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Special Theme Research Article

Effect of a perfluorocarbon-Pluronic F 68-based emulsion on a *Phanerochaete chrysosporium* biofilm immobilised in a membrane gradostat bioreactor

S. K. O. Ntwampe[1]* *and M. S. Sheldon*[2]

[1]*Department of Agricultural and Food Science, Cape Peninsula University of Technology, Cape Town campus, P.O. Box 652, Cape Town 8000, South Africa*
[2]*Department of Chemical Engineering, Cape Peninsula University of Technology, Cape Town campus, P.O. Box 652, Cape Town 8000, South Africa*

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ABSTRACT: The present study highlights the application of perfluorooctyl bromide (PFOB), an oxygen carrier, in a fixed-film membrane gradostat reactors (MGRs) in which biofilms of *Phanerochaete chrysosporium* BKMF-1767 (ATCC 24725) were immobilised. The nutrient medium used in the MGRs was supplemented with PFOB and Pluronic F 68 (PF 68) to alleviate limitations associated with the performance of the immobilised fungus. Lower lignin peroxidase production in PFOB/PF 68 cultures was observed compared to manganese peroxidase production, suggesting an insignificant generation of reactive oxygen species (ROS). Lipid peroxidation, quantified by the formation of malondialdehyde, was lower in the immobilised cultures. After successfully applying PFOB and PF 68 to immobilised *P. chrysosporium* biofilms in the MGRs, the following results were obtained:[1] reduced ethanol production,[2] reduced trace element accumulation,[3] lower β-glucan production and[4] an improved dissolved oxygen penetration ratio in the immobilised biofilms. © 2009 Curtin University of Technology and John Wiley & Sons, Ltd.

KEYWORDS: lignin peroxidase; manganese peroxidase; perfluorocarbon; *Phanerochaete chrysosporium*

INTRODUCTION

The design of continuous fixed-film membrane bioreactors (MBRs) used to produce extracellular secondary metabolites was found to have superior performance compared to batch cultures. In this type of bioreactor, biofilms are immobilised on the external surface of a capillary membrane, organic or inorganic, while liquid-based nutrients are continuously fed to the membrane lumen side. The retentate is continuously recovered when the bioreactor is operated in a dead-end filtration mode. Low nutrient flux is used such that radial nutrient gradients are established in the immobilised biofilms. This is done to keep a section of the biofilms in the idiophase due to nutrient limitations in the biofilms, which are not close to the nutrient-rich areas of the membrane surface. This condition induces extracellular enzyme production in the immobilised biofilms. These biofilms are also continuously exposed to aeration in the extracapillary space (ECS) to improve dissolved oxygen (DO) transport into the biofilms. This type of MBR was classified as a membrane gradostat reactor (MGR).[1–3] For the MGR to perform optimally, the thickness of the immobilised biofilms needs to be sufficient to maintain a nutrient gradostat such that some of the areas in the biofilms experience nutritional limitations. For batch cultures, several external factors that influence and affect the production of enzymes from *Phanerochaete chrysosporium* biofilms are temperature, pH, DO concentration and a fixed nitrogen concentration. These external factors were optimised in batch cultures, and were adopted to be used in fixed-film bioreactors including the MGR, in which the nutrient medium used was that developed by Kirk *et al*.[4] The optimum pH for lignin degradation was determined as 4–4.5, while lignin degradation was suppressed at a pH above 5.5 and below 3.5. Lignin degradation was non-existent under 5% (v/v) oxygen, while its decomposition was two to three times better under 100% (v/v) oxygen compared to air.[4] The source of nitrogen had limited influence, with the nitrogen composition determined to be effective at a concentration of 24 mM, whereby an increase of 25–35% in lignin decomposition was achieved.[4] The optimum growth temperature for the fungus was determined to be 39 °C, although

**Correspondence to*: S. K. O. Ntwampe, Department of Agricultural and Food Science, Cape Peninsula University of Technology, Cape Town campus, P.O. Box 652, Cape Town 8000, South Africa.
E-mail: ntwampes@cput.ac.za

the optimum enzyme production temperature was determined to be 30 °C.[5]

Generally, the conditions that are associated with immobilised biofilms in MGR are nutrient starvation; continuous exposure to trace element ions from the nutrient medium; hyperthermia and shearing caused by continuous aeration. These conditions are unfavourable for prolonged bioreactor and effective biofilm performance. In this paper, conditions optimised for batch culture studies for the production of lignin peroxidase (LiP) and manganese peroxidase (MnP) from *P. chrysosporium*, which were adopted for use in fixed-film bioreactors are analysed to assess related limitations and the use of a perfluorooctyl bromide (PFOB) and Pluronic F 68 (PF 68) mixture as a possible remedy.

