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Research Paper

Laccase-Coated Polyethersulfone Membranes for Organic Matter Degradation and Removal

Phumlile Pretty Mamba [1], Henry Joseph Ogola [2], Titus Alfred Makudali Msagati [1], Bhekie Brilliance Mamba [1], Machawe Mxolisi Motsa [1,*], Thabo Thokozane Innocent Nkambule [1]

[1] *University of South Africa, College of Science, Engineering and Technology (CSET), Institute for Nanotechnology and Water Sustainability (iNanoWS), Florida Science Campus, Private Bag X6, Florida 1709, Johannesburg, South Africa*
[2] *University of South Africa, College of Agriculture and Environmental Sciences (CAES), Florida Science Campus, Private Bag X6, Florida 1709, Johannesburg, South Africa*

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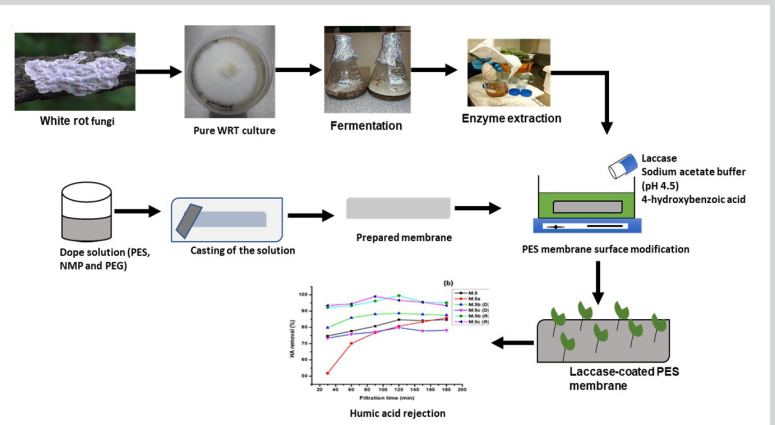
Highlights

- Extraction and isolation of lignolytic enzymes from white rot fungi.
- Enzyme activity was investigated using humic acid solution.
- Surface modification of polymeric UF membranes with enzyme isolates.
- Enzyme deposition formed a water-resistant layer that reduced membrane permeability.
- Modified membranes recorded 90% rejection of humic acid.

Graphical abstract



Abstract

Natural organic matter (NOM) removal from water is getting progressively significant for water treatment plants not only to improve drinking water aesthetics such as taste and smell, but also to avoid disinfection by-products (DBPs) formed during disinfection by chlorine. This study applies the catalytic properties of the wood degrading laccase enzyme produced by white rot fungi (WRF) on breaking down and removing organic matter in drinking water. White rot fungi isolates were collected and examined for their ability to degrade humic acid (HA), a NOM model compound. Highly permeable polyethersulfone (PES) membrane was prepared following the phase inversion process and used as material to support the immobilization of the lignin-degrading enzymes extracted from Perenniporia sp. and Polyporaceae sp. for NOM degradation and removal. A 52 % humic acid removal was recorded for the Polyporaceae sp. The addition of laccase substrate 4-hydroxybenzoic acid showed a great impact on the hydrophilicity of the membranes as a decrease in contact angle measurements of <60 was achieved. Moreover, modified membrane's immobilization yield and enzyme activity also improved. The modified membrane achieved a rejection of greater than 90 % for the model compound. Enzyme activity was a function of contact time and substrate type. The attained results revealed that catalytic membranes can be an efficient alternative for NOM removal and membrane fouling alleviation during water treatment.

1. Introduction

The presence of certain constituents of natural organic matter (NOM) such as humic acids (HAs) in surface water has is of interest to treatment facilities due to their potential to generate chlorine-based disinfection by-products (DBPs). They are also known to be responsible for the odour and colour in drinking water [1,2]. In addition, dissolved organic matter has been reported as a major contributor to bacterial regrowth in water supply pipelines as it serves as a food source for the growth [3]. NOM is generally found in surface water and consists of compounds with different chemical structures and molecular weights, which further complicates its removal in water. Currently, the most reliable conventional water treatment process for NOM

* Corresponding author: motsamm@unisa.ac.za (M.M. Motsa)

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removal is coagulation-flocculation, which involves optimisation of coagulant dosages and water pH. However, this process is associated with increased treatment costs and the production of large quantities of sludge subsequently leading to disposal problems [4,5].

Advanced filtration methods such as membranes have been used over the years as an alternative for NOM removal from water, nevertheless, NOM accumulation on the membrane surface greatly impact the membrane's long-term performance [6,7].

A potential alternative to reduce NOM content in water could be the use of extracellular enzymes generated by white rot fungi (WRF) such as laccase, manganese-dependent peroxidase and lignin peroxidase. These enzymes have the ability to degrade NOM or any carbon source such as lignin into harmless products such as water and carbon dioxide [8,9]. Previous work has shown the capability of WRF enzymes in degrading NOM in waste matter and surface water. One study by Lee and colleagues [10], a 73 % NOM removal efficiency was recorded from wastes constituting NOM due to the high activity of enzyme laccase and manganese-dependent peroxidase from two WRF species *Phanerochaete chrysosporium* and *Trametes versicolor*. In another related work by Solarska [11] a 65 % NOM removal rate by a WRF species *Bjerkandera adusta* was observed. Thus, the potential of WRF in NOM degradation has sparked interest amongst researchers in the water sector. More studies on the use of WRF enzymes on NOM degradation, using mainly laccase are continuously been reported. Zahmatkesh [12] investigated the efficiency of enzyme laccase in the removal of humic acid (HA) as a NOM surrogate in liquid and solid media. In attempt to remove HA, the change in HA concentration and molecular size distribution was monitored [12]. In their study, over 80 %, removal was reported. Zahmatkesh [13] also reported was a 80 % removal rate measured as colour reduction in synthetic and real wastewater. The investigations were carried out under non-sterile and sterile conditions when WRF *Trametes versicolor* was immobilized on sorghum as its sole carbon source [13]. And the removal rate was the result of laccase activity generated by the fungi.

These studies have revealed that the use of enzyme laccase is more efficient than using the whole fungi for NOM removal, as well as illustrating that WRF enzymes has a great potential for use in NOM degradation. However, it has been demonstrated that the free enzymes are not stable and can easily denature if conducive conditions are not provided [14]. Apart from organic matter removal, enzyme laccase was also reported by Simón-herrero [15] to remove pharmaceutical compounds from waste water using polyamide aerogels as the support substrate. Most researchers prefer to use polymeric materials independently or incorporated with nanoparticles because they provide a variety of barrier structures and properties, the energy requirement is low and the operational scale-up can be easily done [16–19]. Therefore, support materials such as porous polymeric membranes e.g. polyethersulfone (PES) are most appropriate support material for enzyme immobilization in order to maintain the enzymes stability [20]. However, it should be noted that the resultant membranes should be strong enough to withstand mechanical forces as well as extreme pH conditions. Generally, PES is one of the ideal polymers due to its inherent properties such as less cost, chemical stability and robustness [20]. Nonetheless, polymeric membranes are hydrophobic in nature, this property make its membranes highly prone to fouling due to favourable adsorption of organic substances on the surface. Therefore, the active sites of the membranes should be modified using certain functional groups to allow the binding of enzymes [21,22]. Moreover, the functional groups introduced on the membrane surface should be designed in such a way that the activity of the enzymes is preserved [14]. Amongst others, 4-hydroxybenzoic acid and gallic acids have been used as enzyme substrates and have proven to induce membrane hydrophilicity [12].

