Influence of Perfluorocarbons on *Phanerochaete chrysosporium* Biomass Development, Substrate Consumption and Enzyme Production

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Limitations placed on aerobic cultures by dissolved oxygen can be alleviated by using oxygen-vectors such as perfluorinated organic oils. In this study, *Phanerochaete chrysosporium* was tested in batch cultures to evaluate the efficacy of perfluorinated oils in improving biomass development, nutrient consumption and extracellular enzyme production. The oils used in an emulsified form in this study were perfluorotripropylamine, perfluorooctyl bromide and bis-(perfluorobutyl) ethene, as they dissolve higher quantities of oxygen compared to others. The surfactant used to stabilise the emulsion was Pluronic F 68, which was used at a concentration of 8.5 % (w/v). The addition of only the surfactant to the medium resulted in a 6.9-fold increase (1.38 mg mL^{-1}) in biomass concentration of *P. chrysosporium*, while a biomass concentration of 0.82 to 1.53 mg mL^{-1} (4.1 to 7.6-fold increase) was observed in the PFC emulsions with an oil-phase fraction in the range of 0.1 to 0.3 (w/v), i.e. 10 to 30 % (w/v). The highest enzyme activity from crude samples was 307 and 410 U L^{-1} for LiP and MnP, respectively, compared to <100 and <50 U L^{-1} enzyme activity for LiP and MnP obtained in control cultures, respectively. Ammonium and glucose utilisation was higher in emulsions with PFC concentrations of 10 % (w/v) for all the perfluorocarbons evaluated than at concentrations of 20 % and 30 % (w/v). It was concluded that the use of the perfluorocarbon emulsions improved the performance of *P. chrysosporium* BKMF 1767 in terms of biomass development, nutrient consumption and enzyme production. Furthermore, PF 68 was found to have growth-promoting abilities for *P. chrysosporium*.

Key words:
Lignin peroxidase, manganese peroxidase, perfluorocarbon, *Phanerochaete chrysosporium*

Introduction

Researchers have screened a large number of *Basidiomycetes* for their ability to degrade lignin and various pollutants in industrial wastewater. The ligninolytic system of the white-rot Basidiomycete, *Phanerochaete chrysosporium*, consists of an extracellular oxidative process initiated by nitrogen, carbohydrate and sulphur limitation.[1] Under ligninolytic conditions the fungus produces extracellular enzymes, lignin peroxidase (LiP) and manganese peroxidase (MnP), which were found to exhibit high rates of lignin degradation.[2,3] Other critical external factors conducive to the improved production of LiP and MnP are temperature, pH and dissolved oxygen (DO) concentration.[2–7]

The conditions necessary for the production of LiP and MnP can be better exploited in continuous fixed-film membrane bioreactors (MBRs), as a section of the biofilm is kept in the idiophase, while the products can be continuously recovered.[8–12] However, in fixed-film MBRs conditions related to nutrient starvation, continuous exposure to trace element ions from the nutrient medium, hyperthermia and shearing forces caused by continuous aeration, prevail. These conditions are unfavourable for prolonged bioreactor- and biomass performance. Furthermore, *P. chrysosporium* biomass has been determined to have poor DO transfer capabilities.[13] This is due to glucan production and storage by *P. chrysosporium* biomass,[14] as a defence mechanism to protect itself against oxidative stress,[15] resulting from the use of high partial pressures of oxygen and continuous aeration. In addition, the ability of the fungus to adsorb and accumulate metal ions can exacerbate and limit overall biomass performance in fixed-film MBRs that are operated for prolonged periods.[16] To overcome these limitations, the use of DO-carrying perfluorinated organic oils (perfluorocarbons/PFCs) emulsified in a poloxamer, Pluronic F 68 (PF 68), to promote growth and enhance the performance of *P. chrysosporium* biofilms, was investigated in this study.