PHANEROCHAETE CHRYSOSPORIUM BIOMASS PERFORMANCE CONSTRAINTS ENCOUNTERED USING THE MGR

P. chrysosporium biofilms have shown to have poor DO transport capabilities.[6] Even though the fungus grows better in air, the production of LiP and MnP is highly dependent on the availability of sufficient quantities of DO. It was further demonstrated that the production of LiP is induced by the presence of reactive oxygen species (ROS) in the biomass of the fungus caused by continuous oxygenation.[7] This led to periodic oxygenation to reduce oxidative stress in the cultures. To induce and improve productivity of LiP and MnP, a combination of aeration and oxygenation was used in the MGR studies.[8–11]

However, prolonged exposure to high partial oxygen pressures in any cell culture leads to oxygen toxicity resulting from an increased generation of ROS, a consequence that does not bode well for prolonged MGR operation. When the fungus was maintained on a glucose-based medium while, simultaneously being exposed to high partial pressures of oxygen, the functioning of mitochondria was impaired,[12] due to the loss of both succinate dehydrogenase and cytochrome oxidase activities. Furthermore, continuous oxygenation was shown to have negligible effects on the increments in DO penetration depth in *P. chrysosporium* biofilms. This led to the conclusion that the immobilised biofilm thickness needs to be controlled for proficient DO transfer and effective penetration depth. This is undesirable for the MGR, as thicker biofilms are required to maintain large sections of the biofilm in the idiophase, in areas away from the membrane surface where there are nutrient limitations. In aerated MGR biofilms, DO penetration depth was dependent on the age of the biofilms. Younger biofilms showed greater DO transfer, while in older biofilms, the transfer of DO was limited.[13] Active biomass near the membrane surface area, where metabolic activity was presumed high due to nutrient availability, was growing under anoxic conditions. As *P. chrysosporium* can survive transient anoxic conditions by producing ethanol, the amount of alcohol produced was determined to be increasing with an increase in biofilm and anaerobic zone thickness. Furthermore, the use of oxygenation resulted in the production of extracellular polysaccharides as the fungus protects itself against oxidative stress, further hampering the transport of DO in the immobilised biofilms. Further research was needed to evaluate methods, which can be used to supply the required quantities of DO. This method should be suitable for the MGR system. Furthermore, it should also not be limited by polysaccharide production and storage observed in immobilised *P. chrysosporium* biomass.

When the nutrient medium for *P. chrysosporium* was developed and optimised for improved fungal performance, the following trace elements were determined to be suitable in batch cultures by Kirk *et al*.[4] and Tien and Kirk[14]: Mg, Mn, Na, Fe, Co, Ca, Zn, Cu, Al and Ca. However, since *P. chrysosporium* has the capacity to absorb and accumulate metal ions including Cu, Mn and Co,[15,16] there is a potential risk of metal accumulation in continuous MGR cultures supplied with this growth medium. As *P. chrysosporium* produces chelators, it was necessary to observe whether the production of chelators reduced trace element ion accumulation in the immobilised fungus with the continuous supply of the nutrient medium to the MGRs. The increase in the polysaccharide mucilage developed to protect cells from oxidative damage was hypothesised as another mechanism, which exacerbates trace element ion entrapment in the biofilm. In the production of extracellular secondary metabolites using batch cultures, some of the identified limitations are not prevalent compared to when continuous MBRs system are used. The relevance of identifying weaknesses in the application of MBRs, while exploring other remedial solutions to enhance the effectiveness in the application of these systems, lies in the fact that continuous systems are being developed to replace semicontinuous and batch bioreactors.

It was observed that the use of the MGR as explained by Leukes *et al*.,[2] with *P. chrysosporium* biofilms has some limitations, as:[1] the DO transport is restricted;[2] oxidative stress is prevalent due to the use of aeration and oxygenation in immobilised biofilms;[3] trace element accumulation and[4] the production of ethanol as the immobilised biofilm forms anoxic regimes. The change in the nutrient medium supply from the bottom to the top of the MGR systems resulted in an increased trace element accumulation and an increased presence of peroxidised lipids.[17] Others have suggested the recycling of the ligninolytic solutions containing secondary metabolites (LiP and MnP) to improve the overall performance of

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the MGR. However, this technique was shown to inhibit and delay extracellular enzyme production when the recovered supernatant was used in freshly inoculated reactors.[18] It is likely that the recycled supernatant was devoid of sufficient quantities of DO to support enzyme production.

THE DEVELOPMENT OF A PERFLUOROCARBON-BASED EMULSION FOR A *PHANEROCHAETE CHRYSOSPORIUM* MGR

A number of features makes perfluorocarbon (PFC)-based emulsions attractive for biotechnological purposes (especially in bioreactors where there is a DO deficiency) including high stability and inertness, gas-dissolving capacity, hydrophobicity and lipophobicity, fluidity and the absence of metabolism. PFCs have a much higher solubility for carbon dioxide; therefore, they can potentially remove toxic gaseous end-products. PFC-based emulsions have been tested and used in clinical trials for medical purposes[19–22] and several bioprocess engineering studies, but not in fixed-film MBRs used for extracellular production of LiP and MnP. It was hypothesised that by introducing a PFC-based emulsion into the MGR system, the DO in the system with the simultaneous removal of metabolic carbon dioxide will greatly improve the functionality of the biofilms.