This work details the fundamental preparation and application of enzyme-modified PES ultrafiltration membranes investigated for HA degradation and removal from water. The enzyme laccase extracted from two WRF species (*Perenniporia* sp. and *Polyporaceae* sp.) were immobilized on the surface of the membranes using a substrate 4-hydroxybenzoic acid. Characterisation of the enzyme-modified membranes was conducted using scanning electron microscope for structural changes. Membrane surface modification was determined through monitoring water permeate fluxes.

2. Materials and Methods

2.1. Materials and reagents

All the chemicals used in this work were supplied by Sigma-Aldrich (Johannesburg, South Africa). The culture media, Zymo fungal/bacterial Quick DNA extraction kit and consumables for molecular experiments were sourced from Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa).

2.2. Isolation and initial screening of fungal isolates

Fruiting bodies of 18 WRF isolates collected from decaying *Eucalyptus* trees in four pulp and paper farms in South Africa and Eswatini were used in this study. For fungal isolation, approximately 0.5 x 1 cm fruiting body was sliced using a sterilized scalpel and transferred to Sabouraud Dextrose Agar (SDA) supplemented with 0.4 g/L chloramphenicol and 0.1 % guaiacol. The solid media cultures were thereafter incubated at 28 °C for a period 7 days. This screening process was adopted based on the optimised concentration levels reported for the screening of the ligninolytic enzymes [23,24]. Colour changes below the colony surface as well as on the periphery were monitored as an indication of the enzyme activity and their ability to oxidize guaiacol. The fungi colonies were periodically sub-cultured into fresh culture media until pure cultures were obtained. The pure cultures were used for identification and screening of the enzymes and humic acid. Agar slants were prepared in 100 ml sterile polystyrene tubes, inoculated with pure cultures and kept for future use at 4 °C.

2.3. Fungal isolates screening for the removal of humic acid (HA)

2.3.1. Preparation of HA stock solution and growth media

Following the method recommended by Zahmatkesh [12], a humic acid stock solution was prepared with slight modifications. Briefly, 4 g of powdered HA was initially dissolved in 200 mL of 0.1 M NaOH. The resultant solution was stirred at room temperature for 30 min before centrifugation at 8 000 rpm for 20 min. The produced supernatant liquid was withdrawn and mixed with approximately 100 mL of 0.5 M potassium hydrogen phthalate and pH was adjusted to 4.5 using HCl. The subsequent solution was centrifuged at 8000 rpm for 20 min to remove particulates, and the resultant supernatant solution was used as HA stock solution. Prior to use, HA stock solution was sieved through a 0.45 µm Whatman® membrane (GE Healthcare, Chicago, USA).

Preparation of the basal media, minimal salts media (MSM) and vitamin mix solutions were prepared as reported by Collins & Thune [25] with slight modification. Briefly, MSM contained a mixture of (0.2 g of KH_2PO_4, 0.005 g of $MgSO_4{\cdot}7H_2O$ and 0.01 g of $CaCl_2$) dissolved in 1 L deionized water was used as major minerals source. The solution was supplemented with 100 µL trace element solution, 26 mM ammonium sulphate as nitrogen source, 100 mL HA stock solution as carbon source and 15 g agar, and pH adjusted to 4.5 using HCl prior to autoclaving for a period of 15 min at 121 °C. The prepared solution was allowed to cool to room temperature and 25 µL filtered (0.45 µm) vitamin mix solution was added as illustrated by Collins and Thune [25] prior to pouring into culture (20 x 100 mm) plates. Liquid media was prepared using similar protocol without addition of agar and dispensed in 250 mL conical flask before autoclaving.

2.3.2. Humic acid removal experiments

The ability of fungal isolates to breakdown HA, was investigated using solid and liquid MSM containing HA as the sole carbon source. Agar plates containing MSM-agar were inoculated with a 1 cm^2 piece of actively growing mycelium growth in triplicates. The inoculated plates, including two uninoculated plates as controls, were natured for 15 days at 28 °C in the absence of light. The diameter of the fungal growth and decolorization of humic acid (halo around the fungal colony) on the plates (top, bottom and vertical) were observed after 4, 7, 10 and 15 days of incubation and were thereafter analysed for qualitative degradation of HA.

When preparing liquid cultures, approximately 1 cm^2 plug of actively growing mycelium was inoculated in 30 mL MSM in 250 mL conical flask, and the mixture incubated in an orbital shaker for effective growth and shaken continuously for 15 days at 28 °C and 150 rpm in the dark. After incubation, the content of the inoculated and uninoculated flasks was filtered through glass fiber filters (0.45µm) before determination of dissolved organic carbon (DOC) using a TOC torch analyzer (Teledyne Tekmar, Mason, Ohio, USA). The filtered samples were also analyzed for light activity (UV-Vis) at a recommended wavelength of 254 nm for NOM detection using a UV-Vis spectroscopy [26].

2.4. Molecular identification of fungal isolates

The extraction of fungal pure isolate DNA was done using Zymo Fungal/Bacterial Quick DNA Extraction Kit (Zymo Research, Irvin, USA), as instructed by the manufacturer. The extracted DNA was then amplified by polymerase chain reaction (PCR) using ITS1 primers (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS 4 primers (5'-TCC TCC GCT TAT TGA TAT GC-3') [27], under following cycling conditions [at 95 °C, 1 min; 35 x (95 °C, 1 min; 53 °C, 1 min; 72 °C,1 min); 72 °C,10 min; and finally 4 °C, infinity]. The resultant amplicons placed on a 1.5% agarose gel and purified using a DNA Clean & Concentrator Kit (Zymo Research, Irvin, USA), before being sent to Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) for Sanger sequencing. These sequences were used to identify the fungi with the help of the BLAST program (www.ncbi.nlm.nih.gov/BLAST). The sequences have been sent to the NCBI database with accession numbers KR907878.1 and KP013025.1 for *Perenniporia* sp. and *Polyporaceae* sp., respectively.

2.5. Enzyme production, partial purification and analysis

2.5.1. Solid state fermentation

Fungal isolates D and R, that exhibited higher HA removal rates, thus, were further studied for enzyme production in solid state fermentation (SSF) using corn husks as carbon source. Firstly, freshly grown cultures of fungi was placed into 250 mL Erlenmeyer flasks comprising of a 100 mL MSM and thereafter incubated for a period of 7 days in duplicates at 30 °C under stirring conditions. The mycelium growth was mixed by stirring at 2.8 rpm using an IKA, T25 digital ULTRA TURRAX mixer (IKA®-Werke GmbH & Co. KG, Staufen, Germany) and poured into 70 g sterilized corn husks moisten with 100 mL MSM basal media and incubated in complete darkness for 21 days at 30 °C. Samples were collected for enzyme assay after 4, 7, 10,15 and 21 days of incubation.

