DEGRADATION OF DYES AND BISPHENOL A PRESENT IN WASTEWATER USING LACCASE-IMMOBILIZED NYLON 66 MEMBRANE

A DISSERTATION

Submitted in partial fulfilment of the requirements for the award of the degree

of

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in

BIOPROCESS ENGINEERING

By

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"DEGRADATION OF DYES AND BISPHENOL A PRESENT IN WASTEWATER USING LACCASE-IMMOBILIZED NYLON 66 MEMBRANE"** in partial fulfilment of the requirements for the award of the Degree of Master of Technology and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out under the supervision of Dr. Anett Werner, Head of Research Group- Enzyme Technology, Technische Universität Dresden, Germany and Dr. Saurav Datta, Assistant Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, India. The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institution.

(LAVIE REKHI)

CERTIFICATE

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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I, after checking the dissertation mentioned above, hereby states my approval of the dissertation submitted in partial fulfilment of the requirements for the degree of Master of Technology in Bioprocess Engineering at Indian Institute of Technology Roorkee, Roorkee, India. I am satisfied with the volume, quality, correctness, and originality of the work.

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Thank you,

Lavie

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ABSTRACT

The industries pollute the environment by releasing dyes and other toxic chemicals as effluents. The reactive dyes and Bisphenol A are of major concern and should be degraded. As the demand for new approaches of water disinfection is increasing, it mandates developments in membrane technology. The development of functionalized membranes with porous support and functional polymer matrices helps in changing the structure of the membrane pore, its selectivity, and reactivity. Due to their microporous structure, which provides high surface area, and low mass-transfer resistance (pressure-driven convective flow), membranes have emerged as a favourable support material for enzyme immobilization. The physical and chemical means used presently for degrading dyes present in water are associated with environmental concerns. As an alternative, approach of bio-inspired remediation, where activity of laccase (obtained from *Funalia trogii*) combined with the versatile behaviour of nylon 66 polymer to prepare the membrane for degradation of Levafix blue E-RA gran, Remazol Turquoise Blue G 133, and Remazol Brilliant Blue R dyes and Bisphenol A was used. The efficacy of these laccase-immobilized membranes toward degradation of the dyes and Bisphenol A was demonstrated under a continuous flow mode. Nearly 94%, 76%, 68% and 49% of breakdown were achieved for Levafix Blue E-RA gran, Remazol Turquoise blue G 133, Remazol Brilliant Blue R dyes and Bisphenol A, respectively. The degraded products were characterized by spectroscopy and fluorometer. The immobilized laccase was active in a varied range of pH and temperature and was found to retain its activity for 3 weeks. The biodegradation by the usage of laccase is an attractive alternative for larger scale applications when compared to the usual and sometimes costly physical and/or chemical means for remediation of industrial textile effluent.

Keywords: Laccase, membrane, dyes, Bisphenol A

INTRODUCTION

Pollution has gripped the globe like never before. It is known that textile industries, which are voracious consumer of water, releases reactive dyes that enter activated sludge sewage treatment plants. Nearly 10-15% of the dyes used in the dyeing process are released into the wastewater, which is the main cause of environmental pollution (Samchetshabam et al, 2017). In addition to destroying of the aesthetic value of the environment, the released chemicals increase the biological and chemical oxygen demand (BOD and COD), and alkalinity level of water, which indirectly harms the aquatic organisms. Some of these compounds are also carcinogenic and allergens to humans. Thus, pollution is a grave issue.

Bioremediation can be accomplished using enzymes, which are of utmost importance in various industries as they are environmentally benign, renewable, and nontoxic. Catalysis by free enzymes is associated with higher initial activity, but they have some major drawbacks, like, difficulty involved in its downstream processing, lack of reusability, and low operational stability (Raghuvanshi and Gupta, 2010). This has led to the development of technologies for enzyme immobilization, which causes an improvement in the cumbersome recovery and reuse of enzymes, without major compromise in activity. The long-term operational stability of the enzyme is also increased. One of the major issues that arise in enzyme immobilization is reduction in the enzyme activity, because of the blocked active sites due to close proximity to the support material (Vishwanath et al., 1995). An approach to evade this problem is to immobilize the enzyme on a functionalized support matrix that actually keeps the enzyme away from the base support material and also allows post immobilization arrangement of enzyme. Another major issue that arises with immobilized enzyme is the slow kinetics of reaction, because of an unfavourable hydrodynamics under diffusive mode of flow through a porous media (Datta et al., 2008). Thus, a support material which offers improved hydrodynamics seems to be of great interest to the scientific community.

In recent years, various microbes have been investigated for decolorization of reactive dyes (Bhatt et al, 2005; Chen, 2002). There are various combined aerobic and anaerobic microbial treatments that have been suggested to enhance degradation of textile dyes (O'Neill et al., 2000). White rot fungi are known for decolourization of a wide variety of synthetic dyes (Wesenberg et al., 2003; Murugesan and Kalaichelvan, 2003; Nilsson et al., 2006). Under anaerobic conditions, azo dyes are usually cleaved by azo-reductases into the corresponding amines, many of which are carcinogenic. Furthermore, these enzymes are very specific, thereby cleaving only azo bonds of selected dyes. By contrast, laccases which are copper-containing oxidoreductases

act oxidatively and less specifically on aromatic rings. Thus, they have potential to degrade a wider range of compounds.

There has been a rise in interest in application of laccase for wastewater treatment, dye decolorization, biobleaching, cathode fuel cells and biosensor due to its low substrate specificity and good intrinsic stability properties (Couta et al., 2004). Laccase has been used in a system where it has been immobilized on the PVDF membranes (functionalized using polyacrylic acid) for the degradation of 2,4,6-trichlorophenol present in water (Sarma et al., 2017). The action mechanism of a laccase-mediator system for degradation of dyes has been extensively studied. It has been observed that the capability of a laccase to decolorize the dye depends on the enzyme-producing species and strain and also on the structure of the dye.

Bisphenol A (BPA) is an intermediate in the production of the process of polycarbonate and epoxy resins. It is an endocrine-disrupting chemical that has detrimental effects on the human health. It can cause breast and prostate cancer, reduction in sperm-count, early sexual maturation in females, as well as immunodeficiency (Chhaya and Gupte, 2013). Environmental pollution caused by BPA can result in fearful environmental concerns. It has been found out that laccases obtained from several fungus, have the potential of degrading BPA (Chairin et al., 2013).

In the recent years, the use of biotechnological processes in the treatment of textile dye effluents has attracted considerable attention and achieved interesting results. The released effluents (Figure 1) are toxic, but the conventional treatments are unable to accomplish new environmental regulations. This issue has become important for the researchers as the legislation about toxic substances in the industrial wastewaters is becoming strict. Thus, there is a need for the development of new technologies for achieving low limits of effluent discharge toxicity. As an alternative to the conventional effluent treatments, enzymes of the class oxidase, especially laccases are now used (Kokola et al., 2007; Cristóvão, 2010; Tilli et al., 2011). The use of laccase has been already described in several studies for the processes of delignification, but less research has been done on its use for degradation of reactive textile dyes (Riva, 2006). In this study, the degradation potential of laccase against reactive textile dyes, which are difficult to decolourize, and BPA often found in the real wastewaters, was assessed.

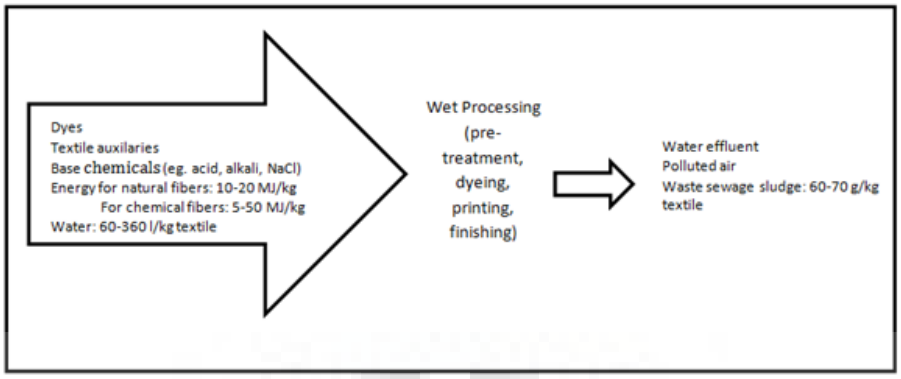


Figure 1: Environmental analysis of textile finishing processes (Toprak and Anis, 2017)

Due to the increase in water pollution, there is a need to develop new approaches of for water disinfection. Hence, membrane technology can be used to treat the toxic components present in water. The structure of the membrane pore, its selectivity, and reactivity can be modified according to the suitability by functionalizing the membranes with functional polymer matrices. Membranes have emerged as a favorable support material for enzyme immobilization because of their microporous structure that provides high surface area, and low mass-transfer resistance. High enzyme loading and a protective environment is provided to the immobilized enzymes by functionalized membranes. There are physical and chemical means for remediating dyes in water which are associated with environmental concerns.