PF 68 was chosen as the surfactant to be used with the PFCs. The choice of the surfactant for the process was based on previous determinations in which the surfactant was determined to protect cells by coating the membrane of microorganisms, thus directly altering the cell membrane, which resulted in the reorganisation of membrane lipids. The surfactant was determined to affect lipid–lipid and lipid–protein interactions, thereby improving the survival rate of microorganisms by inhibiting damaging interactions between the cell membranes and the cells immediate surroundings, fermentation broth and the air–liquid interface.[23,24] PF 68 was shown to protect and prolong survival of low concentrations of biomass suspensions during nutrient starvation. Furthermore, the surfactant prevented death caused by high concentrations of trace element ions, prolonging the survival of cells exposed to higher ion concentrations of Ca^{2+}, Na^{+} and K^{+}. It was effective in the postponement of death caused by trace element ions such as Zn^{2+}, Fe^{3+} and Cu^{2+} and death caused by shearing forces, while prolonging the survival of biomass exposed to hyperthermia.[25] The surfactant protects cells by regulating the permeability and loss of ions from the affected biomass.[26] These conditions were identified as prevalent when using the MGR.

MATERIALS AND METHODS

Microbial strain and inoculum preparation

P. chrysosporium strain BKMF 1767 (ATCC 24 725) was grown at 39 °C on malt agar slants using a spore inducing medium (SIM) described by Tien and Kirk.[14] The spores from the agar plates were harvested after 7–10 days, by suspending them in sterile water. This was followed by passage through a sterile glass wool to entrap suspended mycelia. The inoculum consisted of an estimated 3×10^6 spores for each reactor. The fungal spores were inoculated through the permeate port and were forced into the membranes microvoids using reverse filtration.[27]

Nutrient medium supplemented with Pluronic F 68

The nutrient medium contained 100 ml of a basal medium solution: 100 ml of 0.1 M 2,2-dimethyl-succinate, 100 ml of 55.5 mM glucose, 100 ml of 0.02 M veratryl alcohol, 60 ml trace element solution, 25 ml of 1.1 mM ammonium tartrate, 10 ml of 10 $mg.l^{-1}$ thiamin-HCl, 1 ml of 10% (w/v) PF 68 and 504 ml autoclaved distilled water.[14] For the control experiments, PF 68 was excluded from the nutrient medium. A solution of 10% (w/v) PF 68 (Hangzhou Onicon Chemical Company Ltd, China) was prepared separately. Furthermore, 1 ml of this solution was added per litre of nutrient medium, thus contributing 0.01% (w/v) of PF 68 in the nutrient medium.

Perfluorooctyl bromide oil

The PFC oil, PFOB (Exfluor Research Corp., USA), was used and supplied separately from the nutrient medium to the reactors. The supply rate of the oil was 1.62 $ml.day^{-1}$ and it was such that it composed ~10% (w/v) concentration fraction of the total suspension (nutrient medium and PFOB oil) supplied to the MGRs. The oil was maintained in a reservoir and aerated daily for 10 min. The PFC was filter sterilised before being added to the reservoir.

Nutrient flow rate, airflow rate and bioreactor setup

The flow rate of the nutrient medium combined with the PFOB was ~1.68 $ml.h^{-1}$ for the PFOB experiment, whereas for the control experiment the nutrient flow rate was 1.68 $ml.h^{-1}$. The mixture was supplied through the lumen of the capillary membrane by using a

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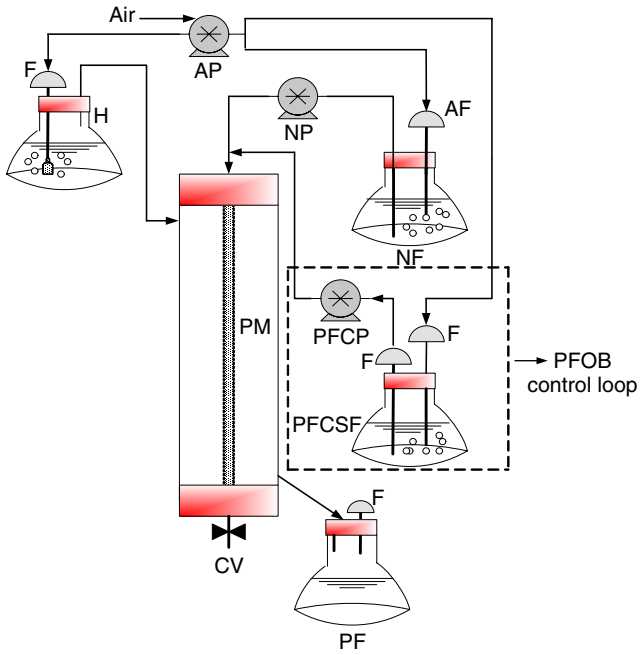

Figure 1. The MGR setup used for application of perfluorooctyl bromide and Pluronic F 68. AF/F, air filter/0.22 μm filter; AP, air pump; CV, closed valve (dead filtration mode applied); H, humidifier; NF, nutrient flask; PF, permeate flask; PFCP, perfluorocarbon pump; PFCSF, perfluorocarbon storage flask and PM, polysulphone membrane. This figure is available in colour online at www.apjChemEng.com.

multi-channel Watson-Marlow 205S pump (Germany) to each MGR system. To avoid sloughing of the biofilm, an airflow rate of 113 ml.min^{-1} was supplied to the ECS of the MGR systems, by using multi-port air pumps with flow control, to ensure that the flow was consistent through all the reactor modules.

