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OXYGEN MASS TRANSFER FOR AN IMMOBILISED BIOFILM OF *Phanerochaete chrysosporium* IN A MEMBRANE GRADOSTAT REACTOR

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Abstract - A novel system, the membrane gradostat reactor (MGR), designed for the continuous production of secondary metabolites, has been shown to have higher production per reactor volume than batch culture systems. The MGR system mimics the natural environment in which wild occurring microorganism biofilms flourish. The biofilms are immobilised on the external surface of an ultrafiltration membrane where substrate distribution gradients are established across the biofilm. The hypothesis that, dissolved oxygen (DO) mass transfer parameters obtained in submerged pellets can be used to describe and model DO mass transfer parameters in the MGR, was refuted. *Phanerochaete chrysosporium* biofilms, immobilised on ultrafiltration capillary membranes in the MGR systems were used to quantify DO distribution using a Clark-type microsensor. The DO penetration depth decreased with increasing biofilm thickness, which resulted in the formation of anaerobic zones in the biofilms. Oxygen flux values of 0.27 to 0.7 g/(m^2.h) were obtained during the MGR operation. The consumption of oxygen and the Monod saturation constants used in the modelling of oxygen distribution in immobilised biofilms were in the range of 894.53 to 2739.70 g/(m^3.h) and 0.041 to 0.999 g/m^3, respectively.
Keywords: Oxygen mass transfer; *Phanerochaete chrysosporium*; Membrane bioreactor; Extracellular enzyme production.

INTRODUCTION

Since the discovery of extracellular enzymes, such as manganese peroxidase (MnP) and lignin peroxidase (LiP) in *Phanerochaete chrysosporium* biofilms (Kirk *et al.*, 1978; Tien & Kirk, 1984; Kuwahara *et al.*, 1985), efficient methods for continuous production of these enzymes have been developed. The use of membrane bioreactors (MBRs), where *P. chrysosporium* biofilms are immobilised on the external surface of support matrices, was shown to have higher enzyme production per reactor volume when compared to submerged batch cultures (Kirkpatrick & Palmer, 1987; Linko, 1988; Venkatadri & Irvine, 1993; Leukes, 1999; Govender *et al.*, 2003; Sheldon & Small, 2005). The continuous production of these enzymes is of great economic importance as it was demonstrated that they are able to metabolise a variety of organic compounds, many of which are pollutants in both liquid effluents and soils (Livernoche *et al.*, 1981; Griselda & Eduardo, 1990;

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Walsh, 1998). The membrane gradostat reactor (MGR) was conceptualised as an alternative system to batch culture systems and was proven to be effective than submerged cultures and other MBRs in terms of LiP and MnP production (Leukes, 1999; Leukes *et al.*, 1999; Govender *et al.*, 2003; Govender *et al.*, 2004; Sheldon & Small, 2005; Ntwampe & Sheldon, 2006).

The most important external factors affecting the production and activity of enzymes produced by *P. chrysosporium* are temperature, pH, dissolved oxygen (DO) concentration and a fixed nitrogen concentration (Fenn *et al.*, 1981; Fenn & Kirk, 1981; Jeffries *et al.*, 1981; Leisola *et al.*, 1984). The morphology of immobilised biofilms is arranged according to the conditions at which they are grown and this affects substrate mass transfer rates and microbial activity (Beyenal & Lewandowski, 2002). A good understanding of DO mass transfer is an essential component of the microbial activity of aerobic biofilms such as that of *P. chrysosporium* (Bishop & Yu, 1999). Measured substrate concentration profiles in immobilised biofilms can be used to quantify mass transfer parameters in the identified biofilm (Lewandowski & Beyenal, 2003b). Any substrate mass transfer in biofilm systems is characterised by three steps: (a) transport from the bulk medium to the biofilm surface; (b) diffusion; and (c) consumption within the biofilm (Zhang & Bishop, 1994). The rate of substrate transport in a biofilm is determined by linking the convective mass transfer rate to the diffusive mass transport rate across the biofilm surface (Beyenal & Lewandowski, 2002). As there is no substrate consumption in the bulk medium, the flux of substrates across the biofilm surface must be conserved, which requires the rate of external (J_f) and internal $(D_f\,(dC/dx))$ oxygen mass transfer, to be equal, as described by Eq. 1 (Beyenal & Lewandowski, 2002; Hibiya *et al.*, 2003; Lewandowski & Beyenal, 2003b). To solve Eq. 1, the oxygen diffusivity coefficient in the biofilm (D_f) and the oxygen diffusivity coefficient in water (D_w) at the incubation temperature, need to be determined.

