioremediation of pulp and paper mill effluent by a novel fungal consortium isolated from polluted soil', *Bioresource Technology,* 98(18), pp. 3647-3651.

Manda, G., Rojo, A.I., Martínez-Klimova, E., Pedraza-Chaverri, J. and Cuadrado, A. (2020) 'Nordihydroguaiaretic acid: From herbal medicine to clinical development for cancer and chronic diseases', *Frontiers in Pharmacology*, 11, pp. 151.

Martínez, A.T., Speranza, M., Ruiz-Dueñas, F.J., Ferreira, P., Camarero, S., Guillén, F., Martínez, M.J., Gutiérrez, A. and del Río, J.C. (2005) 'Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin', *International Microbiology,* 8(3), pp. 195-204https://www.ncbi.nlm.nih.gov/pubmed/16200498.

Martínková, L., Kotik, M., Marková, E. and Homolka, L. (2016) 'Biodegradation of phenolic compounds by Basidiomycota and its phenol oxidases: a review', *Chemosphere,* 149, pp. 373-382.

Martins, S., Teixeira, J.A. and Mussatto, S.I. (2013) 'Solid-state fermentation as a strategy to improve the bioactive compounds recovery from Larrea tridentata leaves', *Applied Biochemistry and Biotechnology*, 171, pp. 1227-1239.

Matheus, M.C., Ekenberg, M., Bassin, J.P., Dezotti, M.W. and Piculell, M. (2021) 'High loaded moving bed biofilm reactors treating pulp & paper industry wastewater: Effect of hydraulic retention time, filling degree and nutrients availability on performance, biomass fractions and nutrients utilization', *Journal of Environmental Chemical Engineering*, 9(1), pp. 104944.

May, A., Narayanan, S., Alcock, J., Varsani, A., Maley, C. and Aktipis, A. (2019) 'Kombucha: A novel model system for cooperation and conflict in a complex multi-species microbial ecosystem', *PeerJ*, 7, pp. e7565.

McAndrew, R.P., Sathitsuksanoh, N., Mbughuni, M.M., Heins, R.A., Pereira, J.H., George, A., Sale, K.L., Fox, B.G., Simmons, B.A. and Adams, P.D. (2016) 'Structure and mechanism of NOV1, a resveratrol-cleaving dioxygenase', *Proceedings of the National Academy of Sciences*, 113(50), pp. 14324-14329.

Meyer, T. and Edwards, E.A. (2014) 'Anaerobic digestion of pulp and paper mill wastewater and sludge', *Water Research*, 65, pp. 321-349.

Mikiashvili, N., Elisashvili, V., Wasser, S. and Nevo, E. (2005) 'Carbon and nitrogen sources influence the ligninolytic enzyme activity of Trametes versicolor', *Biotechnology Letters*, 27(13), pp. 955-959.

Mir-Tutusaus, J.A., Baccar, R., Caminal, G. and Sarrà, M. (2018) 'Can white-rot fungi be a real wastewater treatment alternative for organic micropollutants removal? A review', *Water Research*, 138, pp. 137-151.

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

Mishra, V., Jana, A.K., Jana, M.M. and Gupta, A. (2017) 'Enhancement in multiple lignolytic enzymes production for optimized lignin degradation and selectivity in fungal pretreatment of sweet sorghum bagasse', *Bioresource Technology,* 236, pp. 49-59.

Möbius, C.H. (2006) *Water use and wastewater treatment in papermills* Books on Demand.

Moga, I.C., Bardi, A., Di Gregorio, S., Spennati, F., Munz, G., Batistini, S., Iordache, O.G., Mitran, C.E. and Petrescu, G. (2019) *Improved biofilm carriers for fungal exploitation in wastewater treatment.* IOP Publishing, pp. 012104.

Moilanen, A.M., Lundell, T., Vares, T. and Hatakka, A. (1996) 'Manganese and malonate are individual regulators for the production of lignin and manganese peroxidase isozymes and in the degradation of lignin by Phlebia radiata', *Applied Microbiology and Biotechnology,* 45(6), pp. 792-799.

Moshtaghioun, S.M., Dadkhah, M., Bahremandjo, K., Haghbeen, K., Aminzadeh, S. and Legge, R.L. (2017) 'Optimization of simultaneous production of tyrosinase and laccase by Neurospora crassa', *Biocatalysis and Biotransformation,* 35, pp. 1-10. doi: 10.1080/10242422.2016.1266617 https://www.tandfonline.com/doi/abs/10.1080/10242422.2016.1266617.

Mukherjee, G., Sachan, A., Ghosh, S. and Mitra, A. (2006) 'Conversion of sinapic acid to syringic acid by a filamentous fungus Paecilomyces variotii', *The Journal of General and Applied Microbiology*, 52(2), pp. 131-135.

Nandal, P., Ravella, S.R. and Kuhad, R.C. (2013) 'Laccase production by Coriolopsis caperata RCK2011: optimization under solid state fermentation by Taguchi DOE methodology', *Scientific Reports*, 3(1), pp. 1386.