Air was also supplied to continuously stirred nutrient flasks to improve DO in the nutrient for the control and PFOB experiments. The air supplied to the reactor systems and nutrient flasks was filter sterilised (using a 0.22 μm acetate non-pyrogenic filter). The air supplied to the ECS of the reactors was humidified before passing it through the shell side. Nine MGRs were used for this study. The MGR design and setup was as shown in Fig. 1. Permeate samples were collected daily, which were used to measure pH; redox potential; residual ammonium/glucose concentration; ethanol production and extracellular enzymes LiP and MnP.

Bioreactor and materials sterilisation

All the materials (tubing, bioreactor and flasks) were autoclaved for 20 min before use. Sterile deionised water was used in the humidifiers and in the nutrient medium. After assembling the MGR modules, the bioreactors were chemically sterilised using a 4% (v/v) formaldehyde solution followed by rinsing with sterile distilled water before inoculation.

ANALYTICAL METHODS

Ammonium tartrate and glucose concentration assays

Ammonium tartrate assays were performed using a Spectroquant Ammonium Test Kit (Merck, Germany) to measure the amount of ammonium tartrate present in the permeate samples collected. Because the suite of ligninolytic enzymes from *P. chrysosporium* are only produced under conditions of nutrient limitation, in this case nitrogen limitation, it was necessary to monitor the effective nitrogen source consumption in relation to operation time. The glucose concentration in the permeate samples was determined by using a Roche® (Germany) D-glucose test kit. The glucose concentration assays were performed in permeates of each MGR system to monitor the usage of glucose during biofilm development.

Lignin peroxidase and manganese production assays

LiP activity was measured according to the method of Tien and Kirk.[28] The oxidation of veratryl alcohol to veratryl aldehyde was recorded at 310 nm for 60 s, with one unit defined as 1 μmol of veratryl alcohol oxidised to veratryl aldehyde per minute ($\varepsilon_{310\ nm} = 9300\ M^{-1}.cm^{-1}$) in 1 ml of reaction volume. The reaction mixture contained 0.2 ml of veratryl alcohol (0.01 M), 0.2 ml of tartaric acid (0.25 M), 0.52 ml of enzyme solution and 0.08 ml of hydrogen peroxide (0.005 M). MnP activity was measured by monitoring the change in oxidation state of Mn^{2+} to Mn^{3+} at 420 nm for 60 s ($\varepsilon_{420\ nm} = 36\,000\ M^{-1}.cm^{-1}$). The reaction solution contained 0.35 ml of reagent A and reagent B, with 0.3 ml of enzyme supernatant in a total reaction mixture of 1.0 ml. Reagent A contained sodium succinate and sodium lactate buffers, each at 100 mM with pH of 4.5, 6 mg.l^{-1} of egg albumin, 0.2 ml of manganese sulphate (0.95 mg.ml^{-1}) and 2.2-azino bis-3-ethyl-benzothiazoline-6-sulfonic acid at 80 μg.ml^{-1}. Reagent B contained 100 μM of hydrogen peroxide.[29] Crude samples recovered from the MGRs were used for enzymatic determinations without purification.

Ethanol determination in permeate samples

The ethanol concentration in the permeate samples was determined by using a Roche® (Germany) ethanol

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test kit. The overall ethanol concentration produced was determined using permeate samples recovered from each MGR. The ethanol concentration was determined by following the assay protocol by absorption at 340 nm.

Determination of dissolved oxygen penetration depth and biofilm thickness

The single capillary membrane gradostat reactor (SCMGR) systems were dismantled by removing the glass manifold and exposing the membrane-attached biofilm for easy microsensor measurement. The bioreactors were disconnected in triplicate after 192 h of MGR operation. A Clark-type oxygen microsensor (OX 10, outer tip diameter less than 20 μm) supplied by Unisense (Denmark) was used to measure the DO across the biofilms at interval depths of 50 μm. The setup consisted of a high sensitivity picoammeter connected to the microsensor, which was fixed to a micromanipulator that was used to move the microsensor into the biofilm. The picoammeter was connected to a computer loaded with Profix v1.0 software for data capturing. The microsensors were used to measure the DO concentrations in duplicate from three different MGRs. Measurements were performed at the top and bottom of the active membrane length.

Biofilm thicknesses were determined at the top and bottom of the active membrane length at the same location where the DO profiles were measured. The biofilm thickness was determined by using a Carl Zeiss light microscope and Axiovision digital imaging system equipped with measuring software. The objective used for the measurements was a 2.5× magnification. The calibrated microscope objectives acquired real-size measurements from biofilms attached to the polysulphone membrane surface. The biofilm thickness was calculated by subtracting the clean membranes outer diameter from the membranes with biofilm growth. The value obtained was divided by two and averaged to obtain the actual biofilm thickness.

Accumulation of trace element ions in immobilised biofilms

Dried fungal biomass between 37.9 and 80.1 mg, obtained from MGRs disconnected after 192 h, was weighed into digestion vessels and decomposed in a mixture of 4 ml of a 65% HNO_3 solution and 1 ml of a 30% H_2O_2 solution.[30] The digestion was accomplished using the following microwave program: 7 min at 120 W, 2 min at 460 W and at 700 W for 1 min. The samples were cooled for 1 h and the digests were filtered using glass wool and a 0.22-μm filter. The volume was made up to 30 ml with deionised water. The digests were analysed at the University of Stellenbosch (R.S.A), using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS) for the determination of copper (Cu) and manganese (Mn) in the digested filtrate. Control samples contained only solutions of nitric acid and hydrogen peroxide.