In this study, promising activity of laccase (crude extract obtained from *Funalia trogii*) was combined with the versatile behaviour of nylon 66 polymer to construct the membrane reactor for degradation of textile dyes (Levafix blue E-RA gran, Remazol Turquoise blue G 133, and Remazol Brilliant Blue R) and BPA. The efficacy of these bio-inspired membranes toward degradation of these compounds was demonstrated under a continuous flow mode. The activity of laccase immobilized on the membrane was compared with that of free enzyme in solution at varied conditions of pH and temperature.

REVIEW OF LITERATURE

Decolourization of an acid azo dye in aqueous phase using laccase-membrane reactors

Experimental data has shown that laccase has a great potential for removal of colours without addition of external redox mediators (Katuri et al., 2009). Different types of laccase-membrane reactor configurations such as direct- enzyme contact, enzyme impregnated, and a reactor system based on laccase immobilization in chitosan membranes have actually been used for determining the efficacy of repeated application of laccase on decolourization of dye. Among the different configurations employed, laccase encapsulated in the chitosan membrane have shown advantages like reusability of enzyme for a number of cycles and short-term contact period. In one of the configurations, laccase was covalently bound to PVDF membranes. It was seen that with number of repeated applications of the PVDF-immobilized laccase, contact time increased for getting the maximum efficiency which might be due to the release of covalently bound laccase from the membrane with time or decrease of enzymatic activity with time. But when the laccase was immobilized on the chitosan membrane, advantage was observed in capacity of holding laccase. Decolourization of azo dye (acid black 10 BX) was achieved for multiple runs with the used enzyme.

Enzymatic degradation of BPA with laccase immobilized on TiO_2 sol–gel coated PVDF membrane

To augment the conventional wastewater treatment processes for removal of micro-pollutants like BPA, biodegradation with laccase immobilized on TiO_2 functionalized membrane offers an attractive option (Hou et al., 2014). The coating of TiO_2 nanoparticles on the PVDF membrane surface were done through a low temperature hydrothermal sol–gel process. The enzyme was subsequently immobilized on these membranes by chemical coupling. Enzyme immobilization on nanostructured TiO_2 - coated membranes have been found to address the common issues like the poor enzyme activity and stability that is associated with the free laccase. The loss of laccase has also been seen to be reduced. This bio-catalytic membrane system was also seen to degrade large molecular weight compound, BPA.

Use of polyethyleneimine for enzyme immobilization

One of the most common ways for immobilizing enzymes onto a microreactor is by the usage

of a support that can covalently attach to the enzyme or by using a cross-linking agent that can act as a spacer-arm molecule. Immobilization of enzymes provides many advantages such as their reuse, lower consumption of reagents and samples, higher analysis rate, extended lifetime and greater stability. However, for this purpose, the substrate of the reactor must have some reactive functional groups that can interact with the enzymes. A suitable microenvironment is created for enzyme immobilization that improves its stability, by the usage of polyethyleneimine (PEI) in relation to other amino groups containing reactants (Guisan et al., 2001). PEI prevents direct interaction of the immobilized enzyme with the support. It has also been seen that PMMA functionalization with PEI followed by glucose oxidase immobilization on the microchannel walls, using GTA as the cross- linking agent has enhanced the immobilization process (Cerqueiraa et al., 2014).

Laccase immobilization on polyamide 6,6 fibres

Polyamide matrices, like membranes, gels and non-wovens, have been used as supports for immobilization of enzyme. In one of the studies, a protocol for immobilizing laccasse derived from Trametes hirsuta using woven polyamide 6,6 (nylon) was developed (Silva et al., 2007). The factors like the spacer and enzyme dosage seem to have played a critical role in the laccase immobilization onto nylon support. It is seen that the immobilized laccase has potential for application in the continuous decolourization of various textile effluents, where it can be applied into a membrane reactor.

OBJECTIVES

i. To fabricate the functionalized membranes containing GTA, PEI and laccase
ii. To compare the activity and stability of the immobilized laccase enzyme to those of the free enzyme present in solution
iii. Kinetic Analysis
iv. To evaluate the performance of the laccase-immobilized membranes toward the degradation of dyes and BPA present in wastewater

MATERIALS

Nylon 66 membrane discs (membrane diameter of 47 mm, pore diameter of 0.2 μm, and average thickness of 160 μm) used for all of the experiments were purchased from Supelco (product no. 58060-U). Crude extract of laccase was obtained from *Funalia trogii* (Lot number 2020-058, gift from ASA Spezialenzyme GmbH), Nitrocellulose membrane (membrane diameter of 47 mm and pore diameter of 0.2), Cellulose acetate membrane (membrane diameter of 47 mm and pore diameter of 0.2), Polyethersulphone membrane (membrane diameter of 47 mm and pore diameter of 0.2 μm) (all membranes purchased from Supelco), Glutaraldehyde (GTA), Isopropanol (IPA), Malonic acid, potassium phosphate monobasic, disodium hydrogen phosphate dehydrate, citric acid, sodium hydroxide pellets, Levafix blue E-RA gran (purchased from DyStar, Germany), Remazol Turquoise blue G 133(purchased from DyStar, Germany), and Remazol Brilliant Blue R (RBBR) (purchased from Sigma, product number R8001-25G) dyes, Polyehyleneimine (PEI, product no. 408727, MW ≈ 25 000, purchased from Sigma Aldrich), BPA, beta-cytodextrine, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (purchased from Alfa Aesar, Lot number T24E012). The experiments were conducted with ultrapure water.

METHODOLOGY

Experimental Methods

The experiments were performed in duplicate. The variations which were observed in the experimental results were represented as standard deviation. Ultraviolet-Visible (UV–Vis) spectrophotometer (Beckman DU 640) was used for spectrophotometric and fluorometer (Perkin Elmer) was used for fluorometric measurements of various samples as described below.

Membrane Experiments

The experiments were conducted using two membrane holders (47 mm disc diameter, cross flow type and 10mm disc diameter, dead end type) under convective mode of flow. Inlet of the membrane holder was connected to the feed tank and outlet to the sample collection tank. Desired flow rate was maintained using a peristaltic pump. For the analysis of immobilized

laccase activity, continuous flow was maintained. The porosity (ε) of the membrane was calculated with the help of the given equation:

ε = p/v where, p = porous volume and v = total volume

The porous volume was calculated by measuring the initial weight of a membrane and the final weight after the same membrane was soaked overnight in ultrapure water. Before weighing, the membrane was wiped properly to remove residual surface water. The porous volume of the membrane was then calculated as demonstrated below.

p (m^3) = v × ε = (Weight after soaking − weight before soaking) / Density of water

Initial weight of membrane = 0.1136g,

Final weight of membrane = 0.3262 g

Therefore, p = (0.3262-0.1136) g / 1 g cm−3 =0.2126cm^3 = 21.26× 10−8m^3

Total volume = π × (radius)^2 × thickness = π × (2.35 × 10−^{2})^2× 160 × 10^{-6} = 2774.504× $10^{-10}$$m^3$

Total volume of membrane = 27.75 × 10^{-8} m^3

Thus, porosity (ε) = 21.26 ×10^{-8} m^3 / 27.75 × 10^{-8} m^3 = 0.77

Immobilization of laccase within membranes

10ml of 0.5g/l of laccase solution prepared in water was mixed with 10ml of 0.5g/l PEI solution (acting as a spacer molecule (Figure 2)) prepared in phosphate buffer (pH 7) and incubated in a shaker at 25^{O}C at 150rpm for 15mins. Then 4.25ml of the laccase- PEI solution was mixed with 1.875ml of 100% IPA and 0.125ml of 50% GTA (which works as a cross-linking agent as shown in Figure 3). As soon as the GTA was added to the mixture, different types of membranes (nitrocellulose, nylon 66 and polyethersulphone) were dipped into the solution separately and kept in the shaker at 4^{O}C for 30mins. After the immobilization step, the membranes were stored in water at 4^{O}C for further use.

A recent technique of enzyme immobilization using electron beam has been used for water treatment (Boulares-Pender et al., 2013; Jahangiri, 2018). In this study, the enzyme immobilization by the help of electron beam was carried out by impregnating the nylon 66 membrane in 10ml of 1g/ml of laccase solution and shaking the mixture at 450 rpm for 10 mins at room temperature. The membranes (after drying) were irradiated with 100 kGy electron beam. The enzyme-immobilized membranes were then washed twice with water and 0.1 M

PBS solution (pH 7.4) for 20 mins at 600 rpm. The membranes were stored in PBS solution at 4^{O}C for further use. The enzymatic activity of the laccase immobilized by different methods was then compared.