Nićiforović, N. and Abramovič, H. (2014) 'Sinapic acid and its derivatives: natural sources and bioactivity', *Comprehensive Reviews in Food Science and Food Safety,* 13(1), pp. 34-51.

Nicolella, C., Van Loosdrecht, M. and Heijnen, J.J. (2000) 'Wastewater treatment with particulate biofilm reactors', *Journal of Biotechnology,* 80(1), pp. 1-33.

Norgren, M. and Edlund, H. (2014) 'Lignin: Recent advances and emerging applications', *Current Opinion on Colloidal and Interface Science,* 19, pp. 409-416. doi: 10.1016/j.cocis.2014.08.004 https://dx.doi.org/10.1016/j.cocis.2014.08.004.

Nousiainen, P., Kontro, J., Manner, H., Hatakka, A. and Sipilä, J. (2014) 'Phenolic mediators enhance the manganese peroxidase catalyzed oxidation of recalcitrant lignin model compounds and synthetic lignin', *Fungal Genetics and Biology,* 72, pp. 137-149.

Ntougias, S., Baldrian, P., Ehaliotis, C., Nerud, F., Merhautová, V. and Zervakis, G.I. (2015) 'Olive mill wastewater biodegradation potential of white-rot fungi–Mode of action of fungal culture extracts and effects of ligninolytic enzymes', *Bioresource Technology,* 189, pp. 121-130.

Ogunsile, B.O. and Quintana, G. (2010) 'Modeling of soda-ethanol pulps from Carpolobia lutea', *Bioresources*, 5(4), pp. 2417-2430.

Oikari, A.O. and Nakari, T. (1982) 'Kraft pulp mill effluent components cause liver dysfunction in trout [Salmo gairdneri]', *Bulletin of Environmental Contamination and Toxicology*, 28, pp. 266-270. doi: 10.1007/BF01608505 https://agris.fao.org/agris-search/search.do?recordID=US19830865937.

Palmieri, G., Giardina, P., Bianco, C., Fontanella, B. and Sannia, G. (2000) 'Copper induction of laccase isoenzymes in the ligninolytic fungus Pleurotus ostreatus', *Applied and Environmental Microbiology*, 66, pp. 920-924. doi: 10.1128/AEM.66.3.920-924.2000 https://agris.fao.org/agris-search/search.do?recordID=US201302958452.

Palmieri, G., Giardina, P., Marzullo, L., Desiderio, B., Nitti, G., Cannio, R. and Sannia, G. (1993) 'Stability and activity of a phenol oxidase from the ligninolytic fungus Pleurotus ostreatus', *Applied and Environmental Microbiology*, 39, pp. 632-636. doi: 10.1007/BF00205066 https://www.ncbi.nlm.nih.gov/pubmed/7763931.

Pamidipati, S. and Ahmed, A. (2020) 'A first report on competitive inhibition of laccase enzyme by lignin degradation intermediates', *Folia Microbiol*, 65, pp. 431-437. doi: 10.1007/s12223-019-00765-5 https://link.springer.com/article/10.1007/s12223-019-00765-5.

Pamidipati, S. and Ahmed, A. (2017) 'Degradation of Lignin in Agricultural Residues by locally Isolated Fungus Neurospora discreta', *Applied Biochemistry and Biotechnology*, 181, pp. 1561-1572. doi: 10.1007/s12010-016-2302-6 https://link.springer.com/article/10.1007/s12010-016-2302-6.

Pandey, K.B. and Rizvi, S.I. (2009) 'Plant polyphenols as dietary antioxidants in human health and disease', *Oxidative Medicine and Cellular Longevity,* 2, pp. 270-278.

Papagianni, M. (2004) 'Fungal morphology and metabolite production in submerged mycelial processes', *Biotechnology Advances*, 22, pp. 189-259. doi: 10.1016/j.biotechadv.2003.09.005 https://dx.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. and Huang, Z. (2022) 'Optimizing biocatalytic potential of Dipodascus australiensis M-2 for degrading lignin under laboratory conditions', *Microbiological Research,* 265, pp. 127179. doi: 10.1016/j.micres.2022.127179 https://dx.doi.org/10.1016/j.micres.2022.127179.

Patel, K., Patel, N., Vaghamshi, N., Shah, K., Duggirala, S.M. and Dudhagara, P. (2021) 'Trends and strategies in the effluent treatment of pulp and paper industries: A review highlighting reactor options', *Current Research in Microbial Sciences*, 2, pp. 100077.

Patil, N.D. (2014) *A study of lignin depolymerization by selective cleavage of the Cα-Cβ linkages in lignin model compounds via Baeyer-villiger oxidation & an investigation of the channeling reaction in nitrogen-doped multiwalled carbon nanotubes(N-MWCNTS).* Doctoral Dissertation. University of Kentucky.

Pazarlıoğlu, N.K., Sariişik, M. and Telefoncu, A. (2005) 'Laccase: production by Trametes versicolor and application to denim washing', *Process Biochemistry*, 40(5), pp. 1673-1678.

Pedroza-Rodríguez, A.M. and Rodríguez-Vázquez, R. (2013) 'Optimization of C/N ratio and inducers for wastewater paper industry treatment using Trametes versicolor immobilized in bubble column reactor', *Journal of Mycology*, 2013.