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The use of PFCs decreases mechanical damage to biomass caused by conventional aeration through sparging or continuous stirring in the batch cultures. Controlled concentrations of DO can be used in the PFC emulsions, thus reducing oxidative stress to the immobilised biomass.[17,18] The use of PF 68 as the surfactant was prompted by evidence suggesting that it prolonged the survival of *Tetrahymena* cells during nutrient starvation, exposure to higher concentrations of trace elements and hyperthermia,[19] conditions identified to be prevalent in continuous fixed-film MBRs.[11] Typical surfactant levels used in a PFC-based emulsion had been 1 to 5 % (w/v) for water-in-oil emulsions and 5 to 10 % (w/v) for oil-in-water emulsions.[20] A surfactant (PF 68) concentration in the nutrient medium of ~8.5 % (w/v) was therefore selected for this study. The primary objective of this part of the study was to evaluate the compatibility of three PFCs, perfluorotripropylamine (PFPA), bis-(perfluorobutyl) ethene (PFBE) and perfluorooctyl bromide (PFOB) with *P. chrysosporium*. Furthermore, the effect of the emulsions on biomass development, substrate consumption and enzyme production was assessed.

Materials and methods

Microbial strain and inoculum preparation

P. chrysosporium strain BKMF 1767 (ATCC 24725) was grown at 39 °C on malt agar slants using the maintenance and spore inducing medium (SIM) described by Tien and Kirk.[21] The spores from agar plates were harvested after 7 to 10 days and prepared by suspension in sterile water. This was followed by passage through sterile glass wool to entrap suspended mycelia. The inoculum suspension used in each flask consisted of approximately $3 \cdot 10^6$ spores.

Nutrient medium (continuous phase)

The nutrient medium contained 100 mL of a basal medium solution, 100 mL of 0.1 mol L^{-1} 2,2-dimethylsuccinate, 100 mL of 55.5 mmol L^{-1} glucose, 100 mL of 0.4 mol L^{-1} veratryl alcohol, 60 mL trace element solution, 25 mL of 1.1 mmol L^{-1} ammonium tartrate, 10 mL of 10 mg L^{-1} thiamin-HCl, 376 mL of 22.5 % (w/v) PF 68 and 129 mL autoclaved distilled water.[21] The final concentration of PF 68 in the nutrient medium was ~8.5 % (w/v). For the control experiments, PF 68 was excluded from the nutrient medium and replaced with 376 mL of autoclaved distilled water.

Pluronic F 68 preparation

Pluronic F 68 (PF 68) has been widely used as a cytoprotectant and growth-promoting additive to animal cell and microbial cultures.[22] Hangzhou Onicon Chemical Company LTD (China) supplied the surfactant. The concentration chosen for the preparation of the emulsions was ~8.5 % (w/v).

Perfluorocarbon preparation

Perfluorocarbons, perfluorotripropylamine (Keensun Trade Company LTD, China), perfluorooctyl bromide (Exfluor Research Corp., USA) and bis-(perfluorobutyl) ethene (Oakwood Products Inc., USA) were chosen for the experiments as they exhibit high solubilities for oxygen compared to water, as shown in Table 1. PFC fractions of 0.1 to 0.3 (w/v), i.e. 10 to 30 % (w/v), were used for PFPA and PFOB, while fractions of 0.1 to 0.2 (w/v) were used for PFBE. All PFC emulsions were prepared in an ~8.5 % (w/v) PF 68-based nutrient medium. Appropriate quantities of PFCs were filter sterilised using a 0.22 μm filter, before adding them to the nutrient medium (continuous phase). The PFC emulsions were oxygenated for 10 min before use. The emulsification process for the emulsion was that illustrated by Floyd.[16] The initial and final DO concentration in the culture medium was measured using a DO sensor (Unisense, Denmark). The data obtained was then used to determine the DO accumulated in the broth in order to account for DO enhancement. The PFCs were recovered at the end of the experiment by centrifugation at 4000 rpm for 10 min, as explained by Elibol and Mavituna.[23]