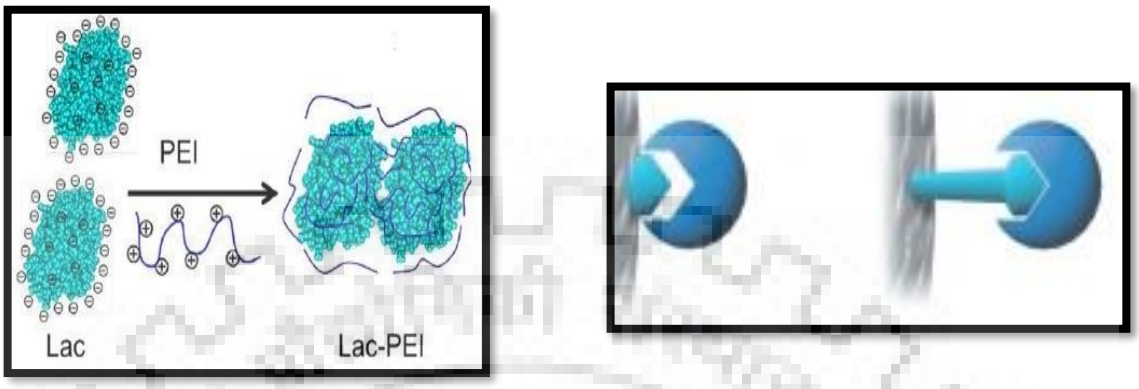


Figure 2: Use of PEI in immobilization

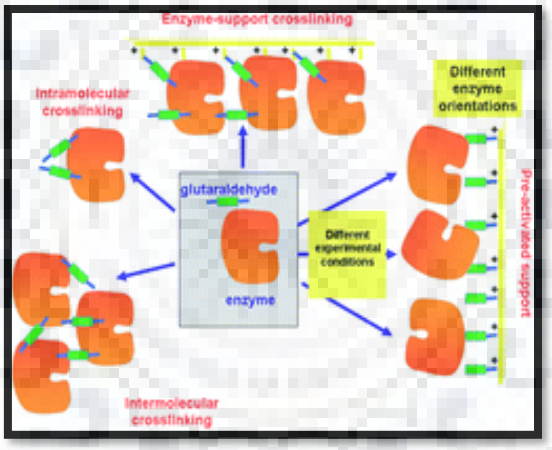


Figure 3: Use of Glutaraldehyde in immobilization

Laccase activity

The activity of laccase was measured using 1 ml of 1 mM ABTS in 0.1 M pH 5 malonate buffer and 20µL aliquots of enzyme. The change in absorbance at 420 nm was noted over time by the spectrophotometer at 25°C. The evaluation of enzymatic activity was done using the molar extinction coefficient of 36.000 M^{-1} cm^{-1} at 420 nm (Akkayaa et al., 2016).

Characterization of the laccase-immobilized membrane

Determination of enzymatic activity by Tecan Plate Reader

A Tecan plate reader was used to determine the enzymatic activity of all the different types of laccase-immobilized membranes. The membranes were inserted into the 96-well microtiter plate and were thoroughly washed twice with 600µl of distilled water. 600µl of 2mM ABTS was then added into the wells and the plate was shaken at 1050rpm for 2mins. The colour of the solution was seen to change from light green to very dark green. After the reaction, 200µl of the reacted solution was analyzed by the help of Tecan plate reader by taking the absorbance at 405nm. Washing was done again before the analysis of the membrane for the next cycle.

Microfluidics for analysis of immobilization

The immobilization of the enzyme on the membrane can be characterized using microfluidics chip (Figure 4). For the continuous measurement, malonate buffer was passed via a peristaltic pump through the built-in membrane microfluidics at a flow rate of 0.5ml/min and forwarded to the flow cell in a photometer. The fixed ABTS concentration of 1mM, 2.5mM and 5mM and volume 200µl was injected into the system; thereby a measurable colour change with time could be traced. The flow of the solution was parallel to the surface of the membrane. The activity of the immobilized enzyme on the membrane was measured. A previous flushing with buffer was used to remove the free substances. The oxidation of the ABTS produced a greenish free radical form of the substrate that could be measured at 420 nm. The chip was also connected with the oximeter by the oxygen electrode and the change in the concentration of oxygen was monitored. The enzyme was immobilized on the membrane over the electrode. In presence of ABTS, the diffusion of oxygen through the gas permeable membrane to the electrode was reduced.

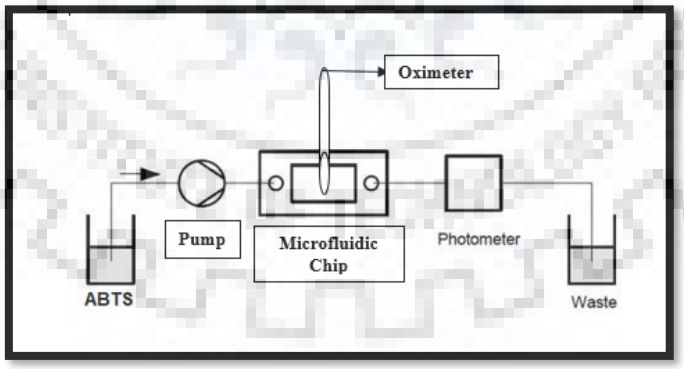


Figure 4: Microfluidics for characterization of the immobilized membrane

Quantification of Immobilization:

The amount of enzyme immobilized on the membrane was quantified using copper test. This

test was used to measure Cu^{2+} ions, which was used for laccase estimation. For the test, membrane with immobilized laccase and known surface area was placed in 5ml water. 1 level of dosing spoon of Cu^{-1} reagent was added into it. After proper mixing, 5 drops of Cu^{-2} reagent was added and mixed. After 5mins of reaction, the sample was analyzed using spectrophotometer at 600nm.

Determination of kinetic parameters of free enzyme in solution

The kinetic parameters of the free enzyme in solution were determined using various substrate concentrations of ABTS. 20µl of the laccase-PEI solution (similar to immobilized method) solution was mixed with 1ml of different substrate concentrations of ABTS in malonate buffer (pH 5). The linear slope gave the initial velocity of the reaction, which could be plotted against their respective substrate concentrations. Thus, the determination of the kinetic parameters (Km and Vmax) could be done from the slope and the intercept of Lineweaver Burk plot.

Determination of Vmax and Michaelis Menten constant (Km (app)) of immobilized enzyme using Lilly-Hornby kinetic model

In this study, the kinetic parameters were analyzed using Lilly Hornby kinetic model. Laccase-immobilized membrane was subjected to convective flow of the substrate solution using a peristaltic pump. The operation was under "once through" mode. ABTS used as a standard substrate that entered the pores of the membrane, got oxidized, and the product stream exited the device. It is used for the continuous flow system, where the membrane works as a packed bed reactor. The Lilly Hornby equation is given by:

$$Ps_o\text{-}Km'\ln(1\text{-}P) = k_3E\beta/Q = C/Q$$

where so is the initial concentration of substrate, P is the fraction of the substrate that is reacted in the column, k_3 is the rate constant for the decomposition of the enzyme-substrate complex, Km is the Michaelis constant, [E] is the concentration of total enzyme respectively, $C=k_3E\beta$ and is the reaction capacity of the column, β is the voidage of the column, and Q is the flow rate. If at a constant flow rate, Q, various initial concentrations of substrate are perfused through the same reactor, then the values of P can be measured. Ps_0 can then be plotted against ln(1- P), which gives a straight line and Km and C are constants at this flow rate. Km can be found out from the slope and the intercept give the value of C/Q. In this study, different concentrations of ABTS (0.05mM, 0.1mM and 0.25mM) were passed through the immobilized nylon 66 membrane at a set of flow rates from 0.2-1.0ml/min by the help of a peristaltic pump.

Dye degradation with immobilized laccase from *Funalia trogii*

The dyes selected for the study were: Levafix Blue E-RA gran, Remazol Turquoise Blue G 133 and RBBR. Specific concentrations of the dyes were prepared in the malonate buffer (pH 5). For preparation of the subsequent tests, their spectra were recorded by the dyes. A feed of 200ml of 0.125g/l of Levafix Blue E-RA, 100ml of 0.1g/l of Remazol Turquoise Blue G, and 25ml of 0.25g/l of Remazol Brilliant Blue R, was prepared in the malonate buffer (pH 5) and was recirculated through the membrane in a cross flow system at a flow rate of 6.7ml/min. The degradation of the dye was detected by taking the readings through the spectrophotometer at an interval of 30 minutes at their respective wavelengths of 610nm, 620nm, and 595nm. As a negative control, heat inactivated enzyme (incubated at 85°C for one hour) was immobilized on the membrane and tested against these dyes. The recirculation was continued till the readings had become constant (when the dye had degraded fully). The decolorization of the dyes could then be calculated by the following equation:

Percent Breakdown= $((C_i-C_f)/C_i) *100$

where C_i= Initial Absorbance of dye

C_f= Final absorbance of dye

Absorption spectroscopy

Results of dye spectra and quantitative measurements were obtained using an UV spectrophotometer (Beckman DU640) for characterization of decolourization processes. All dyes were prepared in malonate buffer (pH 5). The buffer was used as the blank.