To prepare enzymes for immobilization studies, crude enzyme extraction was carried out after 10 days of SSF. The content of the flasks was mixed with 150 mL in 0.1 M citrate phosphate buffer (pH 5) and mixed in orbital shaker at 150 rpm for 1 h before filtering the content using a clean muslin cloth. The solid content was rinsed with 0.1 M citrate phosphate buffer (pH 5) followed by filtering. The filtered mixture was centrifuged by spinning at 10 000 rpm for a period of 30 min to eliminate any particulate matter formed. The supernatants were collected and assayed for enzyme activity. Enzyme purification was carried out using ammonium sulphate precipitation method as described by Othman [28], whereby crude enzyme extracts were initially mixed with ammonium sulphate to achieve a 40 % (w/v) saturation at 0 °C. The mixture was stirred for overnight at 0 °C before being centrifuged at 10 000 rpm for 20 min and the resultant pellet of precipitated protein discarded. The resultant supernatant saturation point was allowed to reach 80 % (w/v) by continuously adding more crystals of ammonium sulphate whilst stirring at 0°C. The precipitate was liquified in 30 mL of 0.05 M citrate-phosphate buffer (pH 5.0) and dialysed in 10 L of the same buffer overnight at 0 °C. The activity of the enzymes was determined prior and after the dialysis process.

2.5.2. Enzyme activity assays

The method for determining enzyme activity was adopted from Ijoma, 2016 [23]. Laccase activity was assayed using guaiacol as a substrate. The increase in ultra-violet (UV) absorbance at 470 nm was measured as laccase activity at 25 °C after incubation at 30 °C for 2 minutes. The mixture contained 1.2 mL guaiacol, 0.4 mL 100 mM sodium buffer at pH 5 and 0.4-mL crude enzymes. Prior to UV measurements, the reaction mixture was agitated by swirling.

A reaction mixture comprising of 0.4 mL of 0.1 M manganese sulphate, 0.4 mL of 50 mM sodium tartrate at pH 5, 0.6 mL autoclaved deionized water, 0.2 mL crude enzyme was used to determine manganese-dependent peroxidase (MnP) activity. The mixture was activated by supplementing 0.4 mL of 5 mM hydrogen peroxide (H_2O_2). The UV absorbances of the reaction mixture were immediately measured at 350 nm at room temperature.

Lignin peroxidase (LiP) was assayed in a reaction mixture which contain 0.5 mL veratryl alcohol as a substrate, 0.4 mL of 0.3 M citrate/phosphate buffer at pH 4.5, 40 µL crude enzyme and 1.26 mL autoclaved deionized water. The mixture was stimulated by supplementing 250 µL of 5 mM H_2O_2 and UV measurements were instantaneously measured at a wavelength of 310 nm at room temperature.

2.6. Enzyme-immobilization experiments

The membrane used in this study was a custom-made polymeric polyethersulfone (PES) ultra-filtration membrane that have shown the most promising performance in terms of water flux and solute rejection. The membrane (M.5) composition was 20 wt.% PES as the polymer, 36 wt.% polyethylene glycol (PEG) as a pore-forming agent. A manuscript which gives the details of the membrane preparation has been submitted for publication to another journal.

Flat sheet PES membranes were cut into small pieces and placed in 500 mL beakers (and were named as M.5- clear membrane as a blank, M.5a-membrane with substrate only, M.5b- membrane with the enzymes only, and M.5c- membrane with enzyme and substrate). The beakers contained 40 mL of 0.1 M sodium acetate buffer (pH 5.0), 50 mL of 28.8 mM 4-hydroxybenzoic acid and 10 mL of crude enzyme laccase with 0.297 U/mL for isolate R and 0.152 U/mL for isolate D. The contents of the beakers were incubated for 24 h at 30 °C under stirring conditions (120 rpm). After the incubation and prior to use, the membranes were kept in desiccators containing silica gel. The change in the colour of the membranes was visually observed after 24 h of incubation.

2.6.1. Characterization of the prepared membranes

2.6.1.1. Scanning electron microscope

The morphology of the modified and unmodified membranes was studied using scanning electron microscopy (SEM) (JSM-IT300 Joel, Tokyo, Japan). The membranes were cut into smaller pieces and coated with 5 nm gold layer prior to examination with SEM.

2.6.1.2. Hydrophilicity

Membrane wettability was probed by measuring contact angles of the membranes using DSA30 Kruss drop shape Analyzer, GmbH (Hamburg, Germany). Membrane samples were initially dried for 12 h at 40 °C and thereafter cut into small pieces on a glass slide prior to analysis using the sessile drop method. The actual measurements were carried out by allowing a minimum of 10 water droplets at various spots across each membrane surface. The average contact angle value of a minimum of 10 drops was reported.

2.6.1.3. Membrane rejection studies

Rejection studies were carried out by filtering ~30 mg/L HA solution through the membranes by means of a dead-end cell system. The permeate flux was monitored at 30 min intervals. The effective area of the membranes was 0.00113 m^2 and three cells were used for each membrane type. The permeate flux was calculated using Equation 1 and water permeability was calculated using Equation 2.

$$J_w = \frac{V}{\Delta t A_m} \tag{1}$$

where J_w is water flux ($L.m^{-2}.h^{-1}$), V is the permeate volume (L), Δt is the time taken to collect the permeate (h) and A_m is the effective membrane area (m^2).

$$A = \frac{V}{A_m \Delta t \Delta P} \tag{2}$$

where A is the membranes permeability ($L/h.m^2.bar$), V is the permeate volume (L), A_m is the membrane area (m^2), Δt is the filtration time (h) and ΔP is the pressure applied (bar).

2.6.2. Immobilized-enzyme HA degradation activity

The permeate analysis was carried out using ultra-violet visible (UV-Vis) spectrometry, with particular attention given to 254 nm as an absorbing wavelength for HA [26] and dissolved organic carbon (DOC). UV-Vis analysis was conducted for all the permeate samples and DOC analysis were conducted after a 30 min filtration of the permeate samples. The removal percentage for HA was calculated according to Equation 3.

$$R(\%) = \frac{C_f - C_p}{C_f} \times 100 \tag{3}$$

where R (%) is the solute rejection, C_f is the feed concentration (mg/L) and C_p is the permeate concentration (mg/L).