Pérez, J., Muñoz-Dorado, J., de la Rubia, T. and Martínez, J. (2002) 'Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview', *International Microbiology*, 5(2), pp. 53-63. doi: 10.1007/s10123-002-0062-3 https://www.ncbi.nlm.nih.gov/pubmed/12180781.

Pessala, P., Schultz, E., Luukkainen, S., Herve, S., Knuutinen, J. and Paasivirta, J. (2004) *Lignin as the cause of acute toxicity in pulp and paper mill effluents*, pp. 319-330 .

Pokhrel, D. and Viraraghavan, T. (2004) 'Treatment of pulp and paper mill wastewater—a review', *Science of the Total Environment*, 333(1-3), pp. 37-58.

Pradeep, N.V., Anupama, S., Navya, K., Shalini, H.N., Idris, M. and Hampannavar, U.S. (2015) 'Biological removal of phenol from wastewaters: a mini review', *Applied Water Science*, 5(2), pp. 105-112.

Prasad, K.K., Mohan, S.V., Rao, R.S., Pati, B.R. and Sarma, P.N. (2005) 'Laccase production by Pleurotus ostreatus 1804: Optimization of submerged culture conditions by Taguchi DOE methodology', *Biochemical Engineering Journal*, 24, pp. 17-26. doi: 10.1016/j.bej.2005.01.019 https://dx.doi.org/10.1016/j.bej.2005.01.019.

Prenafeta-Boldú, F.X., De Hoog, G.S. and Summerbell, R.C. (2018) 'Fungal communities in hydrocarbon degradation', *Microbial Communities Utilizing Hydrocarbons and Lipids: Members, Metagenomics and Ecophysiology; McGenity, TJ, Ed,* , pp. 1-36.

Radjenović, J., Matošić, M., Mijatović, I., Petrović, M. and Barceló, D. (2015) 'Membrane Bioreactor (MBR) as an Advanced Wastewater Treatment Technology'*Emerging Contaminants from Industrial and Municipal Waste* Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 37-101.

Ragunathan, R. and Swaminathan, K. (2004) 'Biological treatment of a pulp and paper industry effluent by Pleurotus spp.', *World Journal of Microbiology and Biotechnology,* 20, pp. 389-393.

Raj, A., Chandra, R., Reddy, M.M.K., Purohit, H.J. and Kapley, A. (2007) 'Biodegradation of kraft lignin by a newly isolated bacterial strain, Aneurinibacillus aneurinilyticus from the sludge of a pulp paper mill', *World Journal of Microbiology and Biotechnology*, 23, pp. 793-799.

Raj, A., Reddy, M.K. and Chandra, R. (2007) 'Decolourisation and treatment of pulp and paper mill effluent by lignin-degrading Bacillus sp.', *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology,* 82(4), pp. 399-406.

Rajesh, J., Kavitha, S., Yukesh Kannah, R., Poornima Devi, T., Gunasekaran, M., Kim, S. and Kumar, G. (2019) 'A review on biopolymer production via lignin valorization', *Bioresource Technology,* 290, pp. 121790. doi: 10.1016/j.biortech.2019.121790 https://dx.doi.org/10.1016/j.biortech.2019.121790.

Rajwar, D., Paliwal, R. and Rai, J. (2017) 'Biodegradation of pulp and paper mill effluent by co-culturing ascomycetous fungi in repeated batch process', *Environmental Monitoring and Assessment*, 189, pp. 1-16.

Ralph, J., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P.F., Marita, J.M., Hatfield, R.D., Ralph, S.A. and Christensen, J.H. (2004) 'Lignins: natural polymers from oxidative coupling of 4-hydroxyphenyl-propanoids', *Phytochemistry Reviews*, 3(1), pp. 29-60.

Ram, C., Rani, P., Gebru, K.A. and Mariam Abrha, M.G. (2020) 'Pulp and paper industry wastewater treatment: use of microbes and their enzymes', *Physical Sciences Reviews*, 5(10), pp. 20190050.

Ramadhani, I., Kanti, A. and Sudiana, I.M. (2019) *Screening of potential lignin-degrading fungi from the tropical forest for lignocellulose biotreatment.* IOP Publishing, pp. 012014.

Rao, R.S., Kumar, C.G., Prakasham, R.S. and Hobbs, P.J. (2008) 'The Taguchi methodology as a statistical tool for biotechnological applications: A critical appraisal', *Biotechnology Journal*, 3(4), pp. 510-523. doi: 10.1002/biot.200700201 https://api.istex.fr/ark:/67375/WNG-Z6MPN36D-2/fulltext.pdf.

Ravichandran, A., Rao, R.G., Thammaiah, V., Gopinath, S. and Manpal Sridhar (2018) 'A novel Versatile Peroxidase from Lentinus squarrosulus (MH172167)towards enhanced delignification and digestibility of crop residues', *BioRxiv*, . doi: 10.1101/394098 https://search.proquest.com/docview/2090649204.

Reid, I.D. (1979) 'The influence of nutrient balance on lignin degradation by the white-rot fungus Phanerochaete chrysosporium', *Canadian Journal of Botany*, 57(19), pp. 2050-2058.