Table 1 – *Properties of PFC liquids compared to those of water at standard pressure and temperature*[18]

Liquid	Dissolved oxygen/ mmol L^{-1}	Density/25 °C	Boiling point
Water	2.2	1.0 g mL^{-1}	100 °C
Perfluorotripropyl-amine	39.6	1.82 g mL^{-1}	130 °C
Perfluorooctyl bromide	44.0	1.93 g mL^{-1}	142 °C
Bis-(Perfluorobutyl) ethene	44.0	1.41 g mL^{-1}	60 °C

Culture conditions

Twelve stationary 250 mL Erlenmeyer flasks containing 15 mL of the emulsions were used for each experiment. The emulsions were oxygenated with ~100 % technical-grade oxygen for 10 min.

The flasks were sealed with rubber stoppers and incubated at 39 °C; the only source of DO was from the PFCs. Mechanical agitation and shear stress have been shown by researchers to deactivate the ligninolytic system,[3,4] therefore, the flasks were not agitated for 192 h. Thereafter, the flasks were shifted to a shearing environment for 24 h using a shaking incubator at 150 rpm, in order to monitor the protective effects of the emulsions on the extracellular enzymes produced. During sampling, 1.5 mL samples were taken daily from three different flask for two consecutive days (48 h), with subsequent flasks used thereafter.

Analytical techniques

Enzyme activity

A determination of the enzyme activity was made at 25 °C using crude permeate samples; i.e. the extracellular fluid used in the assay was not concentrated through ultrafiltration. Enzyme activities were measured in control cultures and compared with activities obtained in surfactant and PFC emulsion-based experimental runs, respectively.

Lignin peroxidase activity

LiP activity was measured according to the method used by Tien and Kirk.[21] The oxidation of veratryl alcohol to veratryl aldehyde was recorded at 310 nm for 60 seconds, with one unit being defined as 1 μmol of veratryl alcohol oxidised to veratryl aldehyde per minute in 1 mL of reaction volume ($\varepsilon_{310\ nm} = 9300\ L\ mol^{-1}\ cm^{-1}$). The reaction mixture contained 0.2 mL of veratryl alcohol (0.01 mol L^{-1}), 0.2 mL of tartaric acid (0.25 mol L^{-1}), 0.3 mL of enzyme solution, 0.22 mL of distilled water and 0.08 mL of H_2O_2 (0.005 mol L^{-1}).

Manganese peroxidase activity

MnP activity was measured by monitoring the change in the oxidation state of Mn^{2+} to Mn^{3+} at 420 nm for 60 s ($\varepsilon_{420\ nm} = 36000\ L\ mol^{-1}\ cm^{-1}$). The reaction solution contained 350 μL of reagent A and reagent B, with 300 μL of enzyme supernatant in a total reaction mixture of 1.0 mL. Reagent A contained sodium succinate, sodium lactate buffers, each at 100 mmol L^{-1} and pH 4.5, 6 mg L^{-1} of egg albumin, 200 μL of $MnSO_4$ (0.95 mg mL^{-1}) and ABTS at 80 μg mL^{-1}. Reagent B contained 100 μmol L^{-1} of H_2O_2.[24]

Determination of ammonium and glucose concentrations in crude samples

A Spectroquant Ammonium Test Kit (Merck, Germany) was used to measure the amount of ammonium present in the broth at different times. Because the suites of ligninolytic enzymes from *P. chrysosporium* are only produced under conditions of nutrient limitation, in this case nitrogen limitation, it was necessary to monitor ammonium consumption. The concentration of glucose in the samples was determined by using a Roche® D-glucose test kit (AEC Amersham, R.S.A). Glucose consumption was evaluated to monitor different consumption rates in flasks. The concentration of ammonium and glucose obtained at different times in the broth was subtracted from the initial concentration in the nutrient medium to yield the corresponding consumption.