$$J = D_w\beta\left(C_b - C_s\right) = D_f\left(dC/dx\right)_{f,x_s} \tag{1}$$

Several published papers, which related the diffusion of oxygen in biofilms to variables such as biofilm dry density and thickness, are available. However, these experiments were performed using microbial biofilms immobilised on flat surfaces (in plate open channel reactors) and the biofilms were submerged in a nutrient medium (e.g. submerged MBR's). These biofilms were not immobilised on the external surface of a capillary membrane or anything that resembles the MGR used in this study. In submerged MBR's, the transfer of DO is from the bulk nutrient medium into the biofilm matrix or oxygen is supplied through the lumen of the membrane to the biofilm. The biofilms immobilised were not of a fungal nature and were not continuously exposed to the gaseous oxygen source. The oxygen was continuously supplied in the extracapillary space (ECS/shell side) of the MGR system. Biofilms positioned horizontally are still commonly used to study kinetic parameters. However, in this study, the MGR's were in a vertical position to enhance radial distribution of substrates (including DO) across the immobilised biofilm, which is a requirement for an effective MGR system (Leukes *et al.*, 1999).

The objectives of the study were to: 1) quantify oxygen mass transfer parameters using measured DO profiles obtained from *P. chrysosporium* biofilms; and 2) use the mass transfer parameters obtained in a mathematical model to reproduce the DO distribution. The study also aims to determine any limitations to oxygen supply within the immobilised biofilms. This will contribute to the understanding of DO transfer in biofilms immobilised in the MGR and on asymmetric capillary membrane systems. As the MGR system is currently being evaluated for its application in the commercial production of secondary metabolites, it was important to establish parameters directly linked to production of the metabolites, in order to optimise the system. The DO mass transfer parameters obtained were compared with those obtained in submerged, batch cultures where mycelia pellets of *P. chrysosporium* were removed, allowed to equilibrate with air and probed with an oxygen microelectrode to determine mass transfer parameters (Michel *et al.*, 1992). The study evaluated the hypothesis that DO mass transfer parameters determined in batch cultures can be used to estimate the DO mass transfer parameters for immobilised microorganisms in the MGR.

Other parameters determined were biofilm thickness (related to growth rate of the fungus) and the DO penetration ratio (the ratio between the DO penetration depth and the increase in biofilm thickness). *P. chrysosporium* BKM-F-1767 produces ethanol from glucose under limited-oxygen conditions. The presence of ethanol in cultures of *P. chrysosporium* leads to deactivation of LiP and MnP (Kenealy & Dietrich, 2004). Therefore, it was

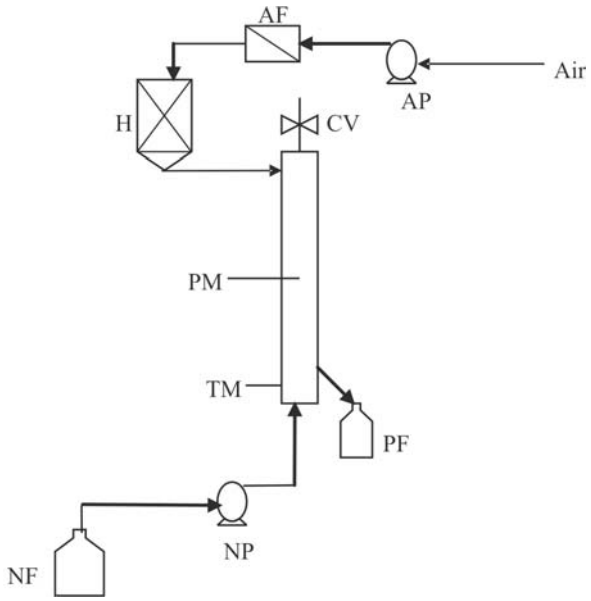
important to identify anaerobic zones in the MGR biofilms over the duration of the study. LiP and MnP activity were not quantified as part of this study, as other researchers have quantified and established patterns of enzyme production using the MGR system (Leukes, 1999; Solomon & Petersen, 2002; Govender *et al.*, 2003; Govender *et al.*, 2004; Sheldon & Small, 2005).