3. Results and discussion

3.1. Screening of fungal isolates

3.1.1. Primary screening of fungal isolates

Guaiacol plates containing media was used for the screening of all the fungal isolates for their ability to produce laccase. The isolates were further screened for their ability to grow in the presence of humic acid (HA) as sole carbon source on solid media. The screening results are shown in Figure 1 and Table 1. Among the 18 fungal isolates screened, only three (D, L and R) gave promising results for enzyme production on 0.1% guaiacol as evidenced by the reddish-brown colour at the top and bottom view of the agar plate (Figure 1). The reddish-brown zone at the bottom and edges of the mycelium growth is due to the oxidation of guaiacol by the fungal enzymes [29,30]. The three isolates showed a colour change within 10 min of inoculating with mycelium disks on agar plates. Guaiacol can be a unique laccase substrate in the absence of hydrogen peroxide (H_2O_2), this will therefore confirm the true activity of the enzyme laccase. However, peroxidases are also able to oxidize guaiacol in presence of hydrogen peroxide as an electron donor [29]. Generally, laccases catalyze in the oxidation reaction that sees the breakdown of guaiacol to phenoxy radicals, which subsequently undergo oxidative polymerization to produce tetraguaiacol. And this reaction is confirmed by the reddish-brown zone formation around laccases positive colonies on agar plates. Thus, guaiacol could be used as a reliable marker for extracellular oxidative enzymes, and the immediate colour change may be an indication of high enzyme activity, either laccases and/or peroxidases produced by the fungi [31]. Similar to this study, Metuku [32] screened for white rot fungi activity by monitoring the polymerization of guaiacol in wood powder agar plates as a result of the produced peroxidases excreted by the fungi. Desai [33] also scanned for laccase activity on potato dextrose agar (PDA) consisting of tannic acid and observed the development of brown coloured precipitate on the plates. In their study, a reddish brown colour on the edges of guaiacol containing plates was observed.

The eighteen (18) isolates were also sub-cultured individually on selective agar plates to screen for their capability to utilize humic acid as the sole carbon source. As indicated in Table 1, the mycelium growth for all isolates were observed visually following 4, 7, 10 and 15 days of incubation. The mycelium growth, in terms of colony radius in mm, was used to monitor the fungal isolate's ability to utilize humic acid as carbon source for growth. As displayed in Table 1, most of the fungal isolates exhibited partial growth on the agar plates containing humic acid. However, the mycelium growth for the three isolates D, L, and R was comparatively intense (>30 mm) than other isolates (Figure 2), illustrating their ability to efficiently use humic acid as a food source. According to Zahmatkesh [12], decolourisation of humic acid in solid media is an indication of its degradation. However, decolourisation does not always corollate to complete degradation. In this work, no humic acid bleaching was observed, possibly due to its presence in low concentrations in the solid media. The same trend in growth behaviour of the three isolates (D, L and R) on humic acid was observed as presented in Figure 2. A scientific ruler was used to measure the growth of the isolates by monitoring the mycelium growth after 4, 7, 10 and 15 days of incubation. Maximum growth was recorded at day 7 followed by a slow, constant growth for the rest of the incubation process. The decreasing rate of the mycelium growth was similar to those obtained in a study by Sou [34]. This could result from a deteriorating carbon source to support the growth of the fungi. The correlation between the intense colour change in guaiacol media (Table 1) and the mycelium growth (Figure 2) could be an indication that the isolates were producing a similar cocktail of lignolytic enzymes for the diminution of humic acid for their growth.

3.1.2. Humic acid degradation in liquid media

According to Zahmatkesh [12], bleaching of humic acids in solid media can be used as an indicator for their degradation/conversion into low-molecular-mass compounds such as fluvic acid (FA) and carbon dioxide. There are several reports for a wide array of white-rot fungi (WRF) species used for the decolorization of HAs under cometabolic conditions. For example, WRF species such as *Trametes versicolor*, *Phanerochaete Chrysosporium*, *Bjerkandera adusta and Collybia dryophila* were found to degrade HA from soil, peat and brown coal [35,36]. However, in this work there was no bleaching of HA observed, possibly due to low concentration of HA used in the solid media. Therefore, *Perenniporia* sp. isolate D, *Polyporaceae* sp isolate R and *Trichoderma koniniopsis* Isolate L were further probed for their ability to decolourise HA in liquid media which served as the sole source of carbon for the fungal growth.

Figure 3 shows decolorization of humic acid following fungal treatment. The side and top view images of the samples were taken after they were passed through a 0.45 µm Whatman filter. High decolorization was observed in cultures inoculated with isolates D and R and the reddish-brown colour of the control changed to a less intense reddish-brown colour (Figure 3). This colour change is an indication of humic acid uptake by the fungal isolates either through degradation or absorption by the fungal mycelia [37]. The decolorization results supports the percentage removal of HA in Figure 2.

Table 1
Screening of fungal isolates for lignolytic enzymes and growth on humic acid as a carbon source.

Isolates	Colour change in 0.1% Guaiacol[a]	Growth on humid acid[b]
A, E, F, G, H, I, and K	-	++
B, C, J, M, N, P, Q and S	-	+
D	+++	+++
L	+++	+++
O	-	-
R	+++	+++

[a] Change in the media colour from colorless to reddish brown: - no change, + slight change, +++ intense colour change.
[b] Colony radius: - no growth, + slight growth (<10 mm), ++ good growth (<30 mm) and +++ Intense growth (>30 mm).

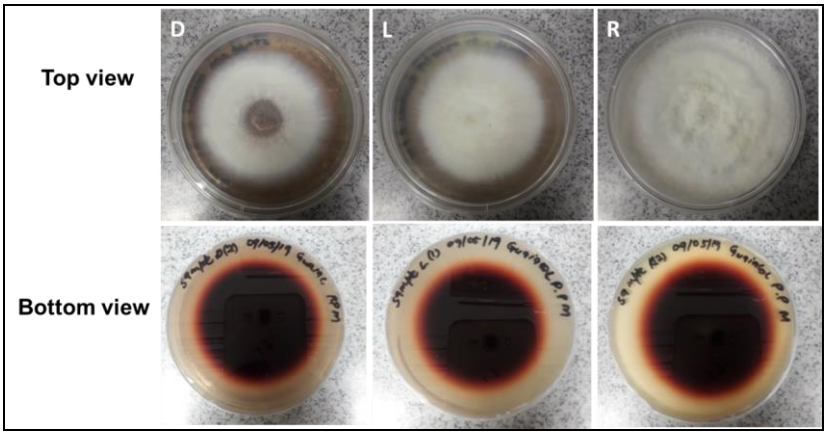

Fig. 1. Culture plates indicating laccase enzyme activity from *Perenniporia* sp. isolate D, *Polyporaceae* sp. isolate R and *Trichoderma koniniopsis* isolate L on guaiacol containing solid media.

3.2. Humic acid removal capacity

Ultraviolet and visible spectrometry (UV-Vis) and dissolved organic carbon (DOC) measurements are some of the characterization methods that are used for studying the change in molecular structure and quantity of natural organic matter in water [38,39]. Humic acid degradation based on UV_{254} and DOC analysis was carried out using samples exposed to *Perenniporia* sp. isolate D, *Polyporaceae* sp. isolate R and *Trichoderma koniniopsis* Isolate L. As displayed by Figure 4, the highest degradation percentage of HA (27.30 % UV_{254}; 52.0 % DOC) was achieved using *Polyporaceae* sp. (isolate R). On the other hand, the lowest HA degradation percentage (11.65 % UV_{254}; 23.05 % DOC) was obtained using *Trichoderma koniniopsis* sp. (isolate L) (Figure 4). These degradation results have indicated a strong correlation between UV_{254} and DOC analysis for the removal of HA.