Dry biomass weight

The generated mycelium was separated by using a screen to separate the pellets from the supernatant after the agitation process. The pellets were washed twice with sterile distilled water. The pellets were dried at 60 °C until the dry weight was constant. The dry biomass weight was determined after 216 h, at the end of the experiment, for all the Erlenmeyer flasks.

Biomass enhancement factor

In any study of enhancing oxygen supply in bioreactors through the addition of PFC emulsions, the effects of the emulsion can be calculated using the enhancement factor, *E*, which is a ratio between biomass generated in bioreactors with and without the PFC emulsion.[25]

$$E = \frac{\text{Biomass generated (PFC emulsion)}}{\text{Biomass generated (control without PF 68)}} \quad (1)$$

Results and discussion

P. chrysosporium cultures were studied in batch flasks for two major reasons: Firstly, to evaluate the compatibility of the fungus with PF 68 and the DO carrying perfluorinated water-immiscible oils (PFPA; PFBE; PFOB). This was because *P. chrysosporium* mycelia were identified to have poor DO transport capabilities even when high partial pressures of oxygen are used. Secondly, to evaluate the overall performance of the *P. chrysosporium* biomass in terms of actual biomass development, nutrient consumption and extracellular LiP and MnP production in order to identify a suitable PFC and concentration.

P. chrysosporium biomasses were grown in three types of nutrient medium: Type 1, was the traditional nutrient medium designed by Tien and Kirk,[21] without any surfactants or PFCs, Type 2 was

the same medium supplemented with ~8.5 % (w/v) PF 68 and Type 3 was the nutrient medium supplemented with different fractions of PFCs using PF 68 as a surfactant. The first two types, without the PFCs, served as the control experiments. All the results of all the assays were calculated as an average of the three parallel flasks.

Effects of Pluronic F 68 on *P. chrysosporium* biomass

PF 68 is a neutral polymer and does not have any nutritional functions for *P. chrysosporium*. However, Table 2 shows that the addition of the polymer increased the biomass from 2.5 mg to above 19.5 mg in the batch cultures. The biomass enhancement factor (*E*), in terms of biomass generated in the PF 68-supplemented medium without PFCs, was determined as 6.9 (Table 2). PF 68 has been identified as a surface-active poloxamer that modifies cellular membranes, thus modifying cellular metabolism events.[26] Although low conidia concentrations of the fungus were used as an inoculum in this study, PF 68 was found to increase the rate at which essential nutrients are utilised by the fungus by modifying the cellular membranes of *P. chrysosporium* mycelia and thus increasing the rate of nutrient usage and biomass development.

Furthermore, it had previously been shown that surfactants with high hydrophilic-lipophilic balance (HLB) values provided significant protection to biomass against shearing effects, while those with low HLB values promoted cell lysis and growth inhibition.[26] Previously, Tween 80 had been shown to have protective effects on LiP and MnP by reducing enzyme deactivation in agitated cultures of *P. chrysosporium*. However, Tween 80 was shown to have only short-term protective effects (Tween has an HLB value of 15, while PF 68 has an HLB value of 24 to 29). As researchers try to develop continuous processes for prolonged LiP and MnP production, non-ionic surfactants such as Tween 80 are normally used as supplements in the nutrient medium. However, they have not been proven to have the capacity to protect immobilised biomass. The enhancement of biomass performance by Tween 80, during conditions where *P. chrysosporium* biomass was continuously exposed to high concentrations of trace element ions, hyperthermia, and nutrient starvation, was not evaluated when studies of Tween 80 were performed in order to evaluate the surfactants effectiveness and suitability for prolonged continuous bioreactor operation.