Rittmann, B.E. and McCarty, P.L. (2001) *Environmental biotechnology: principles and applications* New York: McGraw-Hill Education, pp. 207-730 .

Rodriguez, A., Perestelo, F., Carnicero, A., Regalado, V., Perez, R., De la Fuente, G. and Falcon, M.A. (1996) 'Degradation of natural lignins and lignocellulosic substrates by soil-inhabiting fungi imperfecti', *FEMS Microbiology Ecology*, 21(3), pp. 213-219.

Rodríguez, J., Ferraz, A., Nogueira, R.F., Ferrer, I., Esposito, E. and Durán, N. (1997) 'Lignin biodegradation by the ascomycete Chrysonilia sitophila', *Applied Biochemistry and Biotechnology*, 62, pp. 233-242.

Romero-Olivares, A.L., Taylor, J.W. and Treseder, K.K. (2015) 'Neurospora discreta as a model to assess adaptation of soil fungi to warming', *BMC Evolutionary Biology*, 15, pp. 1-8.

Rosche, B., Li, X.Z., Hauer, B., Schmid, A. and Buehler, K. (2009) 'Microbial biofilms: a concept for industrial catalysis?', *Trends in Biotechnology*, 27(11), pp. 636-643.

Rousk, J., Brookes, P.C. and Bååth, E. (2009) 'Contrasting Soil pH Effects on Fungal and Bacterial Growth Suggest Functional Redundancy in Carbon Mineralization', *Applied and Environmental Microbiology*, 75(6), pp. 1589-1596. doi: 10.1128/AEM.02775-08 https://agris.fao.org/agris-search/search.do?recordID=US201301602556.

Roy-Arcand, L. and Archibald, F.S. (1991) 'Direct dechlorination of chlorophenolic compounds by laccases from Trametes (Coriolus) versicolor', *Enzyme and Microbial Technology*, 13(3), pp. 194-203.

Rughoonundun, H., Mohee, R. and Holtzapple, M.T. (2012) 'Influence of carbon-to-nitrogen ratio on the mixed-acid fermentation of wastewater sludge and pretreated bagasse', *Bioresource Technology*, 112, pp. 91-97.

Ruiz-Dueñas, F.J. and Martínez, ÁT. (2009) 'Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this', *Microbial Biotechnology*, 2(2), pp. 164-177.

Rüttimann-Johnson, C., Salas, L., Vicuña, R. and Kirk, T.K. (1993) 'Extracellular enzyme production and synthetic lignin mineralization by Ceriporiopsis subvermispora', *Applied and Environmental Microbiology*, 59(6), pp. 1792-1797.

Ryan, D.R., Leukes, W.D. and Burton, S.G. (2005) 'Fungal bioremediation of phenolic wastewaters in an airlift reactor', *Biotechnology Progress*, 21(4), pp. 1068-1074.

Saadia, A. and Ashfaq, A. (2010) 'Environmental management in pulp and paper industry', *I Control Pollution*, 26(1).

Saini, S., Tewari, S., Dwivedi, J. and Sharma, V. (2023) 'Biofilm-mediated wastewater treatment: a comprehensive review', *Materials Advances*, 4(6), pp. 1415-1443.

Sainlez, M. and Heyen, G. (2013) 'Comparison of supervised learning techniques for atmospheric pollutant monitoring in a Kraft pulp mill', *Journal of Computational and Applied Mathematics*, 246, pp. 329-334.

Saito, T., Hong, P., Kato, K., Okazaki, M., Inagaki, H., Maeda, S. and Yokogawa, Y. (2003) 'Purification and characterization of an extracellular laccase of a fungus (family Chaetomiaceae) isolated from soil', *Enzyme and Microbial Technology*, 33, pp. 520-526. doi: 10.1016/S0141-0229(03)00158-3 https://dx.doi.org/10.1016/S0141-0229(03)00158-3.

Sakakibara, A. and Sano, Y. (2000) *Chemistry of lignin* Marcel Dekker, Inc. New York, pp. 109-173 .

Sakurai, A., Yamamoto, T., Makabe, A., Kinoshita, S. and Sakakibara, M. (2002) 'Removal of lignin in a liquid system by an isolated fungus', *Journal of Chemical Technology & Biotechnology*, 77(1), pp. 9-14.

Sangha, A.K., Davison, B.H., Standaert, R.F., Davis, M.F., Smith, J.C. and Parks, J.M. (2014) 'Chemical factors that control lignin polymerization', *The Journal of Physical Chemistry B*, 118(1), pp. 164-170.

Sankaran, S., Khanal, S.K., Jasti, N., Jin, B., Pometto III, A.L. and Van Leeuwen, J.H. (2010) 'Use of filamentous fungi for wastewater treatment and production of high value fungal byproducts: a review', *Critical Reviews in Environmental Science and Technology*, 40(5), pp. 400-449.

Saunamaki, R. (1997) 'Activated sludge plants in Finland', *Water Science and Technology*, 35(2-3), pp. 235-243.