Degradation of BPA using laccase- immobilized membrane

BPA was also tested for its degradation using immobilized laccase on the membrane. For the test, 50ml of 40µM of BPA solution was passed through the laccase-immobilized membrane using the cross-flow module for 24 hours. BPA was found to be adsorbed on the membrane. Thus, many runs of the experiment were done to confirm its degradation by comparing the activity of active enzyme with that of heat inactivated enzyme against BPA. The change in the fluorescence was noted every 30 mins taking 100 µl of permeate solution, 100 µl of 3 mM beta cytodextrine (increases the self-fluorescence of BPA, which helps in detecting lower concentrations of BPA) and 800 µl of 2* Mcllvain buffer (pH 5) using fluorescence spectrometer at 25°C with excitation wavelength of 225 nm and emission wavelength of 308 nm.

Storage stability of the immobilized enzyme

The enzyme was immobilized on the membrane and was stored at 4°C for further use. The enzymatic activity was tested by recirculating Levafix Blue E-RA gran dye in a cross-flow module.

Effect of pH and temperature

The enzymatic activity was tested in a varied range of pH (3-7) and temperature (20-80°C) using 1mM syringaldazine in McIlvaine buffer (pH 5) as a substrate. For the pH test, the substrate solution was dissolved in a pH range from 3-7 and for the temperature test, both the membranes and substrate solution were kept separately at 20-80°C for 1hour. The experiment was conducted using a Tecan plate reader where 600µl of substrate was added into the membrane-containing wells of the 96 well plate and was shaken at 1050rpm for 2mins. The colour of the membrane was seen to change from colourless to pink. After the reaction, the membrane was washed with 200µl ethanol and then the pink solution was analysed by taking the absorbance at 545nm. The results obtained were then compared to the free enzyme in solution.

RESULTS AND DISCUSSION

Immobilization of laccase and determination of its activity

To immobilize laccase on membranes we used a combination of polyethylenimine (PEI) and glutaraldehyde (GTA). GTA plays a critical role in immobilization as it is reliable and easy to use (Walt, 1994). PEI has cationic primary, secondary and tertiary amines and is chemically reactive. It can create a more suitable immobilization microenvironment than other amino groups containing reactants (Guisan et al, 2001). Furthermore, enzyme stability can be improved with PEI (Virgen-Ortíz et al, 2017). It creates a network around the enzyme and also acts as a spacer molecule, thereby not allowing a direct contact between the enzyme and the support (Cui et al., 2013). GTA acts as inter- and intra- molecular cross-linking agent and enhances the immobilization process.

In this work, when immobilizing laccase from *Funalia trogii* on nylon 66 membrane using PEI and GTA, it was presumed that GTA provides reactive groups to support binding of amino groups from the laccase with amino groups from the nylon membrane as well as amino groups between different enzyme molecules. In addition, GTA helped in crosslinking amine groups within the same laccase molecule. PMMA functionalization with PEI followed by glucose oxidase immobilization on the microchannel walls, using GTA as the cross- linking agent has enhanced the immobilization process (Cerqueiraa et al., 2014).

Polyamide matrices, like membranes and gels, have been used as supports for immobilization of enzyme. Therefore, different types of membranes were tested for enzymatic activity after immobilizing laccase using the Tecan plate reader. Comparing the enzymatic activities, we observed that using 0.2 µm nylon 66 membranes resulted in highest enzyme activity as shown in Figure 5. For example, in contrast to PES membranes, nylon membranes showed 50 % increase in enzyme activity ($PES_{laccase}$=0.08 mU/mm^2; $Nyl_{laccase}$=0.19 mU/mm^2).

The enzymatic activity of the laccase immobilized membrane using electron beam was determined by ABTS (in a similar way using Tecan Plate Reader). The enzymatic activity was tested for a set of 8 membranes and 4 wash steps were included in between for the ABTS analysis. The activity of laccase immobilized using electron beam was found to be less (nearly 35 µU/mm^2 (Figure 6)) as compared to the one with GTA and PEI (190 µU/mm^2).

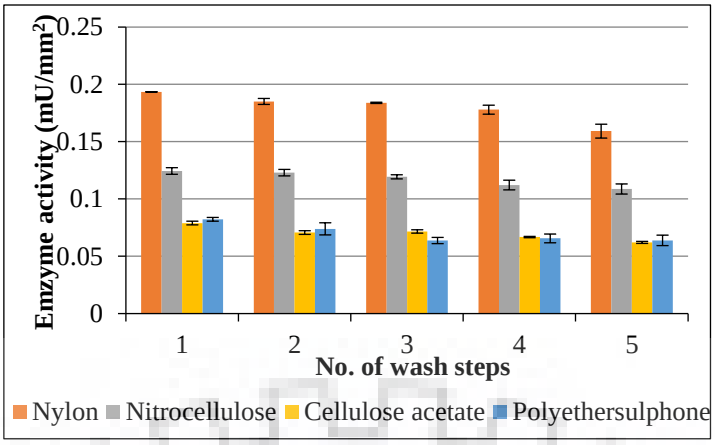


Figure 5: Enzymatic activity of laccase immobilized using GTA and PEI on different membranes (n=8) determined using ABTS. Enzymatic activity was analyzed five times per membrane using washing steps in between ABTS analysis.

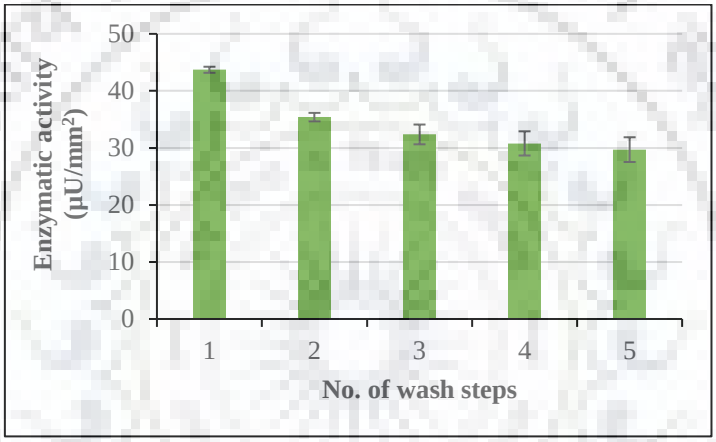


Figure 6: Enzymatic activity of laccase immobilized using electron beam on nylon 66 membranes (n=8) determined using ABTS. Enzymatic activity was analyzed five times per membrane using washing steps in between ABTS analysis.

Additional to enzyme activity, the amount of enzyme immobilized on the membrane was quantified using the Copper test. The enzyme yield was 3 µg/mm² ± 0.00017, determined by the calibration curve of copper sulphate (Figure 7).

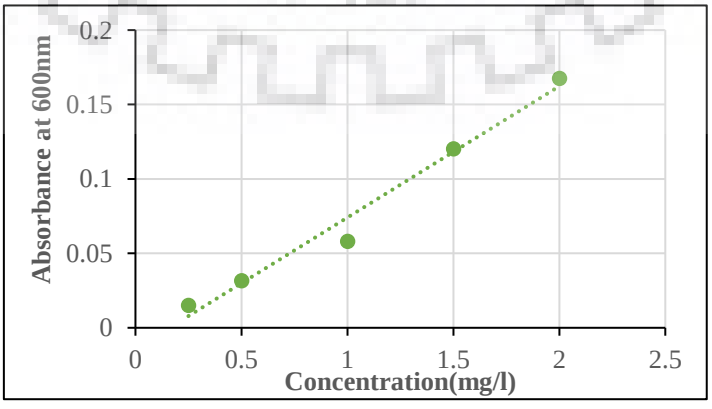


Figure 7: Calibration curve of copper sulphate

Characterization of the membrane by microfluidics

It was seen that the results obtained from the photometer were more sensitive than the oximeter. As the substrate got used up by the enzyme, a decrease in the concentration of oxygen could be seen. In the reaction, oxygen is reduced to water and the ABTS gets oxidized, which gives a green colour. Through the photometer, the enzymatic activity could be obtained from the slope of Figure 8. The oxygen electrode was used as a sensor to measure a decrease in the concentration of oxygen. We see that at a higher substrate concentration, the slope is higher when compared to the lower concentration of the substrate, showing a higher enzymatic activity as shown in Figure 9 (A). The data from the oximeter can be used to plot the rate of the reaction versus substrate concentrations (Figure 9 (B)). The value for Vmax was found to be 0.16 µmol/min/ml of solution.