Table 2 – *Enhancement factor of P. chrysosporium biomass in different culture media supplemented with perfluorocarbons*

Culture medium	Dry biomass formed/mg	Biomass enhancement/*E*	DO enhancement
Control (pure medium)	2.5 ± 0.5	–	–
Control (PF 68)	21.5 ± 3.5	6.9	–
0.1 (w/v) PFPA	18.5 ± 2.5	6.3	6
0.2 (w/v) PFPA	13.5 ± 2.5	4.7	6
0.3 (w/v) PFPA	12.5 ± 3.5	4.1	6
0.1 (w/v) PFOB	20.5 ± 3.5	6.4	6.5
0.2 (w/v) PFOB	14.0 ± 2.0	4.6	7
0.3 (w/v) PFOB	12.5 ± 3.5	4.3	6
0.1 (w/v) PFBE	23.5 ± 4.5	7.6	5.5
0.2 (w/v) PFBE	13.5 ± 2.5	4.7	6.5

P. chrysosporium biomass development in emulsified perfluorocarbons

Table 2 shows a representation of dry biomass achieved in the various media during this study. Overall, biomass development decreased with increasing PFC fractions in the emulsions. A biomass reduction of >45 % was observed as PFC fractions were increased from 0.1 to 0.3 (w/v). The same phenomena was observed by Elibol and Mavituna[23] when they increased the fraction of PFCs in the fermentation medium used in their experiments. One example was that of Actinorhodin production, where *Streptomyces coelicolor* biomass decreased as the fraction of perfluorodecalin was increased from 0.1 to 0.5 (w/v).[23] In this study, low concentrations of *P. chrysosporium* conidia were shown to have poor growth capabilities with increased biomass achieved using PFC emulsions, although PF 68 was identified as the main growth promoter for the cultures. Higher biomass growth was achieved using a fraction of 0.1 (w/v) compared to 0.2 and 0.3 (w/v) for all three PFCs. For a fraction of 0.1 (w/v), PFPA and PFOB produced similar biomass, with the highest biomass achieved using PFBE. Although the increase in PFC fractions decreased the biomass actually formed, the biomass formed in the 0.3 (w/v) emulsions still represented an excess biomass of 400 % compared to control cultures without ~8.5 % (w/v) PF 68. Values of the enhancement factor, (*E*), using the different media and emulsions are listed in Table 2. The values of biomass generation decreased with an increase in the PFC fraction in the medium. The highest *E* value of 7.6 was achieved with 0.1 (w/v) PFBE. It was observed that, after mechanical agitation for 24 h, the PFC phase attached to the formed biomass, leaving a clear continuous phase compared to

the milky emulsions observed before agitation. Furthermore, during the period of oxygenation, the DO in the culture broth was enhanced by 5.5 to 7 fold, when compared to the DO quantities achieved in control cultures. These results showed that the culture broth's DO carrying capacity can be improved by the addition of the PFC's.

Ammonium and glucose consumption

Fig. 1 shows that the control cultures supplemented with PF 68 only also performed better in terms of glucose consumption compared to control cultures without the surfactant during 144 to 216 h, with similarities from 0 to 144 h. This was expected, as an increase in the biomass formed was significant in PF 68-based control cultures. The ammonium concentration in the control cultures was above 30 mg L^{-1}, even after 216 h of biomass incubation, further supporting evidence that poor DO availability led to poor overall nutrient consumption and biomass development. Significantly, 0.2 (w/v) PFPA cultures had a rapid ammonium consumption rate, whereby >80 % of the ammonium was consumed after 48 h even though the biomass enhancement factor was lower than that of 0.1 (w/v) PFPA emulsion.