MATERIALS AND METHODS

Bioreactor Setup and Operation

Biofilms of *P. chrysosporium* strain BKMF 1767 (ATCC 24725) were grown at 39 °C in single capillary MGR systems. A schematic illustration of a vertically positioned single capillary MGR is shown in Figure 1.

The single capillary MGR dimensions and operational conditions are listed in Table 1. MGR's with capillary polysulphone membranes fixed to the centre of the glass modules were used. The polysulphone capillary membrane used was specifically developed to be used in the MGR system. The membranes were manufactured at the Institute of Polymer Science, University of Stellenbosch (Stellenbosch, South Africa) and were coded as IPS 763 (Jacobs & Leukes, 1996). A Teflon mould was used at the bottom of the bioreactor to avoid accumulation of permeate. The experimental setup was chemically sterilised with a 4% (v/v) formaldehyde solution and rinsed with sterile distilled water before the spore inoculation process.



1. AF: air filter;
2. AP: air pump;
3. CV: closed valve (dead-end filtration mode applied);
4. H: humidifier;
5. NF: nutrient flask;
6. NP: nutrient pump;
7. PF: permeate flask;
8. PM: polysulphone membrane TM: Teflon mould. Note: The air was supplied on the shell side and it helps with the flow of permeate to the permeate flask (PF).

Figure 1: Schematic illustration of a vertical single fibre capillary MGR.

Table 1: Dimensions and operating conditions of a vertically orientated single capillary MGR system.

Parameter	Details
Module housing	Glass
Module length	230 mm
Module diameter	12 mm
Active internal volume	20.4 ml
Type of membrane	Polysulphone (IPS 763)
Active membrane length	160 mm
Capillary membrane outer diameter	~1.7 - 1.9 mm
Capillary membrane inner diameter	~ 1.3 mm
Membrane Surface area – Polysulphone	985 mm^2
Operating conditions	
Nutrient supply rate to the bioreactor	1.68 ml/h
Airflow rate	1 $vol.ECS^{-1}.h^{-1}$
*Amount of spores per membrane length	3 million
ECS air pressure	Atmospheric

*Prepared using a spectrophotometer at 650 nm; 1 Abs = 5 million spores per ml (Tien and Kirk, 1988).

A spore solution containing an estimated 3 million spores was used for inoculation. The spores were harvested from mycelia grown on malt agar plates for 5 days at 39°C. The spore solution preparation was done by suspending spores containing mycelia in sterile distilled water followed by passage through a sterile glass wool to free spores from contaminating mycelia (Tien & Kirk, 1988). Spores were immobilised in the cavities of the membrane by reverse filtration using a 40 ml spore solution pumped through the shell side of the MGR (Govender *et al.*, 2004). The nutrient medium used for the growth of *P. chrysosporium* biofilms in the single capillary MGR was similar to that used by Tien and Kirk (1988) to culture *P. chrysosporium* in batch cultures. The nutrient medium contained 56 mM glucose, 1.1 mM ammonium tartrate and supplemented with Veratryl alcohol, Dimethyl succinate, Thiamin and trace elements.

The nutrient medium was supplied to the membrane lumen using a multi channel Watson-Marlow 505S pump (Dune Engineering, Germany). Humidified air was supplied to the MGR's to provide DO to the immobilised biofilms. Air was filter sterilised before being passed through the ECS of the MGR, as shown in Figure 1. Air was supplied using a multi-port air pump (HAILIPAI, ACO-9620 aquarium air pump) with flow control to ensure that the flow was consistent throughout all the bioreactor modules. Air entered at the top of each single capillary MGR system and exited at the bottom through the permeate port, without being pressurised in the ECS. The MGR's were operated in the dead-end filtration mode for 264 h. The MGR's were operated simultaneously and were independent of each other. The permeate pH and redox potential were monitored on a 24 h basis using a Hanna HI 8314 (Hanna Instruments, Portugal) membrane pH meter to determine whether the bioreactors were biochemically similar as shown Figure 2.