Within the WRF species, correlation has been noted between the activities of peroxidases generation and humic acid degradation, with MnP and laccase playing a major role in depolymerizing and mineralizing different HAs in vitro [12]. Thus, the differences observed in humic acid degradation between the isolates is attributed to the variation in the production of this enzyme cocktail.

3.3. Solid state fermentation (SSF) for the production of enzymes

Comparatively, far more superior removal rates of humic acid in liquid media were achieved with *Perenniporia* sp. isolate D, and *Polyporaceae* sp. isolate R than *Trichoderma koniniopsis* Isolate L. Hence, further investigations were focused on the ability of the two isolates (*Perenniporia* sp. isolate D, and *Polyporaceae* sp. isolate R) to produce lignolytic enzymes in an SSF process. Enzyme activity was monitored on the 7th, 10th, 15th and 21st days of incubation to detect for any increase or decrease in the production of enzymes. The results indicated high peroxidase production for both isolates as compared to laccase (see Figure 5a-b). On the 10th and 15th days of incubation, the activity of MnP decreased from 1.523 ± 0.002 U/mL to 0.690 ± 0.003 U/mL for *Perenniporia* sp. isolate D (Figure 5a) and from 3.941 ± 0.004 U/mL to 1.416 ± 0.001 U/mL for *Polyporaceae* sp. isolate R (Figure 5b). The decreased activity could be ascribed to the depletion of carbon source which is responsible for supporting fungal isolate growth. While the highest concentration of LiP was achieved by *Polyporaceae* sp. isolate R (3.738 ± 0.002 U/mL) on day 15, the highest activity levels of LiP (1.397 ± 0.005 U/mL) were obtained only in day 10 for *Perenniporia* sp. isolate D. Interestingly, *Perenniporia* sp. isolate D produced high levels of laccase on day 10 (0.467 ± 0.002 U/mL), which subsequently decreased to 0.178 ± 0.003 U/mL at day 21 (Figure 5a). Following an almost similar trend, *Polyporaceae* sp. isolate R was shown to exhibit high laccase activity on day 7, which subsequently decreased from 0.316 ± 0.002 to 0.068 ± 0.002 U/mL at day 21 (Figure 5b). Results reported from Ozcirak Ergun & Ozturk Urek's [41] work were in contrast to those obtained in this research study; laccase activity was reported the highest compared to the peroxidases produced by *Pleurotus ostreatus* in SSF using potato peel waste [41]. In another study by Damián-Robles [42], white rot fungi species *Irpex lacteus* and *Phlebiopsis* were found to produce lower levels of laccase when basal media was used for the growth of the fungi. The results of this and the above cited studies suggest that the production of lignin degrading enzymes is dependent on the type of species and the fungal growth conditions (e.g. type of carbon source) used.

Since high enzyme activity was obtained during 10 days of incubation, SSF experiment was conducted again over a 10-day period followed by enzyme extraction for purification. The activity of *Perenniporia* sp. isolate D enzyme was found to be 0.311, 0.746 and 0.329 U/mL for laccase, MnP and LiP, respectively. Whilst activities of 0.263, 1.367 and 0.184 U/mL for laccase, MnP and LiP, were recorded for isolate R identified as *Polyporaceae* sp. The enzymes were partially purified according to an ammonium sulphate purification method. Figure 5c shows enzyme activity after purification process. MnP activity was found to be the highest for both isolates. Activity levels of 0.869 U/mL and 0.724 U/mL were recorded for *Perenniporia* sp. Isolate D and *Polyporaceae* sp. isolate R. While the lowest levels of LiP activities were detected for both *Perenniporia* sp. Isolate D and *Polyporaceae* sp. isolate R, recording laccase activity levels of 0.152 and 0.298 U/mL. In some instances, enzyme precipitation through the use of ammonium sulphate increases or decreases enzyme activity depending on the type of method used [28]. In the current work, enzyme activity following purification was slightly increased from 0.746 to 0.869 U/mL (MnP) for *Perenniporia* sp. isolate D, and from 0.263 to 0.298 U/mL (laccase) and from 0.184 to 0.298 U/mL (LiP) for *Polyporaceae* sp. isolate R (Figure 5c).

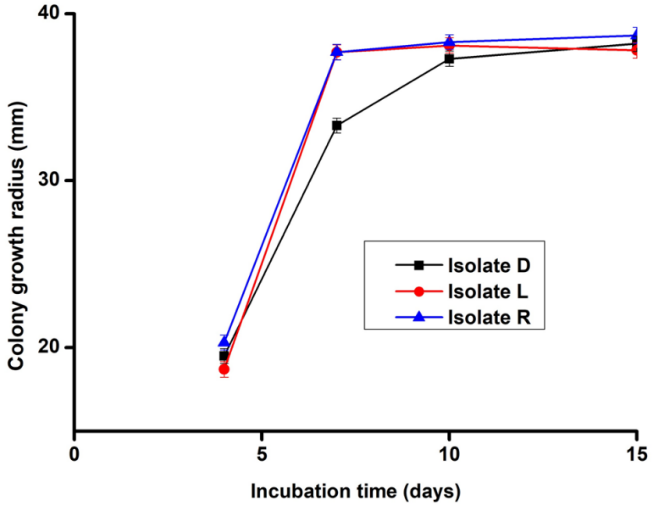

Fig. 2. Growth curve of *Perenniporia* sp. isolate D, *Polyporaceae* sp. isolate R and *Trichoderma koniniopsis* Isolate L on solid media having HA as the sole carbon source.

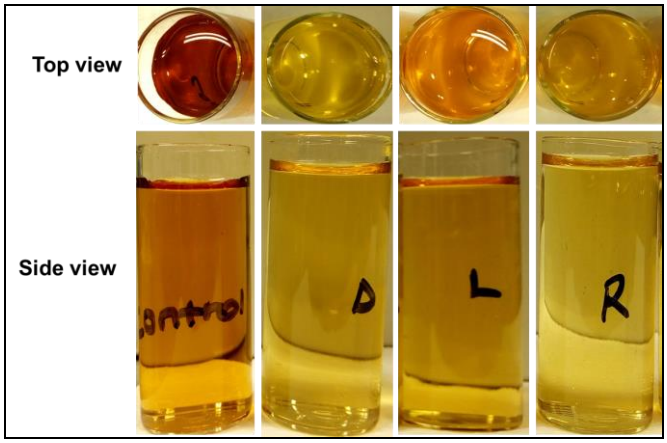

Fig. 3. HA decolourization in liquid media by *Perenniporia* sp. isolate D, *Polyporaceae* sp isolate R and *Trichoderma koniniopsis* Isolate L, and the negative control.