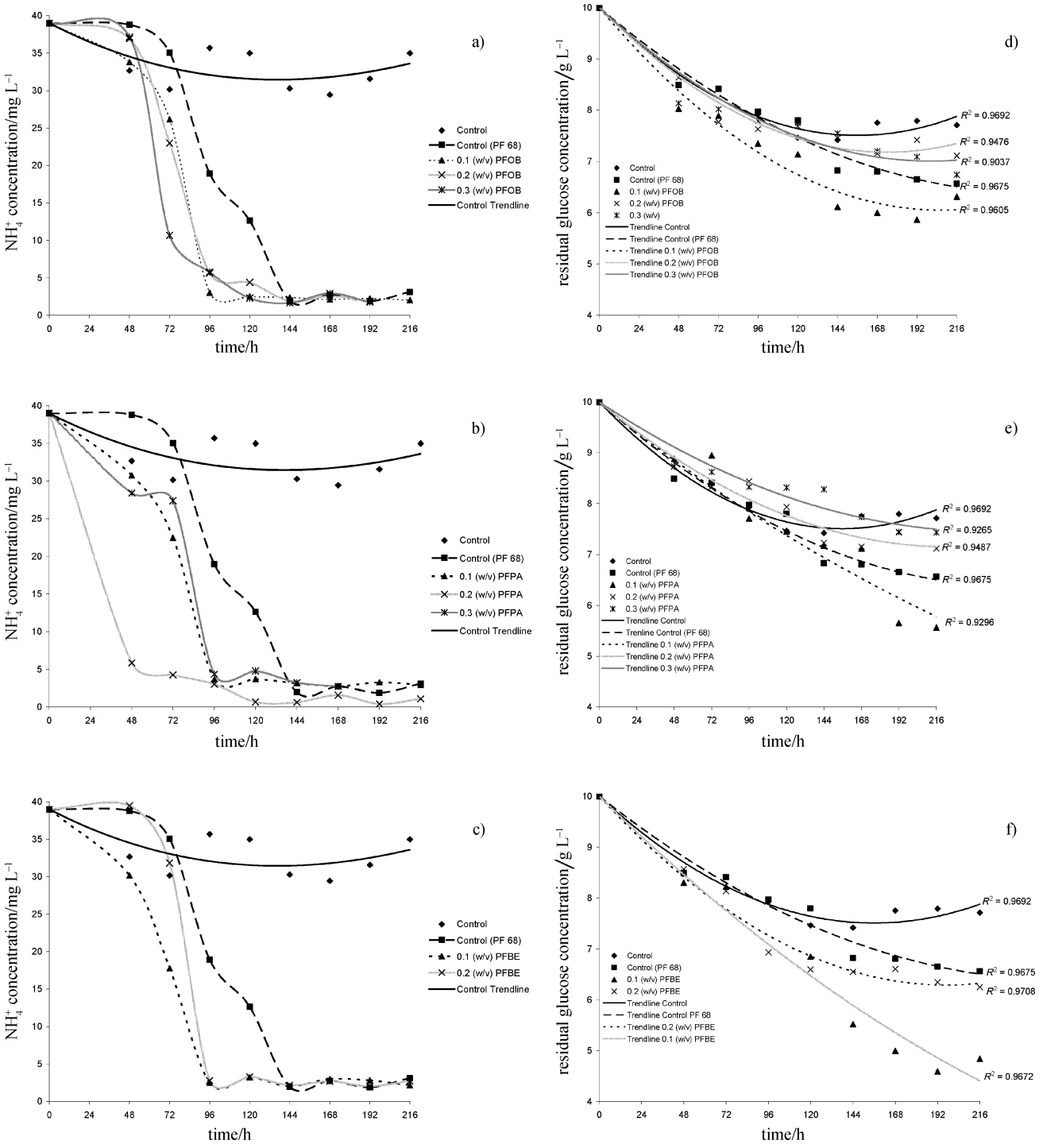
Fig. 1 (a, b, c) shows that the residual ammonium concentration in the PFC emulsions was below 10 mg L^{-1} after 96 h for all emulsion fractions investigated, showing an improved rate of consumption for the ammonium source when compared to the control cultures. The delay in the ammonium source consumption in the emulsions was attributed to the effect of PF 68 on the physiological state and germination of *P. chrysosporium* conidia in the cultures, as the conidia adjusts to the presence of the surfactants and PFCs in the nutrient medium. This was evident for the 0.1 to 0.3 (w/v) PFOB and 0.2 (w/v) PFBE emulsions. However, no evident lag phase was observed for the 0.1 to 0.3 (w/v) PFPA emulsions and 0.1 (w/v) PFBE emulsions. As PF 68 was shown to increase fluorescence uptake by *Saccharomyces cerevisiae*,[27] the uptake rate during this study of the ammonium source and glucose in the control cultures supplemented with PF 68 was lower compared to cultures supplemented with PFC emulsions. This was attributed to the low availability of DO in the control cultures, as the PFC emulsions had greater oxygen availability.