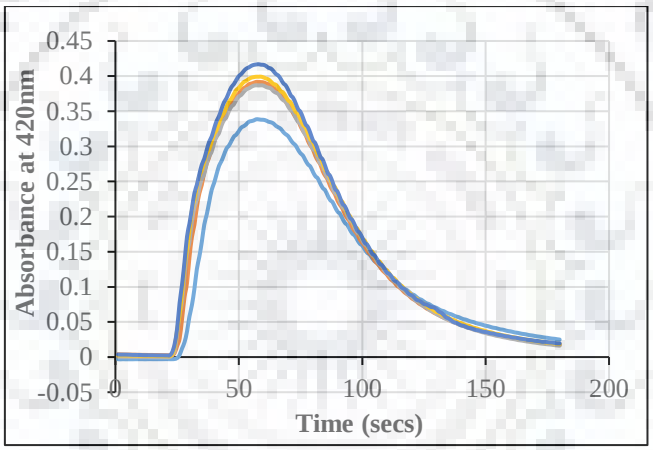


Figure 8: Change in absorbance of 2mM ABTS determined using photometer (5 replicas)

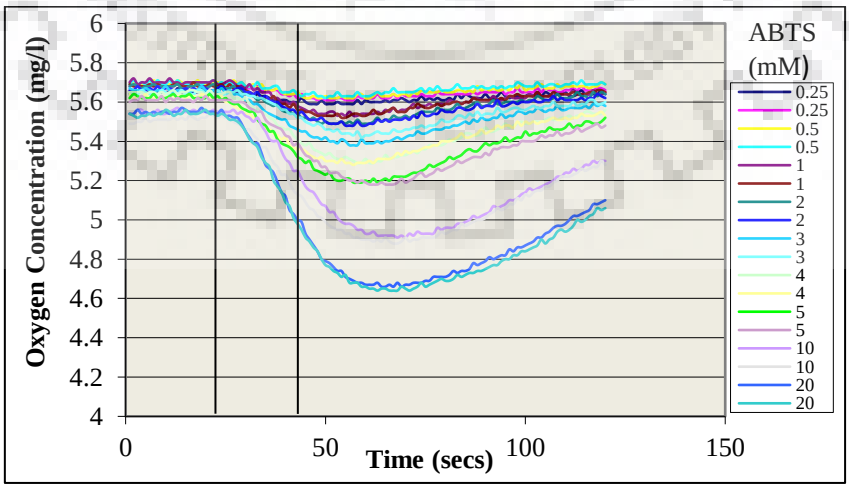


Figure 9 (A): Change in oxygen concentration with time at different substrate concentrations determined using oximeter

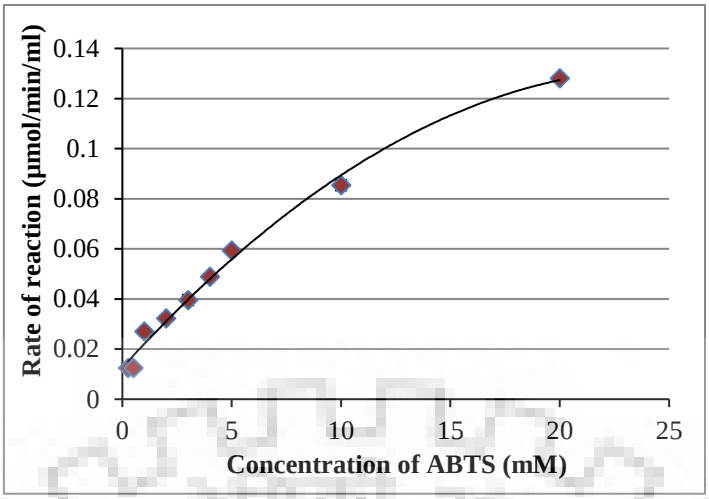


Figure 9(B): Dependence of rate of reaction on substrate concentration

Determination of kinetic parameters of free enzyme in solution

From the Lineweaver Burk plot (Figure 10), the kinetic parameters of free enzyme in solution were obtained, based on the Michaelis Menten equation. The slope of the straight line gives the value of Km/Vmax and intercept gives the value of 1/Vmax. Thus, Vmax was found to be 1.84 µmol/ml/min and Km was 18 µM.

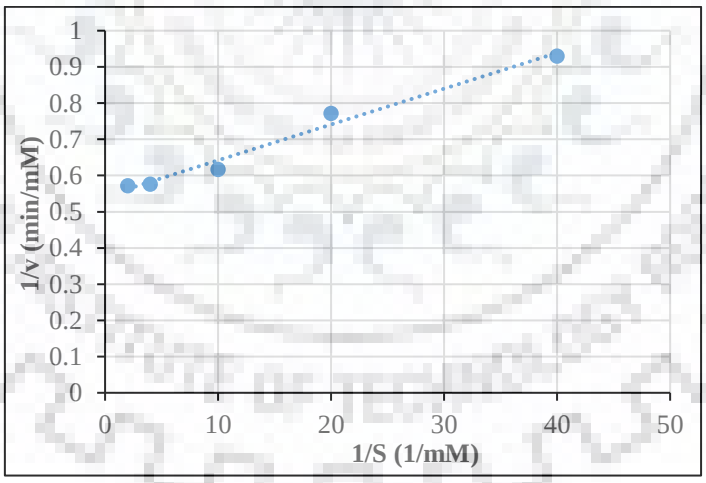


Figure 10: Lineweaver Burk plot

Determination of kinetic parameters of immobilized enzyme using Lilly-Hornby kinetic model

We use the Lilly Hornby kinetic model for determination of the kinetic parameters involving the continuous flow. The model was used to plot the product of initial substrate concentrations

($[S]_0$) with the fraction of substrate utilized (X) versus –ln(1-X). From the equations of the line, C/Q could be obtained from the intercept, whereas the slope gives the value for Km (app) (Figure 11). Vmax of immobilized laccase on the membrane was found to be 2.4 µmol/min/ml of reactor bed volume at the highest flow rate of 1 ml/min. The value for Vmax was also determined in terms of per mg of the crude extract and was found to be 0.13 µmol/min/mg. The Km(app) is dependent on the flow rate at low rates of perfusion, through the columns (Lilly and Hornby, 1966). At higher flow rates, the value of Km(app) decreased, thereby increasing the substrate affinity for the enzyme (Figure 13). At higher flow rate, the diffusion of the substrate towards the enzyme was enhanced, thereby decreasing the value of Km(app). There is an increase in mass transfer at higher flow rates due to convective transport and diffusion. As the flow rate was increased, the Km(app) approached that of the free enzyme in solution (Seong et al., 1981). At a flow rate of 1ml/min, the Km (app)was found to be 20.2 µM. Membrane was blocked at flow rates higher than 1 ml/min and there was a higher pressure on the membrane.

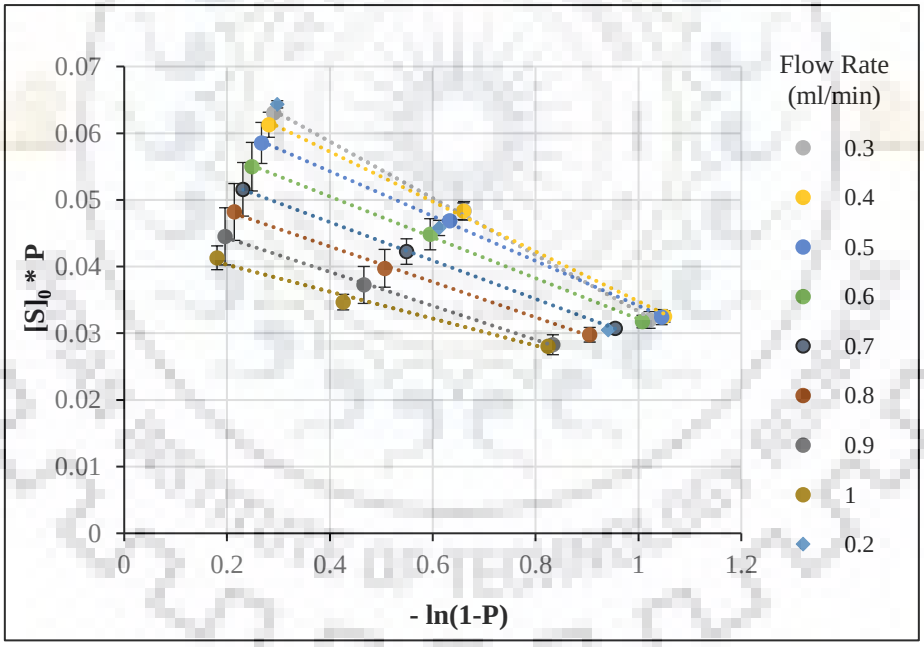


Figure 11: The kinetic parameters at different flow rate was determined using Lilly Hornby kinetic model.