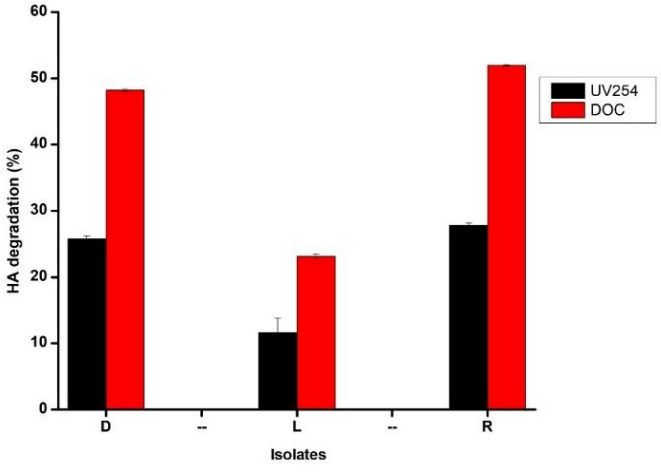

Fig. 4. Percent HA degradation by three selected fungal isolates based on UV absorption spectra (UV_{254nm}) and DOC analysis.

3.4 Membrane surface modification

3.4.1. Colour observation

A visual change in colour of the modified membrane surface was considered as confirmation for effective grafting of the enzymes and the substrate. The change in colour was particularly intense for M.5c when compared with M.5b, especially with enzymes extracted from isolate D (see Figure 6a). The change in colour for M.5b was ascribed to the adsorption of enzymes onto the membrane surface. The intense colour change in M.5c is indicative of an enzymatic free radical reaction involving the substrate and the sulfonate groups of the membranes [43]. The membranes were kept for 24 h in a desiccator prior to use and no further color change was observed after storage.

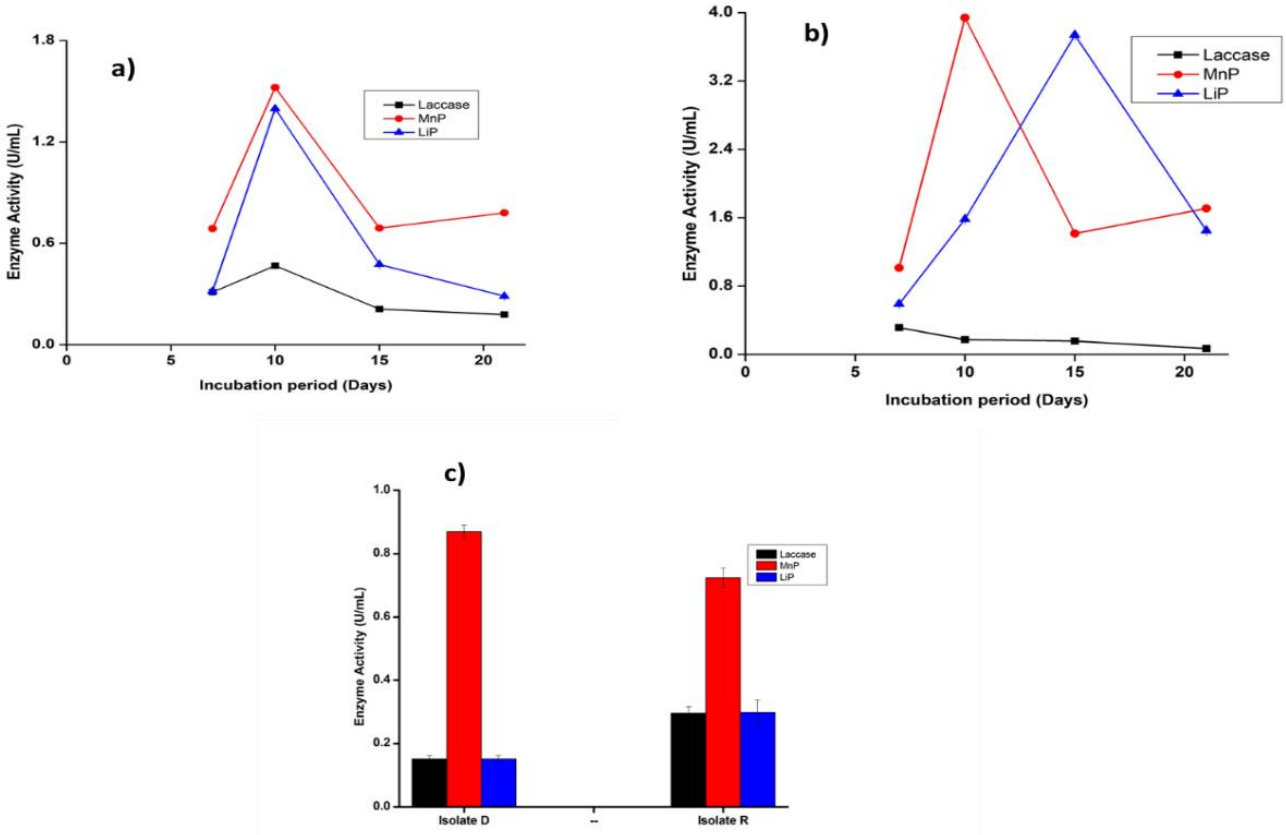

Fig. 5. Change in lignolytic enzyme production during solid state fermentation by *Perenniporia* sp. isolate D (a) and *Polyporaceae* sp isolate R (b), and the final enzyme activities of partially purified extracts (c).

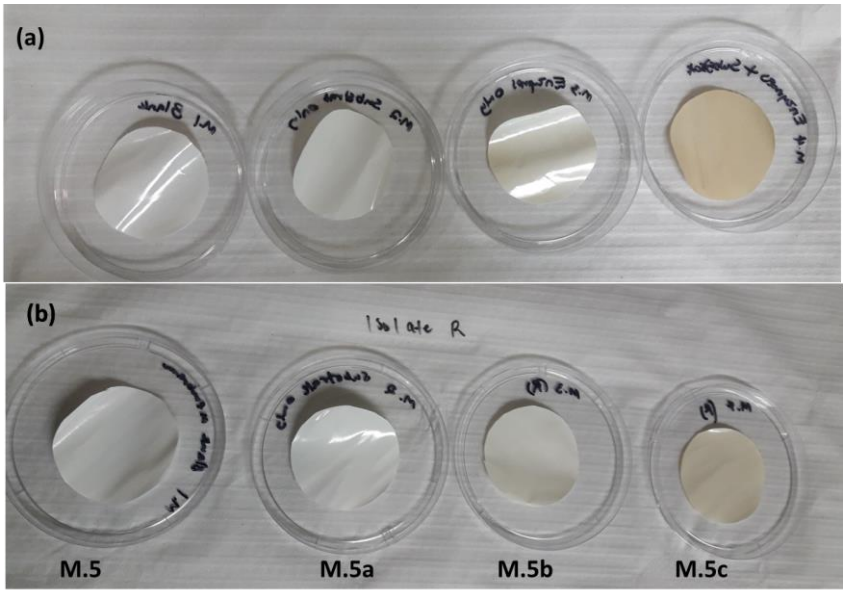

Fig. 6. Images of unmodified and modified membranes using enzymes produced by *Perenniporia* sp. isolate D (a) and *Polyporaceae* sp isolate R (b).