Scheel, T., Höfer, M., Ludwig, S. and Hölker, U. (2000) 'Differential expression of manganese peroxidase and laccase in white-rot fungi in the presence of manganese or aromatic compounds', *Applied Microbiology and Biotechnology,* 54, pp. 686-691.

Schilling, B., Linden, R.M., Kupper, U. and Lerch, K. (1992) 'Expression of Neurospora crassa laccase under the control of the copper-inducible metallothionein-promoter', *Current Genetics,* 22, pp. 197-203.

Schueffler, A. and Anke, T. (2014) 'Fungal natural products in research and development', *Natural Product Reports,* 31(10), pp. 1425-1448.

Sedighi, M., Karimi, A. and Vahabzadeh, F. (2009) 'Involvement of ligninolytic enzymes of Phanerochaete chrysosporium in treating the textile effluent containing Astrazon Red FBL in a packed-bed bioreactor', *Journal of Hazardous Materials,* 169(1-3), pp. 88-93.

Seesuriyachan, P., Kuntiya, A., Kawee-ai, A., Techapun, C., Chaiyaso, T. and Leksawasdi, N. (2015) 'Improvement in efficiency of lignin degradation by Fenton reaction using synergistic catalytic action', *Ecological Engineering,* 85, pp. 283-287.

Selinheimo, E., Saloheimo, M., Ahola, E., Westerholm-Parvinen, A., Kalkkinen, N., Buchert, J. and Kruus, K. (2006) 'Production and characterization of a secreted, C-terminally processed tyrosinase from the filamentous fungus Trichoderma reesei', *The FEBS Journal,* 273(18), pp. 4322-4335.

Sharma, P., Iqbal, H.M. and Chandra, R. (2022) 'Evaluation of pollution parameters and toxic elements in wastewater of pulp and paper industries in India: A case study', *Case Studies in Chemical and Environmental Engineering,* 5, pp. 100163.

Shary, S., Ralph, S.A. and Hammel, K.E. (2007) 'New insights into the ligninolytic capability of a wood decay ascomycete', *Applied and Environmental Microbiology,* 73(20), pp. 6691-6694.

Sigoillot, J., Berrin, J., Bey, M., Lesage-Meessen, L., Levasseur, A., Lomascolo, A., Record, E. and Uzan-Boukhris, E. (2012) 'Fungal strategies for lignin degradation', *Advances in Botanical Research,* 61, pp. 263-308.

Simstich, B. and Oeller, H. (2010) 'Membrane technology for the future treatment of paper mill effluents: chances and challenges of further system closure', *Water Science and Technology,* 62(9), pp. 2190-2197.

Singh Arora, D. and Kumar Sharma, R. (2010) 'Ligninolytic fungal laccases and their biotechnological applications', *Applied Biochemistry and Biotechnology,* 160, pp. 1760-1788.

Singh, A.K., Bilal, M., Iqbal, H.M. and Raj, A. (2021) 'Lignin peroxidase in focus for catalytic elimination of contaminants—A critical review on recent progress and perspectives', *International Journal of Biological Macromolecules,* 177, pp. 58-82.

Singh, D. and Chen, S. (2008) 'The white-rot fungus Phanerochaete chrysosporium: conditions for the production of lignin-degrading enzymes', *Applied Microbiology and Biotechnology,* 81(3), pp. 399-417.

Singh, P., Katiyar, D., Gupta, M. and Singh, A. (2011) 'Removal of pollutants from pulp and paper mill effluent by anaerobic and aerobic treatment in pilot-scale bioreactor', *International Journal of Environment and Waste Management,* 7(3-4), pp. 423-431.

Singh, R., Paul, D. and Jain, R.K. (2006) 'Biofilms: implications in bioremediation', *Trends in Microbiology,* 14(9), pp. 389-397.

Singh, S. (2018) 'White-rot fungal xylanases for applications in pulp and paper industry', *Fungal Biorefineries,* , pp. 47-63.

Singhal, A. and Thakur, I.S. (2009) 'Decolourization and detoxification of pulp and paper mill effluent by Emericella nidulans var. nidulans', *Journal of Hazardous Materials,* 171(1-3), pp. 619-625.

Siqueira, G., Milagres, A.M., Carvalho, W., Koch, G. and Ferraz, A. (2011) 'Topochemical distribution of lignin and hydroxycinnamic acids in sugar-cane cell walls and its correlation with the enzymatic hydrolysis of polysaccharides', *Biotechnology for Biofuels,* 4, pp. 1-9.

Skulcova, A., Majova, V., Kohutova, M., Grosik, M., Sima, J. and Jablonsky, M. (2017) 'UV/Vis Spectrometry as a Quantification Tool for Lignin Solubilized in Deep Eutectic Solvents', *Bioresources,* 12. doi: 10.15376/biores.12.3.6713-6722 .

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. and Crocker, D. (2008) 'Determination of structural carbohydrates and lignin in biomass', *Laboratory Analytical Procedure,* 1617(1), pp. 1-16.

Spellman, F.R. (2013) *Water & wastewater infrastructure: Energy efficiency and sustainability* Florida,USA: Crc Press, pp. 360-361 .