The increases in redox potential in the recovered permeate samples shows that the solution has high oxidative capabilities, which is a sign of extracellular enzyme production. The high variation in pH and redox potential observed at the beginning of the experiments was attributed to the spore's acclimatisation to the conditions in the single capillary MGR systems. The pH and redox potential values stabilised after 72 h of bioreactor operation.

Dissolved Oxygen Measurements

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 intervals of 10 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 concentration in triplicate in the immobilised biofilms at time intervals of 72, 120, 168, 216 and 264 h. The DO measurements were measured at atmospheric pressure in three independent experiments. The MGR's were dismantled by removing the glass manifold and exposing the membrane attached biofilm for easy microsensor measurement.

The computational methods used to interpret the mass transfer parameters from DO profiles are explained in the analytical methods section. The available computational procedures for determining the mass transfer parameters from the substrate concentration profiles require the assumption that biofilms are homogeneous (uniform), not heterogeneous. For current conceptual models of biofilms and the use of mathematical models available to interpret microsensor measurements, the researcher can minimize the effect of biofilm heterogeneity by selecting the locations of microsensor measurements (De Beer & Schramn, 1999; Lewandowski & Beyenal, 2001; Lewandowski & Beyenal, 2003a). Even though biofilms in the single capillary MGR's were of a similar thickness across the active membrane length, an area closer to the bottom of the bioreactor where sporulation was not evident (~ 2cm above the Teflon mould), was chosen as suitable for measurement of the oxygen profiles. Biofilm thicknesses were determined at this point of measurement. During microsensor calibration, the DO concentration in water was measured as ~ 6.5 ± 0.2 g/m^3 at room temperature.

Biofilm Thickness, Oxygen Penetration Depth and Ratio

The biofilm thickness was determined by using a Carl Zeiss Axiovision light-microscope digital imaging system equipped with measuring software. The objective used for the measurements was a 2.5 X magnification objective. The calibrated microscope objectives acquired real-size measurements from biofilms attached to the polysulphone membrane surface. The average thickness of a clean membrane was determined to be 1.73 mm on average, which falls within the manufacturing range of 1.7 to 1.9 mm, as shown in Table 1. The average biofilm thickness was measured by determining the overall thickness of the membrane-attached biofilm and then subtracting the thickness of the clean membrane, to get the overall biofilm thickness.

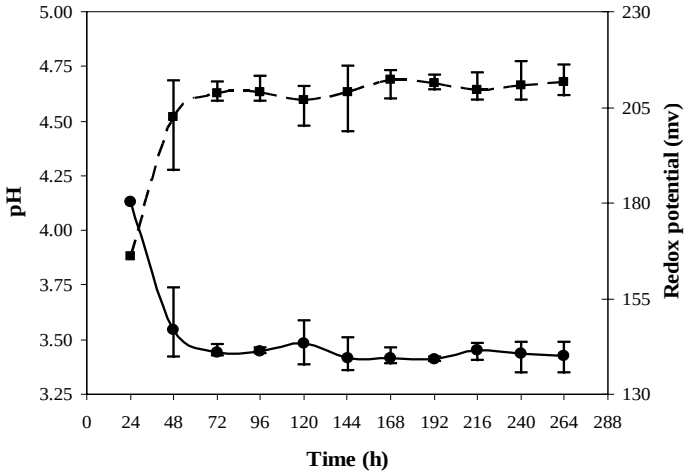


Figure 2: pH reduction (-●-) and increasing redox potential (-■-), in permeate samples collected from single capillary MGR systems.

ANALYTICAL METHODS

Model Assumptions

a. The rates of DO mass transfer in the biofilm were proportional to the concentration difference across the biofilm thickness and the proportionality factors were directly related to DO mass transfer constants.

b. DO mass transfer was assumed to be one-dimensional, from the gaseous ECS, across the biofilm thickness towards the substratum (polysulphone membrane). Reproducible DO profiles (three profiles per location) were measured several minutes apart showing negligible magnitude of error between the profiles. Figure 3 shows examples of reproduced profiles from three independent experiments measured at ~ 2cm above the Teflon mould after 120 h of single capillary MGR operation. This showed that no DO radial flows (steady or unsteady) perpendicular to the biofilm surface occurred.

c. As *P. chrysosporium* is an aerobic microorganism, the oxygen uptake rate through biological reaction was assumed to be described by the Monod's equation.

d. The immobilised biofilms are at pseudo steady state with respect to the transport of DO into the inner mycelia, as shown in Figure 4. Pseudo steady state $\left(dC_s/dt = 0\right)$ was defined as a condition where oxygen concentration does not change for a period of time at the biofilm surface and aerial mycelia, when oxygen is transported from the gaseous phase into the mycelia. The distribution of DO in the aerial mycelia, where the biofilm thickness was 0 µm to 40 µm, was determined to be similar across all three experiments over a period of 264 h as shown in Figure 4.