Measurement of oxidative damage determined as malondialdehyde formation

The formation of malondialdehyde (MDA) was determined with an MDA assay kit (Northwest Life Sciences Specialties, Vancouver, WA), following the manufacturers directions. Preserved biofilms stored at −80 °C were homogenised with glass beads in 2 ml Eppendorf tubes using a vortex mixer until the mixture was well homogenised. The biofilm homogenates were centrifuged at $10\,000 \times g$ for 10 min to obtain a clear supernatant before being used in the assay. The MDA in homogenates was determined by following the assay protocol by absorption at 532 nm.

After DO measurements, a section of the biofilm was surgically separated and rinsed in a 5 mM butylated hydroxyl toluene (BHT) solution supplemented with a 20 mM hydroxymethyl amino methane–hydrochloride (Tris–HCl), before storing at −80 °C in the Tris–HCl solution. This was done to prevent new lipid peroxidation during biofilm storage. This solution was also used in the homogenisation and biomass lysis process for MDA and glucan determinations.

Extracellular polysaccharide storage measured as glucan in immobilised biofilms

Glucan concentration in homogenates was measured using a mixed linkage β-glucan assay kit acquired from Megazyme (Ireland). The supernatant used for MDA determination was also used for glucan determination. The concentration of β-glucans in homogenates was determined by following the assay protocol by absorption at 510 nm using 100 μl of the homogenised biofilms.

RESULTS AND DISCUSSION

Ligninolytic activity and biomass performance of *P. chrysosporium* using PFOB oil in combination with a PF 68-supplemented nutrient medium in the MGR system were evaluated. The application of the mixture to the MGRs was conceptualised to increase

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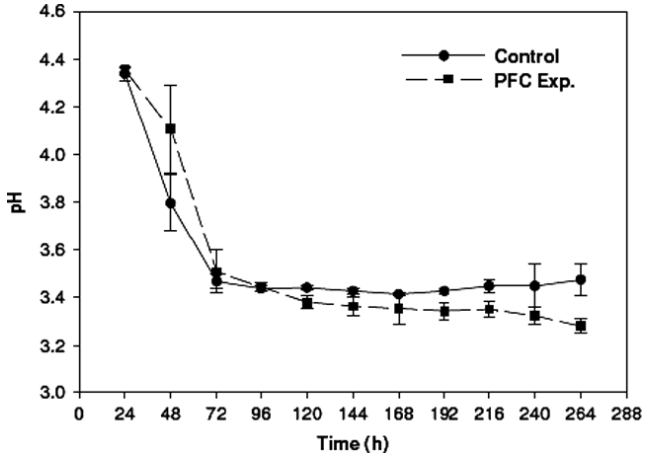

Figure 2. Average pH profile observed for permeate collected from MGRs in which PFOB and PF 68 were used.

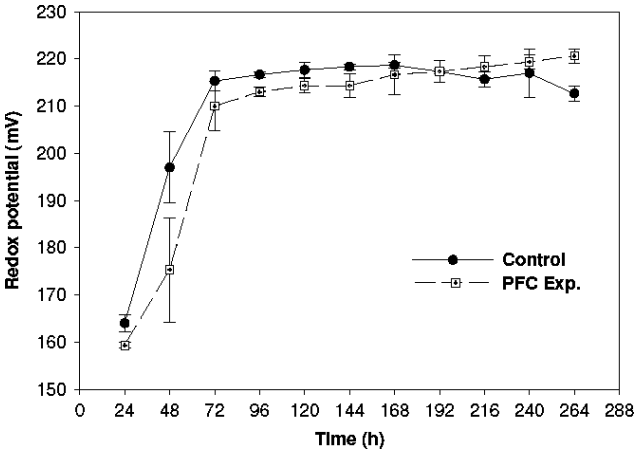

Figure 3. Average redox potential of permeate samples collected from the MGRs indicating active enzyme production by immobilised biofilms.

and improve DO in the immobilised biofilms, to improve enzyme production from the biofilm during the secondary metabolic stage, while alleviating identified limitations.

Monitoring of pH and redox potential in the permeate

Figure 2 shows that a similar trend in the reduction of pH was observed between the permeate collected from the MGRs for control experiments and the experiments in which PFOB/PF 68 was utilised. After 72 h, the average pH of control experiments was ~3.8, while the pH for PFOB/PF 68-based MGRs was ~4.15. This was expected as the fungus adjusted to the presence of the PFC oil and the surfactant in the bioreactor. It was previously determined that MnP and LiP had stable pH ranges of 2.6–4.5 and 2.0–5.0, respectively.[31] Overall, the bioreactors operated within the range suitable for MnP and LiP production, and the pH range in which LiP and MnP are stable.

The redox potential of purified MnP and LiP were determined to be 1510 and 1450 mV, respectively.[31] By monitoring the redox potential of recovered permeate, the presence of ligninolytic enzymes could be forecasted with the increase in the redox potential of the permeate. An increase in the redox potential shows the presence of enzymes in the permeate, while a decrease or stagnant redox potential shows inefficient enzyme production. Figure 3 shows the redox potential of the PFOB/PF 68 emulsion compared with that of control MGRs. The redox potential of the control and PFC-based experiments was greater than 200 mV after 72 h of MGR operation. The performance of the PFOB/PF 68 bioreactors showed a higher redox potential than control experiments from 216 to 264 h. During the same period, the averaged redox potential of control experiments declined. This suggested that the PFOB/PF 68-based MGRs might be suitable for prolonged operation as a steady trend of increasing averaged redox potential was observed.