Sperling, M.v. and de Lemos Chernicharo, C.A. (2005) *Biological wastewater treatment in warm climate regions* IWA London.

Sridhar, M. (2016) 'Versatile Peroxidases: Super Peroxidases with Potential Biotechnological Applications-A Mini Review', *Journal of Dairy, Veterinary & Animal Research*, 4(2). doi: 10.15406/jdvar.2016.04.00116 .

Srivastava, V.C., Mall, I.D. and Mishra, I.M. (2005) 'Treatment of pulp and paper mill wastewaters with poly aluminium chloride and bagasse fly ash', *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 260(1), pp. 17-28. doi: 10.1016/j.colsurfa.2005.02.027 https://dx.doi.org/10.1016/j.colsurfa.2005.02.027.

Subowo, Y.B. (2019) *Screening of Basidiomycetes with laccase activity for lignin degradation on POME*. IOP Publishing, pp. 012013.

Sun, R., Tomkinson, J. and Bolton, J. (1999) 'Separation and Characterization of Lignins from the Black Liquor of Oil Palm Trunk Fiber Pulping', *Separation Science and Technology*, 34(15), pp. 3045-3058. doi: 10.1081/SS-100100821 https://www.tandfonline.com/doi/abs/10.1081/SS-100100821.

Suryadi, H., Judono, J.J., Putri, M.R., Eclessia, A.D., Ulhaq, J.M., Agustina, D.N. and Sumiati, T. (2022) 'Biodelignification of lignocellulose using ligninolytic enzymes from white-rot fungi',

Heliyon, 8(2), pp. e08865. doi: 10.1016/j.heliyon.2022.e08865 https://dx.doi.org/10.1016/j.heliyon.2022.e08865.

Svobodová, K. and Novotný, Č (2018) 'Bioreactors based on immobilized fungi: bioremediation under non-sterile conditions', *Applied Microbiology and Biotechnology*, 102, pp. 39-46.

Taboada-Puig, R., Lú-Chau, T., Moreira, M.T., Feijoo, G., Martínez, M.J. and Lema, J.M. (2011) 'A new strain of Bjerkandera sp. production, purification and characterization of versatile peroxidase', *World Journal of Microbiology and Biotechnology*, 27(1), pp. 115-122. doi: 10.1007/s11274-010-0435-2 https://link.springer.com/article/10.1007/s11274-010-0435-2.

Tabraiz, S., Aiswarya, N.M., Taneja, H., Narayanan, R.A. and Ahmed, A. (2022) 'Biofilm-based simultaneous nitrification, denitrification, and phosphorous uptake in wastewater by Neurospora discreta', *Journal of Environmental Management*, 324, pp. 116363. doi: 10.1016/j.jenvman.2022.116363 https://dx.doi.org/10.1016/j.jenvman.2022.116363.

Tarlan, E., Dilek, F.B. and Yetis, U. (2002) 'Effectiveness of algae in the treatment of a wood-based pulp and paper industry wastewater', *Bioresource Technology*, 84(1), pp. 1-5.

Tezel, U., Guven, E., Erguder, T.H. and Demirer, G.N. (2001) 'Sequential (anaerobic/aerobic) biological treatment of Dalaman SEKA pulp and paper industry effluent', *Waste Management*, 21(8), pp. 717-724.

Thakur, I.S. (2004) 'Screening and identification of microbial strains for removal of colour and adsorbable organic halogens in pulp and paper mill effluent', *Process Biochemistry*, 39(11), pp. 1693-1699.

Thompson, G., Swain, J., Kay, M. and Forster, C.F. (2001) *The treatment of pulp and paper mill effluent: a review* Oxford: Elsevier Ltd, pp. 275-286 https://dx.doi.org/10.1016/S0960-8524(00)00060-2 .

Thurston, C.F. (1994) 'The structure and function of fungal laccases', *Microbiology,* 140(1), pp. 19-26.

Toczyłowska-Mamińska, R. (2017) 'Limits and perspectives of pulp and paper industry wastewater treatment–A review', *Renewable and Sustainable Energy Reviews*, 78, pp. 764-772.

Todhanakasem, T., Salangsing, O., Koomphongse, P., Kaewket, S., Kanokratana, P. and Champreda, V. (2019) 'Zymomonas mobilis biofilm reactor for ethanol production using rice straw hydrolysate under continuous and repeated batch processes', *Frontiers in Microbiology,* 10, pp. 1777.

Ulmer, D.C., Leisola, M.S., Schmidt, B.H. and Fiechter, A. (1983) 'Rapid degradation of isolated lignins by Phanerochaete chrysosporium', *Applied and Environmental Microbiology*, 45(6), pp. 1795-1801.

Upadhyaya, J.S. and Singh, B. (1991) 'Decolourisation of effluent from pulp and paper industry', *Indian Journal of Environmental Health,* 33(3), pp. 350-356.

Vargas-Straube, M.J., Beard, S., Norambuena, R., Paradela, A., Vera, M. and 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', *Journal of Proteomics*, 225, pp. 103874.

Vashi, H., Iorhemen, O.T. and Tay, J.H. (2018) 'Degradation of industrial tannin and lignin from pulp mill effluent by aerobic granular sludge technology', *Journal of Water Process Engineering,* 26, pp. 38-45.