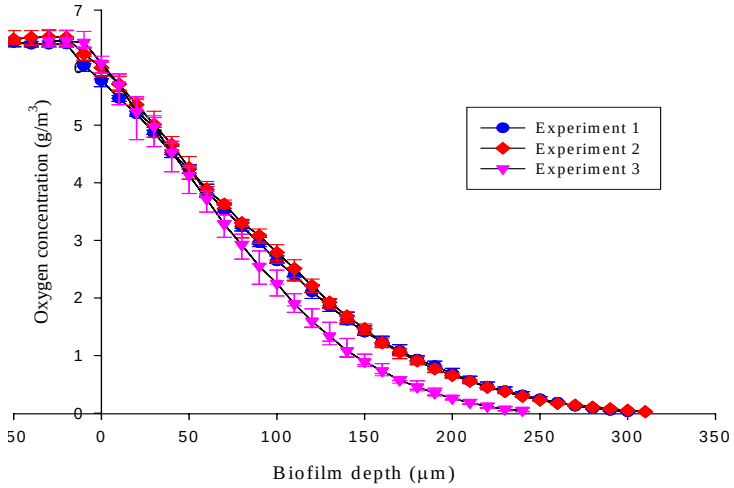


Figure 3: DO profiles in immobilised *P. chrysosporium* biofilms measured from three independent experiments after 120 h of single capillary MGR operation.

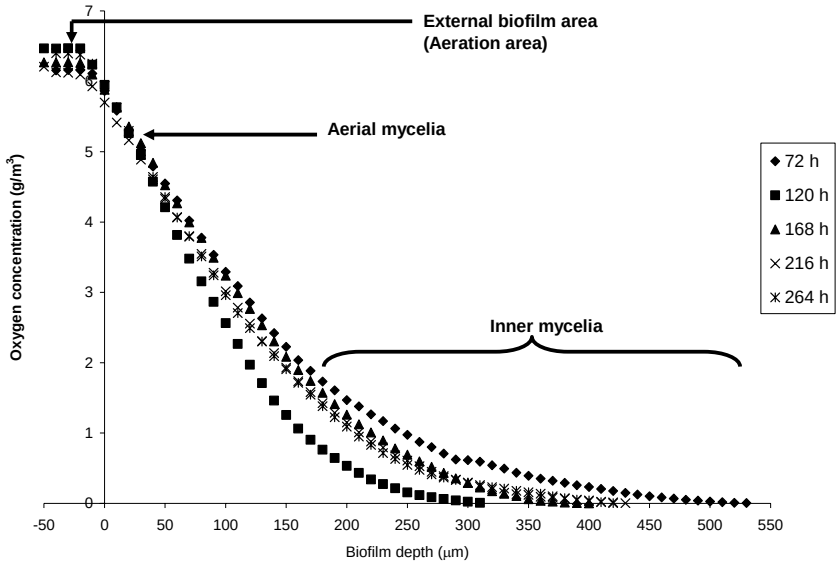


Figure 4: A combination of averaged DO profiles in biofilm of *P. chrysosporium* determined at different times during single capillary MGR operation.

Model Validation

It was important to determine whether DO transport into the biofilm was by convection or diffusion. This parameter is important as it determines the type of model to be used to determine DO mass transport parameters in the immobilised biofilms. The experimental coefficient, β_a from Eq. 1 was determined in the aerial mycelia, 0 to 40 µm from the biofilm surface using an averaged profile from the five average DO profiles (shown in Figure 4) measured at different times, as DO transport in the region was similar. This was done by plotting an empirical exponential function $\ln[1-(C-C_s)/(C_b-C_s)]$ against penetration depth $(x-x_s)$ in the aerial mycelia region, using $C_b = 6.5 g/m^3$. The experimental coefficient (β_a) was determined from the slope of the graph (R^2 = 0.98). By using Eq. 1, with known values of oxygen diffusivity coefficient in water with 3% salinity (D_w), and the DO concentration gradient in the aerial mycelia $(dC/dx)_{a,f,xs}$, parameters such as the DO flux, including the diffusion coefficient of oxygen in the aerial mycelia region could be determined. The parameters obtained are shown in Table 2. The ratio between the oxygen diffusivity coefficient in the nutrient medium and oxygen diffusivity coefficient in the aerial mycelia was equivalent to 0.97. This showed that DO transport into the biofilm was diffusional.