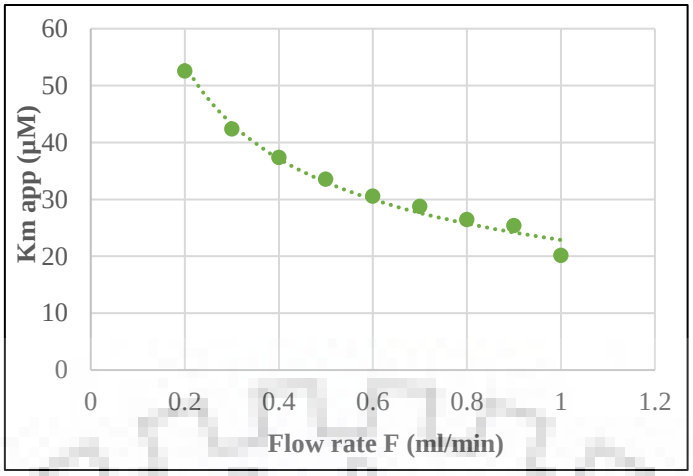


Figure 12: Km(app) at different flow rate was found using Lilly Hornby kinetic model

Degradation of dyes

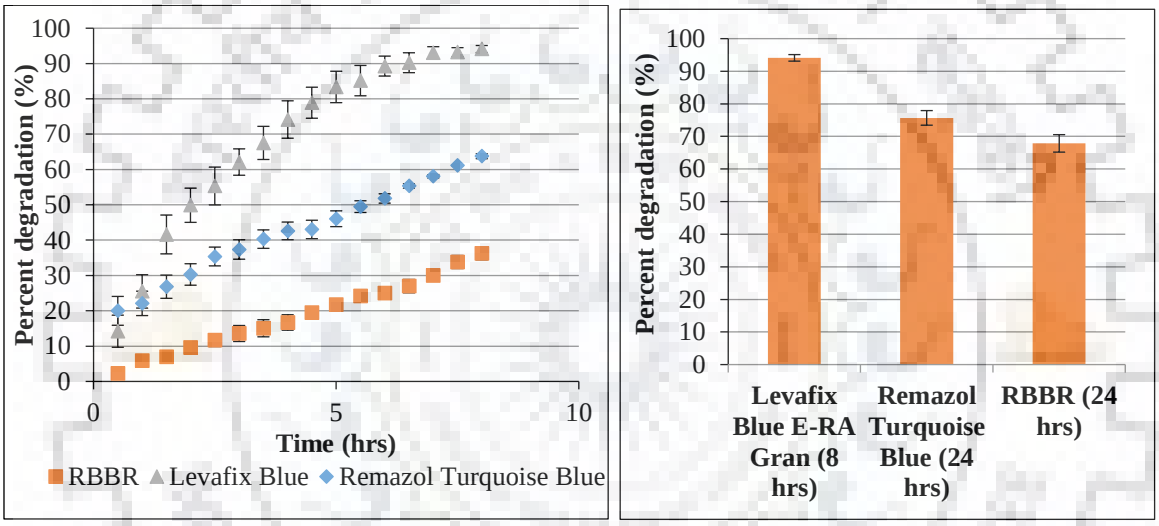
The effect of the immobilized laccase was evaluated in degradation of the dyes. The absorption maxima were found based on the spectrum for the reactive dyes. Levafix blue E-RA gran dye degraded in 5 hours and the color changed from dark blue to yellow (Figure 14). Remazol Turquoise blue changed its color from blue to very light blue in 24 hours. RBBR was found to degrade slowly and change from blue to colorless in 24 hours. The spectrum obtained from the degraded dye solution was different when compared to the initial one. As a negative control, immobilized heat inactivated enzyme was tested with these dyes and a very little change in the absorbance was detected. It can hence be confirmed that there was very little amount of dye that was adsorbed on the membrane and the rest is degraded. The percent degradation obtained for the dyes by the activated laccase, when compared to the heat inactivated one, is shown in Table 1 and Figure 15. The change in colour of the respective dyes was achieved due to its degradation by immobilized laccase (Figure 16-18).

Table 1: Dye Degradation

DYE	PERCENT DEGRADATION
Levafix blue E-RA gran	94.1 % ± 1.004
Remazol Turquoise Blue	75.68 % ± 2.236
RBBR	67.86 % ± 2.674

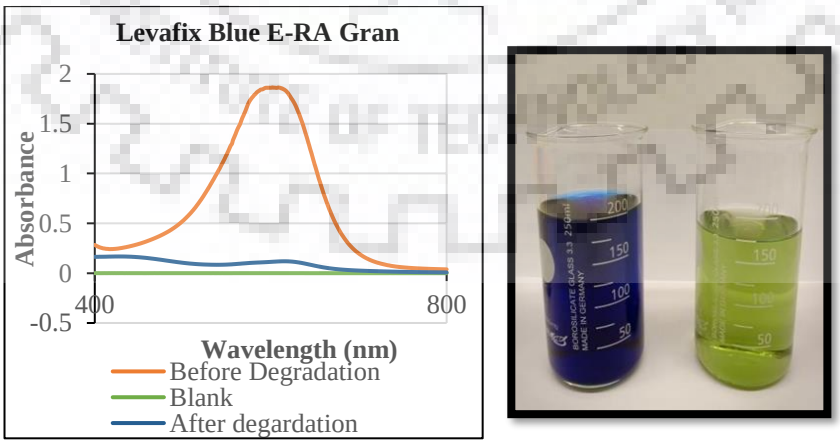
(A) (B)

Figure 13: Cross flow filtration. A) Nylon membrane used in a cross-flow module for degradation of reactive dye B) Cross flow module



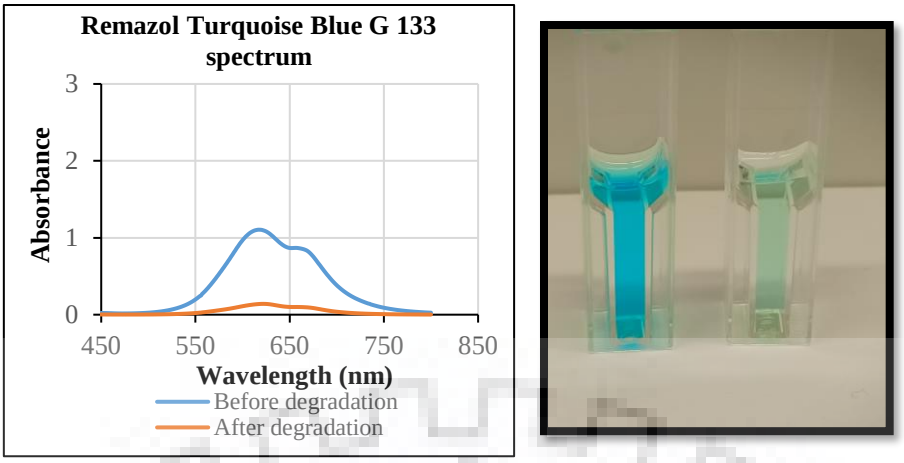
(A) (B)

Figure 14: (A) Degradation of dyes with respect to time in 8 hours (B)in 24 hours



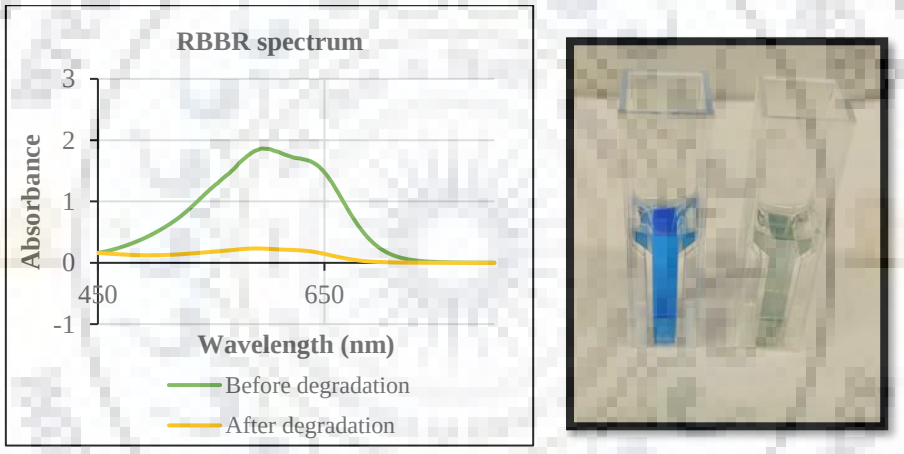
(A) (B)

Figure 15: (A) Change in the spectrum (B) decolourization of Levafix Blue-ERA gran dye



(A) (B)

Figure 16: (A) Change in the spectrum (B) decolourization of Remazol Turquoise Blue G133 dye



(A) (B)

Figure 17: A) Change in the spectrum **B)** decolourization of RBBR dye

Degradation of BPA

BPA was found to be adsorbed more on the membrane. To confirm its degradation by enzyme, fresh solution of BPA having the same concentration and volume was consecutively recirculated five times in 1h interval. The difference in the fluorescence obtained between the activated and the inactivated enzyme immobilized on the membrane was due to enzymatic activity of laccase on BPA, which was found to be nearly 0.5mU/cm² as shown in Figure 19. Nearly 39% of the BPA got degraded in the first five and a half h, but 49% of BPA in the given solution got degraded using laccase immobilized membrane in 24 h (Figure 20).