Glucose and ammonium consumption in the MGRs

The average glucose concentration measured in the nutrient solution using an enzymatic assay kit was 10.5 $g.l^{-1}$ compared to the theoretical 10 $g.l^{-1}$. The ammonium (NH_4^+) concentration in the nutrient feed was determined as 39.7 $mg.l^{-1}$ compared to the theoretical 39 $mg.l^{-1}$. The production of peroxidases by *P. chrysosporium* takes place as an extracellular process using mechanisms dependent on nutrient availability.[32] The production of enzymes, LiP and MnP, can be induced by nitrogen and carbohydrate (carbon source) limitation in the immobilised biofilms.[33] As shown in Fig. 4, the ammonium (ammonium tartrate) source was depleted after 72–96 h of MGR operation, representing a 95% depletion.

The rapid consumption of ammonium source obtained in this study was similar to those determined in batch cultures by Keyser *et al*.[34] and Rothschild *et al*.[35]. Ammonium depletion after 72–96 h coincides with an increase in redox potential, which was determined to be above 200 mV after 72 h.

The addition of PFOB and PF 68 to the MGRs had a negligible effect on the consumption of glucose as shown in Fig. 5, when comparing the control MGRs to the PFOB/PF 68-based MGRs. These results were in contradiction with results obtained from batch cultures, where the addition of PFOB resulted in an increase in glucose consumption (unpublished data). A difference was observed in the periods of 24–144 h, where control cultures showed higher glucose consumption when compared to PFOB/PF 68 MGR cultures.

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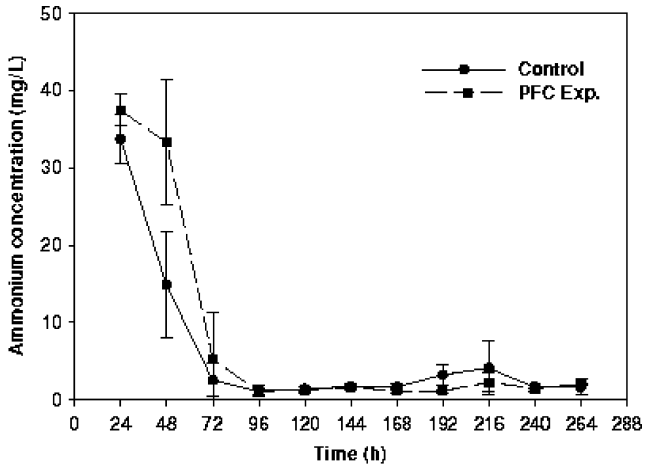

Figure 4. Ammonium (NH_4^+) concentration in the permeate samples collected from MGRs operated for 264 h.

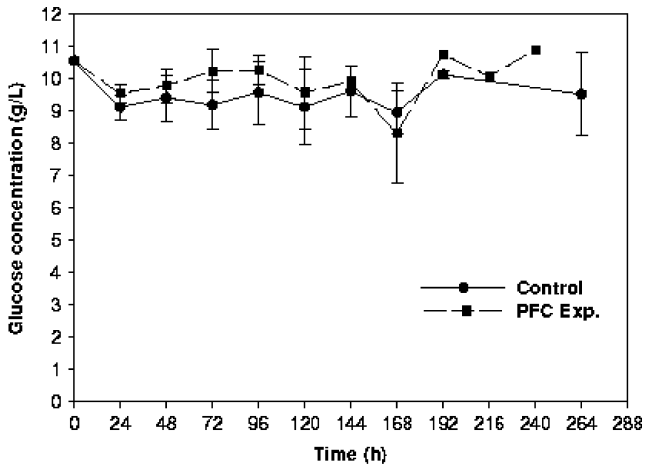

Figure 5. A comparison of averaged glucose consumption for PFOB-based MGRs and control cultures.

Glucose consumption averaged at 0.7 g.day^{-1} for PFOB MGRs compared to 1.27 g.day^{-1} for the control cultures, representing a 55% decline. This was attributed to the presence and continuous supply of PFOB/PF 68 to the MGRs, which reduced the amount of biomass generation at the bottom of the reactor, while higher biomass increases were observed at the top of the bioreactor. This phenomenon was not observed previously in MGR studies.

Determination of biofilm thickness in PFOB/PF 68-based MGRs

As biofilm thickness can be used to monitor the growth kinetics and activity of microorganisms in MBRs, the thickness of *P. chrysosporium* was monitored to determine the effect of PFOB and PF 68 on the immobilised fungus. As the design of the MGRs was changed to improved nutrient medium and PFOB/PF 68 hydraulic residence time, the growth of the fungus was evenly distributed in control cultures. However, the introduction of PFOB and PF 68 resulted in an increase in the biofilm thickness at the top of the bioreactor. In control MGRs, averaged biofilm thickness was ~2250 μm, which was less than the biofilm thickness at the top of the PFOB and PF 68-based bioreactors that was determined at an average of ±2516 μm. The observed biofilm reduction at the bottom of the MGRs was attributed to the settling of PFOB at the bottom of reactor, as high concentrations of PFCs were observed to reduce biomass production in batch cultures.