3.4.2. Surface morphological examination

Membrane surface morphology was conducted to verify the enzyme adhesion on the membrane surface. The presence of large rough spherically shaped amorphous substances on the surface of modified membranes is evident in Figure 7 c,d,g and h. A clean, neat surface was observed on the surface of the blank membrane M.5 (Figure 7a). Membranes incubated with enzymes from isolate D (M.5b and M.5c) were found to display sparsely dispersed enzymes on the membrane surface (Figure 7c,d) when compared with membranes modified with enzymes from isolate R (Figure 7g,h). The good distribution of the enzymes could be attributed to complete physical adhesion of isolate D enzymes on the membranes surface since all the membranes were subjected to the same incubation conditions. The morphological changes observed on the enzyme modified membranes were similar to that obtained by Nady [44]. To verify if enzyme leaching occurred during filtration, the morphology of the enzyme modified membranes were examined after filtration. As depicted in Figure 7 e,f,i and j, there was no significant changes in the surface appearance of enzyme modified membrane samples. This could be due to the successful attachment of the enzymes on the membranes; however, it could also mean that saturation point of the enzymes was achieved following the observed stable permeabilities after 60 min of filtration.

3.4.3. Membrane wettability

The measured water contact angles for all membranes are presented in Figure 8. The contact angles were found to be greater than 60°, indicating that the membranes are slightly hydrophobic. Malczewska & Żak [45], stated that a hydrophobic membrane is characterised by a contact angle measurement of > 60°. The grafting of the enzymes on the membranes surface increased the contact angles from 68.1°± 5.8 (M.5) to 71.94°± 2.41 (M.5b) and 72.84°± 4.72 (M.5b) for the isolate D and R enzymes, respectively. The increased measured contact angles are mainly due to enzymes containing hydrophobic functionalities which results in poor affinity for water. However, upon supplementation of the substrate, the contact angles decreased slightly to 59.3°± 4.2, 63.83°± 2.07 and 61.25°± 5.11 for the M.5a, M.5c isolate D enzyme and M.5c isolate R enzyme, respectively. In addition, the slight decrease in the contact angles indicates an improvement in hydrophilicity which can be accompanied by enhanced water permeability of the membranes. The contact angles obtained in this work are in agreement with those reported by Nady [46]. The increase in hydrophilicity is attributed to the polar carbonyl groups present in the 4-hydroxybenzoic acid substrate, which are covalently bonded to the sulfonate groups of polyethersulfone membranes [43].

3.4.4. Permeability and Humic acid rejection experiments

Pure water permeability is often describes as the quantity of water that is transported through a membrane area per unit time at a certain transmembrane pressure [7]. Figure 9 illustrates the filtration performance of the membranes studied in terms of water permeability. Upon modification, membrane permeability declined due to the presence of the extra enzyme layer. The decrease in water permeabilities further confirms the successful modification of the membrane surface. The results obtained revealed high permeabilities of 30.09 L/h.m^2.bar (M.5) and 21.24 L/h.m^2.bar (M.5a) for the unmodified and enzyme modified membranes (Figure 9). However, the respective permeabilities of the same membranes decreased to 3.98 L/h.m^2.bar (M.5) and 2.65 L/h.m^2.bar (M.5a) after 180 min of filtration. During the first 15 min, permeabilities of 6.73 L/h.m^2.bar (M.5b) and 4.96 L/h.m^2.bar (M.5c) were recorded from isolate D modified membranes and further decreased over time. The gradual decrease in permeabilities was due to reduced membrane porosity caused by the grafting of the enzymes on the membranes as well as the formation of an extra permeate flow resistant layer [47,48]. A reduction in water permeability was also reported in a study by Nady [49] whereby PES membranes were modified with enzyme laccase for the rejection of bovine serum albumin. In their study, the reduction was attributed to the membrane modification and irreversible protein adsorption under different modification conditions [49].

A similar trend was observed for the isolate R enzyme modified membranes. The contact angle measurements obtained for M.5b (R) and M.5c (R) corresponded to the observed increase in permeability from 4.956 to 6.726 L/h.m^2.bar. The increase in permeability is attributed to the incorporation of the substrate 4-hydroxybenzoic acid onto the membrane M.5c (R) which provided favorable forces for the interaction of water molecules with the membrane surface. A rise in membrane permeabilities was also reported by Koloti [48] when glutaraldehyde was used as a substrate for enzyme laccase to bind the enzyme to dendritic membranes for the removal of bisphenol-A from water. However, there was no significant difference in the permeabilities amongst the membranes modified with D and R enzymes. This implies that the extent of enzyme grafting does not affect the permeability of membranes. Overall, humic acid removal by the enzyme grafted membranes was an interplay between foulant-enzyme interactions, permeate drag force and flow rate.

All the membranes investigated in this research work were evaluated for their performance in rejecting humic acid rejection, and the results are presented in Table 2. Upon addition of the enzymes, the removal rate for HA in terms of UV_{254} increased from 54 % (M.5) to 84.8 % (M.5b (D)) as presented in Table 2. The increase in HA removal efficiency can be associated with sufficient active sites of the enzymes which enables high interactions with the compound leading to improved degradation rate.