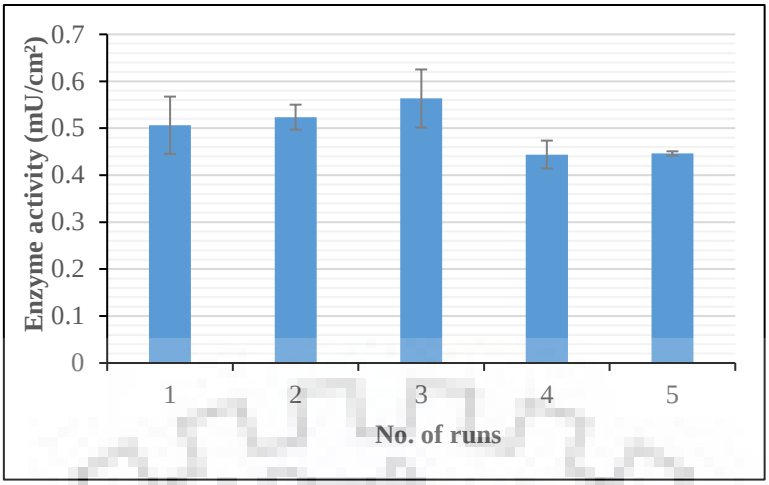
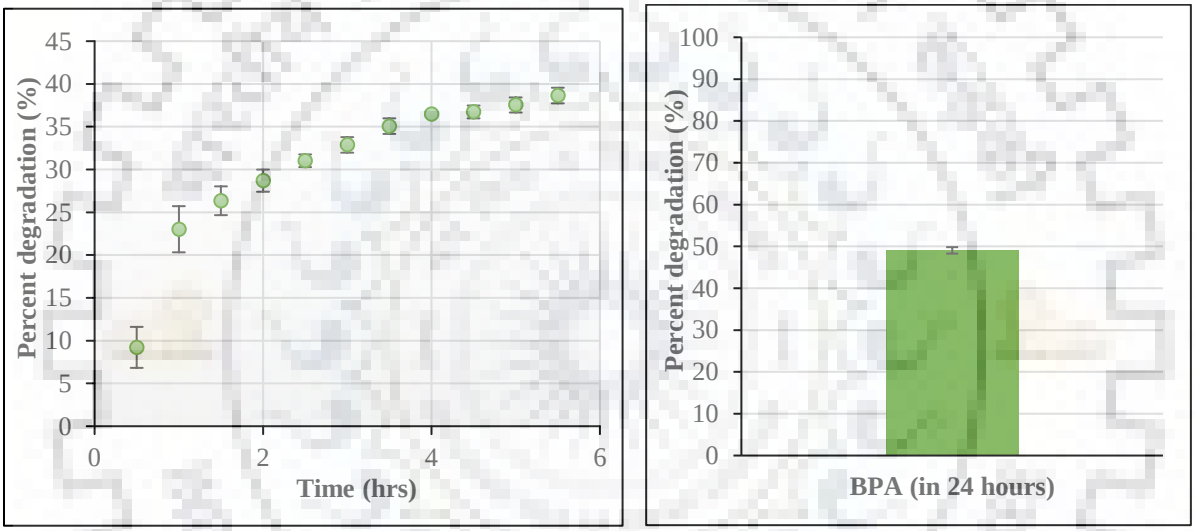


Figure 18: Enzymatic activity against BPA



(A) (B)

Figure 19: (A) Degradation of BPA with respect to time in 5.5 hours (B) In 24 hours

Storage stability of the immobilized enzyme

To determine stability of the membrane-immobilized enzyme, membranes have been analyzed for dye degrading ability using levafix blue E-RA gran. We quantified stability for 22 days using the described method. We could show that the enzyme activity towards dye degradation only reduced slightly over 3 weeks (Figure 8). On the first day, 93.4% of the dye degraded (8.5 hours) and after 3 weeks 68.53% (8.5 hours), resulting in decreased activity of 25% over 3 weeks as seen in Figure 21.

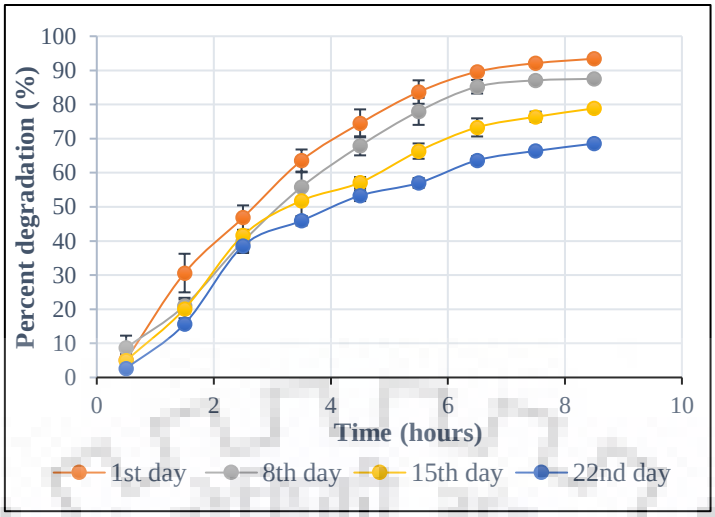


Figure 20: Storage stability of the immobilized laccase tested against Levafix blue dye

Effect of pH and temperature

Enzyme activity depends severely on pH and temperature. To achieve optimal conditions for enzyme functionalized membranes and to compare the ranges with free enzyme, we have tested a wide range of pH and temperature. The immobilized enzyme was active in a wider range of pH (4-7), and even at pH 8, a residual activity of 38 % was achieved compared to the loss of activity of free enzyme at this pH value (Figure 22). The temperature range also increased to 20 – 70 °C for immobilized enzyme as compared to the free enzyme with an optimal range of 30 – 60 °C (Figure 23). At 70 °C immobilized enzyme showed an activity of 66 % compared to 22 % for free enzyme. The results obtained are similar to that already published by Batal et al., 2015.

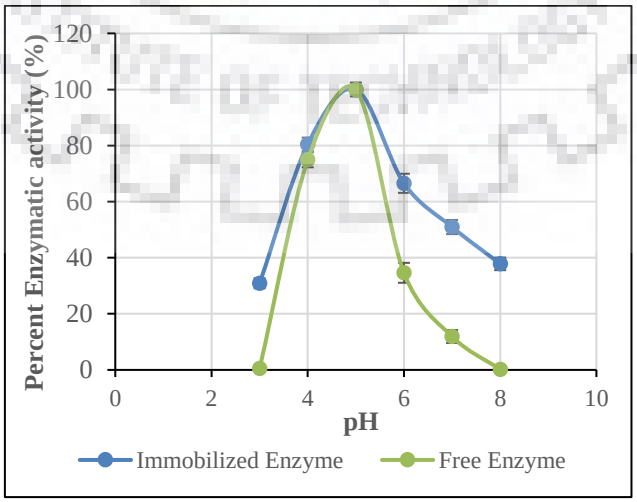


Figure 21: Effect of pH on free laccase in solution and immobilized laccase on membrane

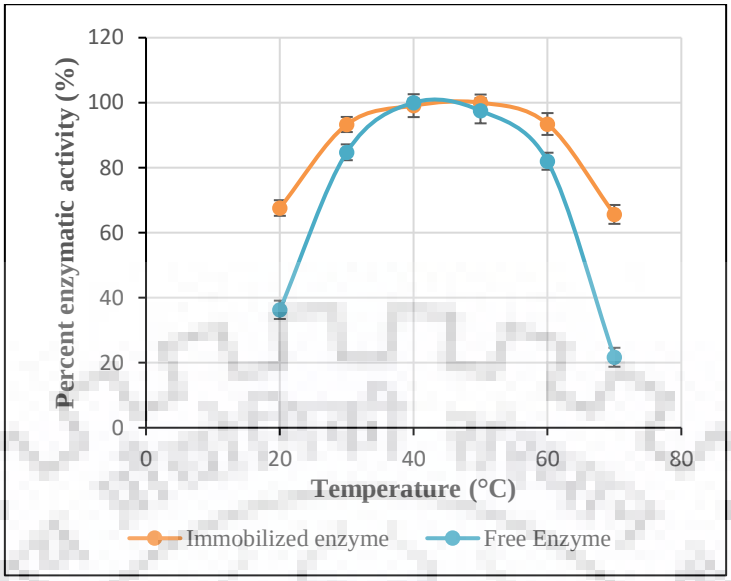


Figure 22: Effect of temperature on free laccase in solution and immobilized laccase on membrane

CONCLUSIONS

The effect of the immobilization conditions on the properties of laccase and characterization of the immobilized enzyme were conducted, also showing its suitability for continuous colour removal from the effluents of textile industries. It was found to be an efficient method for treating the reactive dyes and Bisphenol A, which are harmful for the humans and aquatic organisms. The biodegradation by the usage of commercial laccase is an attractive alternative for larger scale applications, when compared to the usual and sometimes costly physical and/or chemical means for remediation of industrial textile effluent. The immobilized enzyme had higher storage stability at 4°C and was active over a wider range of pH and temperature compare to the free enzyme. This method designates its potential for industrial applications in the field of wastewater bioremediation. Laccase obtained from *Trametes versicolor* has been found to degrade more than 90 % BPA as BPA increases the laccase production (Zeng et al., 2017). Hence, these fungi can also be used to obtain the enzyme for testing.