Dissolved oxygen distribution in immobilised biofilms

DO in the PFOB and PF 68-based immobilised biofilms was determined at 192 h, both at the top (thicker biofilms) and at the bottom (thinner biofilms) in the MGRs. The averaged DO penetration depth for biofilms at the top of the reactor was 250 μm. From the DO penetration depth and biofilm thickness obtained, the presence of anaerobic zones in biofilms at the top of the MGRs was irrefutable. Previously, the oxygen penetration ratio, which is the ratio between DO penetration depth and biofilm thickness obtained after 192 h of MGR operation was determined as <0.3. However, the introduction of PFOB resulted in an improved DO penetration ratio of more than 0.36 for biofilm at the bottom of the MGRs, while penetration ratio of 0.21 was determined in thicker biofilms (at the top of the MGRs). The high variability of DO profiles obtained at 192 h at the top of the bioreactors was attributed to differential biofilm heterogeneity caused by shearing, as the area was an entry point for the humidified air. The shape of the DO profiles at the top of the MGRs, indicated limited oxygen consumption, and was assumed that *eddy* currents with the assistance of the PFOB were the driving force of DO presence and distribution in the biofilms. However, at the bottom of the reactors, higher DO penetration depth in excess of 650 μm was observed that was an improvement from an averaged maximum DO penetration depth of less than 550 μm, determined previously in MGRs. As shown in Fig. 6, unconventional DO profiles were obtained at the bottom of the reactor, indicating that the DO concentration started increasing at penetration depths exceeding 650 μm, which was not previously observed in profiles obtained at the top. This further ratified the reasoning that PFOB settling in the lumen of the MGRs was prevalent.

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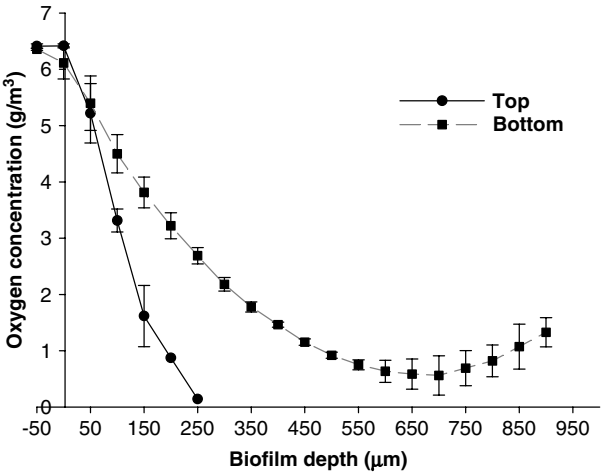

Figure 6. Dissolved oxygen distribution in PFOB/PF 68-based MGRs after 192 h.

Continuous ligninolytic enzyme production in perfluorooctyl bromide MGRs

Crude samples were used for the determination of LiP and MnP, without purification. Generally, the production of low extracellular enzymes LiP and MnP (shown in Fig. 7A,B) was observed. As LiP was determined to be influenced and induced by ROS,[7] the production of LiP might have been hampered by the reduced presence of ROS. MnP production was higher in PFOB/PF 68 MGRs than LiP. LiP production was higher in control cultures compared to when PFOB/PF 68 was used in the MGRs.

Contribution of the PFOB/PF 68-based medium to immobilised biofilm performance

Table 1 is a summary of results obtained using the PFOB/PF 68-based medium, in relation to the immobilised biofilm performance after 192 h, which were compared to parameters obtained by Ntwampe *et al*.[17]

As DO limitation in *P. chrysosporium* cultures leads to ethanol production, the determination of ethanol production was of paramount importance, as illustrated in Table 1. Even with aerated nutrient medium, ethanol production was similar to cultures in which aeration was applied only to the ECS of the MGRs. A reduction in ethanol production was determined for the PFOB/PF 68 experiments. Ethanol production increased but the concentration was less than 1 $mg.l^{-1}$. This was observed during the period 144–192 h, which is a period previously identified as a phase characterised by decelerated and stagnating growth for *P. chrysosporium* immobilised in the MGR.[36,37]

Furthermore, the values of glucan concentration were observed among both sets of studies, with glucan storage reaching 1.2 $mg.ml^{-1}$ after 192 h of MGR operation. Therefore, the change in the design to accommodate and improve nutrient distribution including hydraulic residence time for the medium in immobilised biofilms can increase glucan storage. However, the availability of PFOB and PF 68 contributed to a reduction in the presence of glucan. As glucan restricts DO transport in mycelia, the availability of PFOB/PF 68 should further counteract the resistance to transfer DO, as shown in this study.

Increased polysaccharide storage was observed to entrap trace element ions. Manganese (Mn) and copper (Cu) ion accumulation was monitored during this study. As expected, by increasing the nutrient hydraulic residence time in the biofilms, there was a noticeable increase in Mn and Cu accumulation compared to cultures studied previously. Reduced amounts of Mn and Cu were observed in cultures where a PFOB/PF 68-based medium was used.