Venkatadri, R. and Irvine, R.L. (1993) 'Cultivation of Phanerochaete chrysosporium and production of lignin peroxidase in novel biofilm reactor systems: hollow fiber reactor and silicone membrane reactor', *Water Research,* 27(4), pp. 591-596.

Verma, P. and Madamwar, D. (2002) 'Production of ligninolytic enzymes for dye decolorization by cocultivation of white-rot fungi Pleurotus ostreatus and Phanerochaete chrysosporium under solid-state fermentation', *Applied Biochemistry and Biotechnology,* 102, pp. 109-118.

Villegas, L.G.C., Mashhadi, N., Chen, M., Mukherjee, D., Taylor, K.E. and Biswas, N. (2016) 'A short review of techniques for phenol removal from wastewater', *Current Pollution Reports,* 2(3), pp. 157-167.

Vishtal, A.G. and Kraslawski, A. (2011) 'Challenges in industrial applications of technical lignins', *BioResources,* 6(3), pp. 3547-3568.

Viswanath, B., Rajesh, B., Janardhan, A., Kumar, A.P. and Narasimha, G. (2014) 'Fungal Laccases and Their Applications in Bioremediation', *Enzyme Research,* 2014(2014), pp. 163242-21. doi: 10.1155/2014/163242 .

Vogel, H.J. (1964) 'Distribution of Lysine Pathways Among Fungi: Evolutionary Implications', *The American Naturalist,* 98(903), pp. 435-446. doi: 10.1086/282338 https://www.jstor.org/stable/2459146.

Vrsanska, M., Buresova, A., Damborsky, P. and Adam, V. (2015) 'Influence of different inducers on ligninolytic enzyme activities', *Journal of Metallomics and Nanotechnologies,* 3, pp. 64-70.

Wang, K., Hu, J., Guo, C. and Liu, C. (2016) 'Scale-up laccase production from Trametes versicolor stimulated by vanillic acid', *Bioprocess and Biosystems Engineering,* 39(7), pp. 1041-1049.

Wang, L.K. (2006) *Waste treatment in the process industries* Boca Raton [u.a.]: CRC/Taylor & Francis.

Wang, L.K., Shammas, N.K. and Hung, Y. (2007) *Biosolids treatment processes* Springer.

Wang, T., Jiang, M., Yu, X., Niu, N. and Chen, L. (2022) 'Application of lignin adsorbent in wastewater Treatment: A review', *Separation and Purification Technology,* , pp. 122116.

Wang, Y., Chen, H. and Yu, O. (2010) 'Metabolic engineering of resveratrol and other longevity boosting compounds', *BioFactors,* 36(5), pp. 394-400.

Watanabe, I., Miyata, N., Ando, A., Shiroma, R., Tokuyasu, K. and Nakamura, T. (2012) 'Ethanol production by repeated-batch simultaneous saccharification and fermentation (SSF) of alkali-treated rice straw using immobilized Saccharomyces cerevisiae cells', *Bioresource Technology,* 123, pp. 695-698.

Watanabe, T., Teranishi, H., Honda, Y. and Kuwahara, M. (2002) 'A selective lignin-degrading fungus, Ceriporiopsis subvermispora, produces alkylitaconates that inhibit the production of a cellulolytic active oxygen species, hydroxyl radical in the presence of iron and H2O2', *Biochemical and Biophysical Research Communications,* 297(4), pp. 918-923.

Waye, A., Annal, M., Tang, A., Picard, G., Harnois, F., Guerrero-Analco, J.A., Saleem, A., Hewitt, L.M., Milestone, C.B. and MacLatchy, D.L. (2014) 'Canadian boreal pulp and paper feedstocks contain neuroactive substances that interact in vitro with GABA and dopaminergic systems in the brain', *Science of the Total Environment*, 468, pp. 315-325.

Worrall, J.J., Anagnost, S.E. and Zabel, R.A. (1997) 'Comparison of wood decay among diverse lignicolous fungi', *Mycologia*, 89(2), pp. 199-219.

Wu, J., Xiao, Y. and Yu, H. (2005) 'Degradation of lignin in pulp mill wastewaters by white-rot fungi on biofilm', *Bioresource Technology*, 96(12), pp. 1357-1363.

Xie, X. and Dai, C. (2015) 'Degradation of a model pollutant ferulic acid by the endophytic fungus Phomopsis liquidambari', *Bioresource Technology*, 179, pp. 35-42.

Xie, X., Huang, C., Fu, W. and Dai, C. (2016) 'Potential of endophytic fungus Phomopsis liquidambari for transformation and degradation of recalcitrant pollutant sinapic acid', *Fungal Biology*, 120(3), pp. 402-413.

Yadav, S., Tripathi, S., Purchase, D. and Chandra, R. (2023) 'Development of a biofilm-forming bacterial consortium and quorum sensing molecules for the degradation of lignin-containing organic pollutants', *Environmental Research*, 226, pp. 115618.