FUTURE PROSPECTS

- The detoxification of the biodegraded dye can be determined using the phytotoxicity and microbial toxicity tests.
- The enzyme can be obtained from different fungi and the activity can then be compared after immobilizing on the membranes.
- The degrading potential of the enzyme can be tested on different pollutants.

REFERENCES

1. Akkayya A, Ozsekera EE and Akdoganb HA (2016) Degradation of Dyes by Laccase. *Analytical Letters*, **49(6),** 790–798.
2. Batal AI, ElKenawy NM, Yassin ASY, Amin MA (2015) Laccase production by Pleurotus ostreatus and its application in synthesis of gold nanoparticles. *Biotechnology Reports* **5:** 31-39.
3. Bhatt N, Patel KC, Keharia H and Madamwar D (2005) Decolorization of diazo-dye Reactive Blue 172 by Pseudomonas aeruginosa NBAR12. *Journal of Basic Microbiology* **45:**407–18.
4. Boulares-Pender A, Thomas I, Prager A, Schulze A (2013) Surface Modification of Polyamide and Poly(vinylidene fluoride) Membranes. *J Appl Polym Sci* **128:** 322-331
5. Cerqueira MR, Grasseschi D, Matos RC, Angnes L (2014) A novel functionalisation process for glucose oxidase immobilisation in poly(methyl methacrylate) microchannels in a flow system for amperometric determinations. *Talanta* **126:** 20-26
6. Chairin T, Nitheranont T, Watanabe A, Asada Y, Khanongnuch C, Lumyong S (2013) Biodegradation of bisphenol A and decolorization of synthetic dyes by laccase from white-rot fungus, Trametes polyzona. *Applied biochemistry and biotechnology* **169:** 539-545
7. Chen BY (2002) Understanding decolorization characteristics of reactive azo dyes by Pseudomonas luteola: toxicity and kinetics. *Process Biochemistry* **38:** 437-446
8. Chhaya U, Gupte A (2013) Possible role of laccase from Fusarium incarnatum UC-14 in bioremediation of Bisphenol A using reverse micelles system. *J Hazard Mater* **254:** 149-156
9. Cristóvão RO (2010) Degradation of dye-containing textile effluents by enzymatic catalysis, porto (2010). Thesis
10. Cui C, Tao Y, Li L, Chen B, Tan T (2013) Improving the activity and stability of Yarrowia lipolytica lipase Lip2 by immobilization on polyethyleneimine-coated polyurethane foam. *Journal of Molecular Catalysis B: Enzymatic* **91:** 59-56
11. Datta S, Cecil C, Bhattacharyya D (2008) Functionalized membranes by layer-by-layer assembly of polyelectrolytes and in situ polymerization of acrylic acid for applications in

enzymatic catalysis. *Ind Eng Chem Res* **47:** 4586-4597

12. Guisan JM, Sabuquillo P, Fernandez-Lafuente R, Fernandez-Lorente G, Mateo C, Halling PJ, Kennedy D, Miyata E, Re D (2001) Preparation of new lipases derivatives with high activity-stability in anhydrous media: adsorption on hydrophobic supports plus hydrophilization with polyethylenimine. *J Mol Catal B-Enzym* **11:** 817-824
13. Hou J, Dong G, Ye Y, Chen V (2014) Enzymatic degradation of bisphenol-A with immobilized laccase on TiO2 sol–gel coated PVDF membrane. *Journal of Membrane Science* **469:**19-30
14. Jahangiri E, Thomas I, Schulze A, Seiwert, Cabana H., Abd Schlosser D (2018) Characterisation of electron beam irradiation-immobilised laccase for application in wastewater treatment. *Sci Total Environ*., **624:** 309-322.
15. Katuri K, Mohan SV, Sridhar S, Pati BR, and Sarma PN (2009) Laccase-membrane reactors for decolorization of an acid azo dye in aqueous phase: Process optimization. *Water Research* **43(15):** 3647-3658.
16. Kokola V, Doliškaa A, Eichlerováb I, Baldrianb P, Nerud F (2007) Decolorization of textile dyes by whole cultures of Ischnoderma resinosum and by purified laccase and Mn-peroxidase. *Enzyme Microb Tech* **40:** 1673-1677
17. Lilly MD, Hornby WE, Crook EM (1966) Kinetics of Carboxymethylcellulose-Ficin in Packed Beds. *Biochem J* **100:** 718-&
18. Murugesan K, Kalaichelvan PT (2003) Synthetic dye decolourization by white rot fungi. *Indian journal of experimental biology* **41:** 1076-1087
19. Nilsson I, Moller A, Mattiasson B, Rubindamayugi MST, Welander U (2006) Decolorization of synthetic and real textile wastewater by the use of white-rot fungi. *Enzyme Microb Tech* **38:** 94-100
20. O'Neill C, Lopez A, Esteves S, Hawkes FR, Hawkes DL, Wilcox S (2000) Azo-dye degradation in an anaerobic-aerobic treatment system operating on simulated textile effluent. *Applied microbiology and biotechnology* **53:** 249-254
21. Raghuvanshi S, Gupta R (2010) Advantages of the Immobilization of Lipase on Porous Supports Over Free Enzyme. *Protein Peptide Lett* **17:** 1412-1416
22. Riva S (2006) Laccases: blue enzymes for green chemistry. *Trends Biotechnol* **24:** 219-226
23. Samchetshabam G, Hussan A, Gon Choudhury T, Gita S, Soholars P, Hussan A (2017) Impact of Textile Dyes Waste on Aquatic Environments and its Treatment.. *Environment*

& Ecology **35 (3C) :** 2349—2353.

24. Sarma R, Islam MS, Miller AF, Bhattacharyya D (2017) Layer-by-Layer-Assembled Laccase Enzyme on Stimuli-Responsive Membranes for Chloro-Organics Degradation. *ACS applied materials & interfaces* **9:** 14858-14867
25. Seong BL, Han MH, Park JM, Choi CY (1981) A graphical method of determining the Michaelis-Menten constant free of external mass transfer resistance for immobilized enzymes in a packed bed reactor. *Biotechnology Letters* **3:** 607-612
26. Silva C, Silva CJ, Zille A, Guebitz GM, Cavaco-Paulo A (2007) Laccase immobilization on enzymatically functionalized polyamide 6,6 fibres. *Enzyme Microb Tech* **41:** 867-875
27. Tilli S, Ciullini I, Scozzafava A, Briganti F (2011) Differential decolorization of textile dyes in mixtures and the joint effect of laccase and cellobiose dehydrogenase activities present in extracellular extracts from *Funalia trogii. Enzyme Microb Tech* **49:** 465-471
28. Toprak T, Anis P (2017) Textile industry's environmental effects and approaching cleaner production and sustainability, an overview. *J Textile Eng Fashion Technol* **2(4):**429-442.
29. Vishwanath S, Bhattacharyya D, Huang W, Bachas LG (1995) Site-Directed and Random Enzyme Immobilization on Functionalized Membranes - Kinetic-Studies and Models. *Journal of Membrane Science* **108:** 1-13
30. Walt DR, Agayn VI (1994) The Chemistry of Enzyme and Protein Immobilization with Glutaraldehyde. *Trac-Trend Anal Chem* **13:** 425-430
31. Wesenberg D, Kyriakides I, Agathos SN (2003) White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnology advances* **22:** 161-187
32. Zeng S, Zhao J, Xia L (2017) Simultaneous production of laccase and degradation of bisphenol A with Trametes versicolor cultivated on agricultural wastes. *Bioprocess and biosystems engineering* **40:** 1237-1245