The presence of lipid peroxidation was evident as measured by the formation of MDA. In the case of

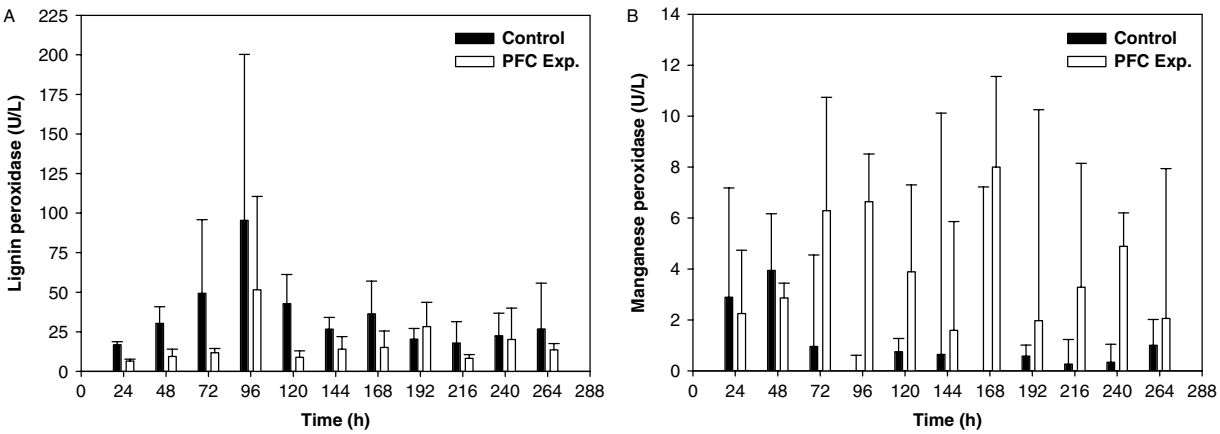

Figure 7. Lignin peroxide and manganese peroxidase production determined from crude samples obtained in MGRs supplied with PFOB/PF 68.

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Table 1. Significant contribution of the PFOB/PF 68-based nutrient medium on the performance of immobilised *P. chrysosporium* biofilms in MGRs after 192 h.

Parameter	Dimension/quantity		Comment/consequence
	Control	PFOB/PF 68	
Ethanol production due to anoxic conditions	5–10 $mg.l^{-1}$	<1 $mg.l^{-1}$	Reduced ethanol production was detected in permeate samples collected, with ±1 mg/l ethanol production during 168 and 192 h, which was identified as a stationary phase of immobilised biofilms in the MGR.
Oxidative damage (MDA/ml homogenised biomass)	28 $\mu M.ml^{-1}$ control	26 $\mu M.ml^{-1}$	Reduced lipid peroxidation was observed in the biofilms representing a ~7% reduction.
Cu/Mn accumulation (mg per g dry biomass)	±13 mg/±68 mg	±9.2 mg/±29.7 mg	Cu accumulation is associated with oxidative damage as it induces ROS generation. Significant reduction in Cu can improve prolonged biomass performance. Even though minute concentration of Mn^{2+} prevents chemically supported lipid peroxidation, high concentrations hamper biofilm development in continuous MBRs.
β-Glucan storage	2 $mg.ml^{-1}$	1.2 $mg.ml^{-1}$	Overall reduction of β-glucan is desirable as it restricts DO transport. The presence of the polysaccharide can also entrap trace element ions, further exacerbating ROS generation. The application of PFOB/PF 68 was shown to reduce glucan storage in the immobilised biofilms.

MGR operating conditions: 1 atm, aerated (21% v/v O_2), 192 h bioreactor operational time, continuous nutrient feed, dead-end filtration mode.

MDA formation, the orientation of the nutrient supply line had negligible effect to lipid peroxidation in the MGRs. The application of 10% (w/v) PFOB and 0.01% (w/v) PF 68 to the MGRs showed a small but significant improvement in terms of MDA presence in the harvested biofilms. MDA formation in the control cultures was 28.26 $\mu M.ml^{-1}$ of homogenised biomass in control cultures and 26.34 $\mu M.ml^{-1}$ of homogenised biomass in PFOB/PF 68 cultures was observed after 192 h of operation.

CONCLUSIONS

The application of PFOB and PF 68 to the MGRs contributed a small but significant difference into the performance of the immobilised biofilm in these bioreactors. The reduction in pH and an increase in the redox potential were similar for control experiments in which a defined medium was used. Glucose and ammonium consumption was higher in control experiments during the initial stages of the bioreactor operation, as *P. chrysosporium* adjusted to the presence of PFOB in the experiment. DO distribution was higher at the bottom of the reactor, where increased DO availability was measured to improve towards the membrane surface with anaerobic zones observed in biofilms immobilised at the top of the membranes in the reactors. Although anaerobic zone formation was evident, the DO penetration and distribution was improved, resulting the reduction of ethanol production and improved MnP production. LiP presence in control cultures was high compared to PFOB/PF 68 cultures. This attributed to a low generation of ROS corroborated by less MDA formation in PFOB/PF 68 cultures, for which MDA quantification was used as a measure for lipid peroxidation for the cultures. The presence of glucan was reduced when a PFOB/PF 68 medium was used in immobilised cultures. From the results, the application of PFOB and PF 68 to the immobilised biofilms resulted in the reduction of both Cu and Mn ion presence.

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