Fig. 1 (d, e, f) illustrates residual glucose concentrations in the batch cultures studied. The rapid rate of glucose consumption in the emulsions was similar to that of the ammonium consumption. The PFC emulsions with lower oil fractions, 0.1 (w/v), performed better than those with a higher oil fraction. After 168 h, the residual glucose concentration in the cultures containing PFV fractions of 0.1 (w/v), was observed to be below 6.5 g L^{-1}, compared to the control cultures where the residual concentration was above 7.5 g L^{-1} after 216 h of biomass incubation. The fungus grown in 0.1 (w/v) PFPA emulsion showed poor ability to use the carbon source, as the residual glucose concentration was high during the 0 to 192 h period, when compared to 0.1 (w/v) of PFOB and PFBE emulsions. This was further corroborated by the enhancement factor as the increments were in the order of PFPA < PFOB < PFBE for an emulsion fraction of 0.1 (w/v).

Extracellular secondary metabolite production

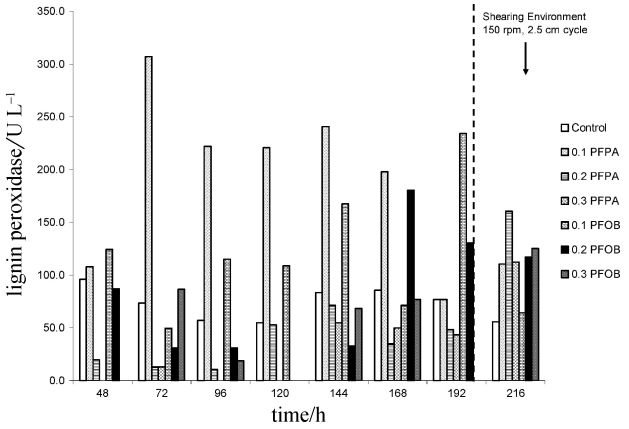
LiP and MnP production were measured for different emulsions at different concentrations, and the results are presented in Figs. 2 and 3, respectively. Previously, the addition of PF 68 to the fermentation medium without DO carrying water-immiscible oils was shown to decrease the DO transfer coefficient, even under fermentation broth agitation speeds exceeding 500 rpm.[28] That was why the LiP production was negligible in the control experiment for this study, where the medium was supplemented with ~8.5 % (w/v) PF 68 only. Negligible LiP was detected in the control, with PF 68; 0.1 and 0.2 (w/v) fractions of PFBE, respectively. This could have been caused by the production and interaction of volatile compounds with the enzyme, attributable to the PFCs' low boiling point (60 °C) and culture-incubation temperature (39 °C). However, at this point, further experimentation is required to verify this hypothesis. Furthermore, the presence of PFPA had a positive effect, as higher activity rates were determined when compared to the control cultures.

It was observed that moderate agitation (150 rpm) had negligible effects on enzyme activity deactivation in the presence of PFC emulsions. In the control cultures, average LiP and MnP rates were 77 and 12.6 U L^{-1}, respectively, after 192 h. After 24 h of broth agitation, the enzymes were reduced to an average of 55.8 U L^{-1} for LiP, while MnP was reduced to 0.8 U L^{-1} in the cultures. However, no significant changes in MnP activity were observed in the different fractions of the PFOB- and PFPA emulsions. Increases in MnP activity was observed in all fractions of the PFBE emulsions. All PFPA emulsions contributed significantly to higher LiP increases after the broth agitation, with 0.1 and 0.2 (w/v) PFOB emulsions showing decreasing activity, while the 0.3 (w/v) PFOB emulsion showed an increase. The highest activity obtained from crude samples during the study was 307 and 410 U L^{-1} for LiP and MnP respectively, as shown in Figs. 2 and 3.