Yamada, S., Nabe, K., Izuo, N., Nakamichi, K. and Chibata, I. (1981) 'Production of L-phenylalanine from trans-cinnamic acid with Rhodotorula glutinis containing L-phenylalanine ammonia-lyase activity', *Applied and Environmental Microbiology*, 42(5), pp. 773-778.

Yamauchi, K., Yasuda, S. and Fukushima, K. (2002) 'Evidence for the biosynthetic pathway from sinapic acid to syringyl lignin using labeled sinapic acid with stable isotope at both methoxy groups in Robinia pseudoacacia and Nerium indicum', *Journal of Agricultural and Food Chemistry,* 50(11), pp. 3222-3227.

Yang, Q., Angly, F.E., Wang, Z. and Zhang, H. (2011a) 'Wastewater treatment systems harbor specific and diverse yeast communities', *Biochemical Engineering Journal,* 58, pp. 168-176.

Yang, Y.S., Zhou, J.T., Lu, H., Yuan, Y.L. and Zhao, L.H. (2011b) 'Isolation and characterization of a fungus Aspergillus sp. strain F-3 capable of degrading alkali lignin', *Biodegradation,* 22(5), pp. 1017-1027.

Yesilada, O., Yildirim, S.C., Birhanli, E., Apohan, E., Asma, D. and Kuru, F. (2010) 'The evaluation of pre-grown mycelial pellets in decolorization of textile dyes during repeated batch process', *World Journal of Microbiology and Biotechnology,* 26, pp. 33-39.

Yoruk, R. and Marshall, M.R. (2003) 'Physicochemical properties and function of plant polyphenol oxidase polyphenol oxidase: A review', *Journal of Food Biochemistry,* 27, pp. 361-422. doi: 10.1111/j.1745-4514.2003.tb00289.x .

Yuzugullu, Y. (2015) *Functional And Structural Analysis Of Catalase-phenol Oxidase From Scytalidium Thermophilum.* . METU. Available at: http://etd.lib.metu.edu.tr/upload/3/12611463/index.pdf (Accessed: .

Zahmatkesh, M., Spanjers, H. and van Lier, J.B. (2018) 'A novel approach for application of white rot fungi in wastewater treatment under non-sterile conditions: immobilization of fungi on sorghum', *Environmental Technology,* 39(16), pp. 2030-2040.

Zakzeski, J., Bruijnincx, P.C., Jongerius, A.L. and Weckhuysen, B.M. (2010) 'The catalytic valorization of lignin for the production of renewable chemicals', *Chemical Reviews,* 110(6), pp. 3552-3599.

Zhang, Q. and Chuang, K.T. (1998) 'Kinetics of wet oxidation of black liquor over a Pt–Pd–Ce/alumina catalyst', *Applied Catalysis B: Environmental,* 17(4), pp. 321-332.

Zhang, S., Jiang, M., Zhou, Z., Zhao, M. and Li, Y. (2012) 'Selective removal of lignin in steam-exploded rice straw by Phanerochaete chrysosporium', *International Biodeterioration & Biodegradation,* 75, pp. 89-95.

Zhang, Z., Ni, B., Zhang, L., Liu, Z., Fu, W., Dai, X. and Sun, J. (2022) 'Medium-chain fatty acids production from carbohydrates-rich wastewater through two-stage yeast biofilm processes without external electron donor addition: Biofilm development and pH impact', *Science of the Total Environment,* 828, pp. 154428.

Zheng, Y., Zhang, W., Tang, B., Ding, J. and Zhang, Z. (2018) 'Membrane fouling mechanism of biofilm-membrane bioreactor (BF-MBR): Pore blocking model and membrane cleaning', *Bioresource Technology,* 250, pp. 398-405.

Zhu, D., Zhang, P., Xie, C., Zhang, W., Sun, J., Qian, W. and Yang, B. (2017) 'Biodegradation of alkaline lignin by Bacillus ligniniphilus L1', *Biotechnology for Biofuels,* 10(1), pp. 1-14.

Znameroski, E.A., Coradetti, S.T., Roche, C.M., Tsai, J.C., Iavarone, A.T., Cate, J.H. and Glass, N.L. (2012) 'Induction of lignocellulose-degrading enzymes in Neurospora crassa by cellodextrins', *Proceedings of the National Academy of Sciences,* 109(16), pp. 6012-6017.

Znameroski, E.A., Li, X., Tsai, J.C., Galazka, J.M., Glass, N.L. and Cate, J.H. (2014) 'Evidence for transceptor function of cellodextrin transporters in Neurospora crassa', *Journal of Biological Chemistry,* 289(5), pp. 2610-2619.

Żuchowski, J., Pecio, Ł, Jaszek, M. and Stochmal, A. (2013) 'Solid-state fermentation of rapeseed meal with the white-rot fungi Trametes versicolor and Pleurotus ostreatus', *Applied Biochemistry and Biotechnology,* 171, pp. 2075-2081.

Zwain, H.M., Hassan, S.R., Zaman, N.Q., Aziz, H.A. and Dahlan, I. (2013) 'The start-up performance of modified anaerobic baffled reactor (MABR) for the treatment of recycled paper mill wastewater', *Journal of Environmental Chemical Engineering,* 1(1-2), pp. 61-64.