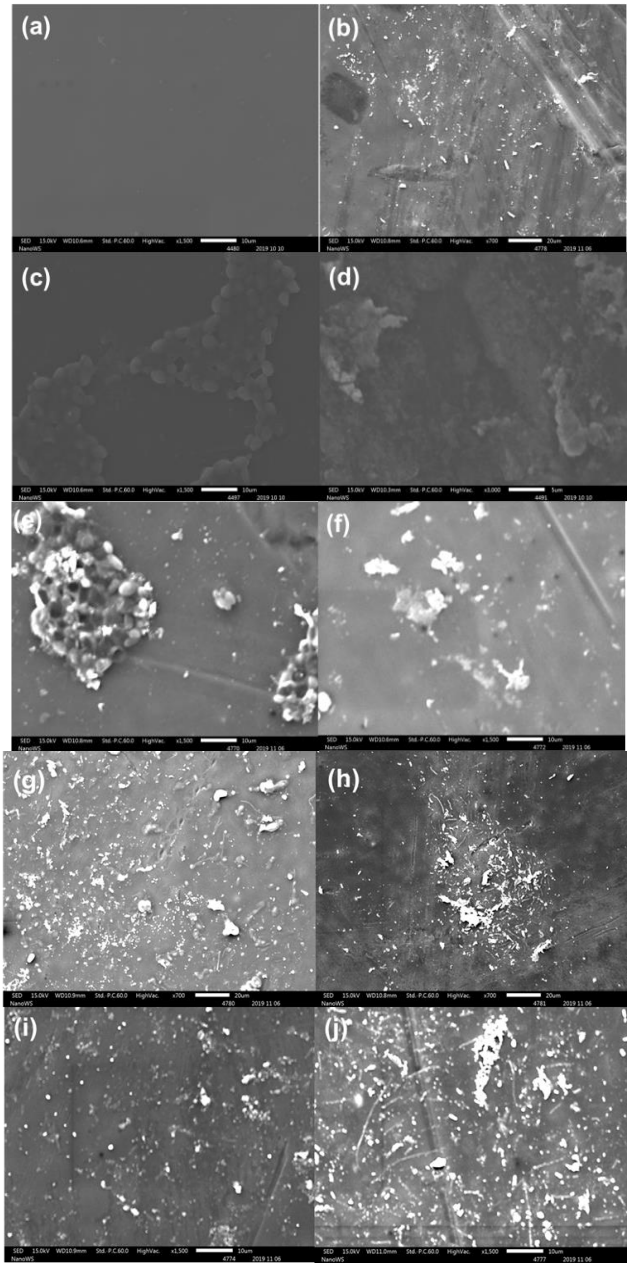

Fig. 7. SEM images of all membranes investigated (a) M.5 blank membrane, (b) M.5a membrane with substrate only, (c) M.5b(D) membrane with enzyme only, (d) M.5c(D) membrane with enzyme and substrate, (e) M.5b(D) used membrane, (f) M.5c(D) used membrane, (g) M.5b(R) membrane with enzyme only, (h) M.5c(R) membrane with enzyme and substrate, (i) M.5b(R) used membrane and (j) M.5c(R) used membrane.

The other effect is linked to size exclusion where there is reduction in pore size because of enzymes accumulation on the surface of the membranes resulting in increased humic acid rejection. However, upon addition of the enzyme substrate (4-hydroxybenzoic acid), the removal rate decreased from 84.8 % to 55 % with membrane M.5b (D). The decrease in HA removal could be attributed to reduced HA adsorption on the membrane surface or insufficient interaction sites that may have been hindered by the substrate. There was also increase in permeate fluxes (Figure 8) for M.5c (D) that might have interfered with contact time and interaction between humic acid compounds and the enzyme sites. The same trend was observed when enzymes from *Polyporaceae* sp. isolate R were used.

The highest HA removal rate in terms of DOC was achieved from M.5c (R) (93.4%) membrane modified with enzymes extracted from *Polyporaceae* sp. isolate R. Whilst M.5a recorded the lowest removal efficiency of 51.8 % as reported in Table 2. These removal percentage figures are similar to those obtained by Barrios-Estrada [22] and Bilal & Iqbal [51], which involved the breakdown of Bisphenol-A (BPA) using laccase produced from white rot fungi species. The results have illustrated that humic acid removal by the modified membranes are higher than those of the other membranes investigated. This suggests that enzymes are much more effective in the removal of HA when used without the substrate on membranes as a support material for immobilisation.

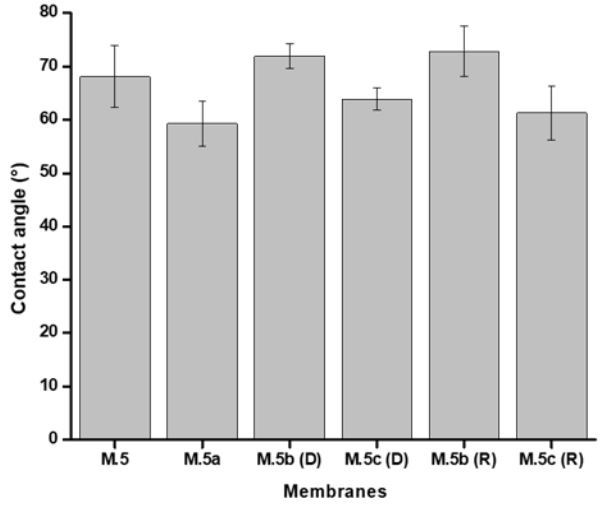

Fig. 8. Measured water contact angle for all the membranes studied.

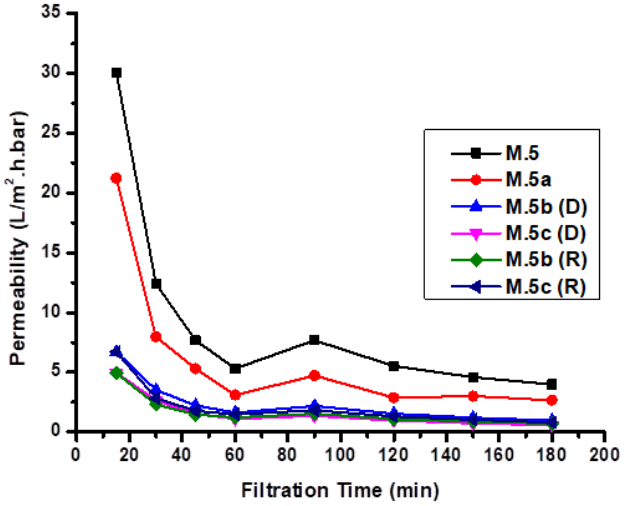

Fig. 9. The permeabilities of all the membranes investigated in this study.

Table 2
Humic acid (HA) removal efficiency in terms of UV_{254} nm and DOC for all the membranes studied.

Membranes	UV_{254} removal %	DOC removal %
M.5	54	74.7
M.5a	53.9	51.8
M.5b (D)	84.8	79.8
M.5c (D)	55	73.4
M.5b (R)	81.1	92.2
M.5c (R)	55.7	93.4

4. Conclusions

This study sought to develop fundamental information of the immobilization of white rot fungi enzymes on polymeric ultrafiltration membranes to induce catalytic functionality. The laccase enzymes extracted from two isolates D and R of *Perenniporia* sp. and *Polyporaceae* sp. were tested for the degradation of humic acid as a model compound of NOM.
It was observed that the substrate led to an improvement of the hydrophilicity of PES membranes modified with enzymes extracted from isolate R. The intense colour of the enzyme modified membranes confirmed the interaction between the enzyme, substrate and membranes. The appearance of the spherically shaped structures on the SEM images of M.5b and M5.c enzyme modified membranes was used as confirmation for the successful immobilization of the enzyme. Moreover, a reduction in the permeability of the modified membranes served as confirmation for the enzyme immobilization, which could have resulted from the reduced porosity (clogged pores) of the membranes. Rejection results suggest that the enzyme modified membranes can be used for the removal of HA and other similar compounds from water since 93.4 % HA removal rate was achieved when the isolate (R) membrane was used. The immobilization of WRF enzymes of polymeric ultrafiltration membranes showed great potential for the removal of organic compounds such as humic acid. It was clear that enzyme activity and membrane transport properties were influenced by surface modification. Thus, this hybrid membrane still needs to be optimized for effective degradation and further work is being conducted to confirm the extent of degradation.

Furthermore, it has been demonstrated that polymer type is important when choosing the immobilisation substrate. The polymer should be favorable to protein adhesion. In addition, the performance of the enzyme depends on the organic matter type and concentration. Traditional pressure driven filtration doesn't allow for efficient breakdown of compounds and it's also difficult to isolate the impact of filtration from degradation.

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Compliance with Ethical Standards

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Conflict of Interest: The authors declare that they have no conflict of interest.

Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study (Reactor design and evaluation).

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