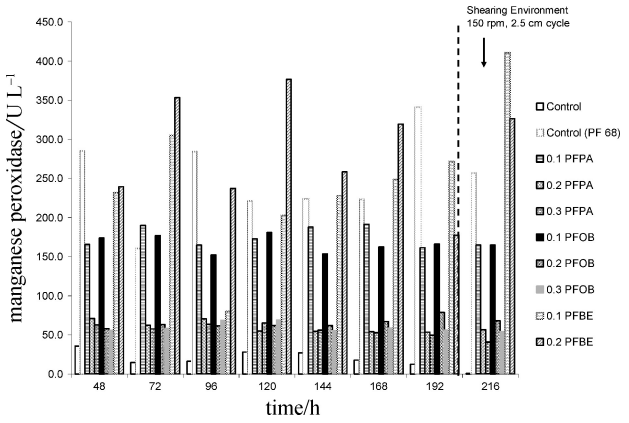


F i g . 1 – *Residual ammonium (NH_4^+) concentration (A – PFOB, B – PFPA and C – PFBE) and residual glucose concentration (D – PFOB, E – PFPA and F – PFBE) in batch cultures over 216 h*

It was difficult to observe a clear enzyme production trend over time. However, there was a noteworthy difference in enzyme productivity observed for cultures containing PFC emulsions when they were compared to the control cultures. For LiP, maximum enzyme activity was observed after 72 h. This was attributed to easier DO transport in younger biofilms of *P. chrysosporium*, which was shown to influence LiP production. Furthermore, this coincided with the depletion of the ammonium source in the culture broth. However, this pattern was not evident with the presence of MnP in the cultures, in which maximum activity was observed at different time intervals during the course of this part of the study.



F i g . 2 – *Lignin peroxidase activity obtained using: 1) pure medium, 2) medium supplemented with PF 68 and 3) with emulsified PFCs. Negligible LiP activity was found in 1) control (PF 68), 2) 0.1 PFBE and 3) 0.2 PFBE experiments.*



F i g . 3 – *Manganese peroxidase activity obtained using: 1) pure medium; 2) medium supplemented with PF 68; and 3) with emulsified PFCs. The activity was determined in crude samples.*

Conclusion

In this study, the possibility of developing sufficient biomass and enhancing extracellular enzyme production from low inoculum concentrations of *P. chrysosporium* was experimentally analysed using DO carrying oils and a poloxamer, PF 68, as the emulsifier. PFBE, PFOB and PFPA were successfully used as oxygen carriers for *P. chrysosporium*. The PFCs tested improved overall biomass formation, substrate consumption and enzyme production compared to the control cultures. The use of PFC emulsions reduced the effects of agitation on enzyme deactivation, as no significant decrease in enzyme activity was observed after agitation. Although some of the PFCs performed better than others, sufficient amounts of biomass were achieved when compared to the cultures grown without the emulsions and PF 68 surfactant. LiP production rates of >200 U L^{-1} were observed over the period of 72 to 168 h, with 0.1 (w/v) PFPA. Overall, a shearing environment resulted in reduced LiP activity in cultures with different PFC concentrations. Higher fractions of 0.3 (w/v) PFC showed greater enzyme protection under shearing conditions. However, the increase in PFC concentration resulted in lower MnP production (<60 U L^{-1}). It was demonstrated that for effective enzyme production, biofilm immobilisation and supplementation of the nutrient medium with essential additives is a viable option for improving biomass performance. Despite the fact that PF 68 had not previously been observed to enhance biomass growth, it was shown in this study that it significantly enhances *P. chrysosporium* biomass generation.

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