Table 2: Parameters determined in the aerial mycelia (0 to 40 µm).

Parameter	Value
Experimental coefficient (β_a)	25889 m^{-1}
$\beta_a(C_s-C)$	30421.18 g/m^4
D_w (3% salinity)	1.161E-05 m^2/h
$J_{a,m}$ (averaged flux in the aerial mycelia)	0.35 $g/m^2.h$
$(dC/dx)_{a,f,xs}$	29376.55 g/m^4
$D_{a,f}$	1.202E-05 m^2/h
$D_w/D_{a,f}$	0.97

*Nutrient media contained 3% salinity, measured using a hydrometer.

Computing Mass Transfer Parameters from DO Profiles

By considering all the model assumptions, Eq. 2 can be used to model mass transport and reaction in biofilms (De Beer & Schramn, 1999; Lewandowski & Beyenal, 2001; Hibiya *et al.*, 2003; Lewandowski & Beyenal, 2003a).

$$D_f\left(d^2C/dx^2\right)_f = \frac{r_m C}{K_m + C} \tag{2}$$

The mass transfer parameters were determined using Taylor's expansion method (explained in section 3.3.1), as demonstrated by Lewandowski and Beyenal (2003b). The mass transfer parameters obtained were tested using the mass balance equation in porous materials to reproduce distribution of dissolved profiles in the biofilms of *P. chrysosporium* (Frank-Kamenetskii, 1969). Hibiya *et al.* (2003) also demonstrated the use of this method.

a) Taylor's Expansion [Lewandowski & Beyenal, (2003b)]

Using Taylor's expansion of the function describing the concentration profiles around the point, $x = x_s$, positioned at the biofilm surface, the substrate concentration profiles near this point are described by Eq. 3.

$$C = C_s + \left(\frac{dC}{dx}\right)_{xs}\left(x - x_s\right) + \frac{1}{2!}\left(\frac{d^2C}{dx^2}\right)_{xs}\left(x - x_s\right)^2 + \frac{1}{3!}\left(\frac{d^3C}{dx^3}\right)_{xs}\left(x - x_s\right)^3 + ... + \frac{1}{n!}\left(\frac{d^nC}{dx^n}\right)_{xs}\left(x - x_s\right)^n \tag{3}$$

Since there are three unknown parameters in Eq. 2, D_f, r_m and K_m, calculating the first three derivatives, in Eq. 3, was suitable as only three equations are required to calculate the parameters. As the flux of substrate across the biofilm has to be continuous, the flux described by Eq. 1, and represented as $J = J_{w,xs} = J_{f,xs}$ will hold. Eq. 4 and Eq. 5 describe the flux at the biofilm interface and nutrient-film, respectively.

On the biofilm surface side

$$J_{f,xs} = D_f\left(dC/dx\right)_{f,xs} \tag{4}$$

On the nutrient-film side

$$J_{w,xs} = D_w\left(dC/dx\right)_{w,xs} \tag{5}$$

Therefore $\left(dC/dx\right)_{f,xs}$ can be estimated using the oxygen flux across the nutrient-film layer and the diffusion coefficient in the biofilm as shown in Eq. 6, which is the solution to the first derivative in Eq. 3.

$$\frac{J_{w,xs}}{D_f} = \left(dC/dx\right)_{f,xs} \tag{6}$$

As the derivatives were evaluated from the biofilm surface, the higher derivatives are:

2nd order derivative:

$$\left(\frac{d^2C}{dx^2}\right) = \frac{r_m}{D_f}\frac{C_s}{\left(K_m + C_s\right)} \tag{7}$$

3rd order derivative:

$$\left(\frac{d^3C}{dx^3}\right) = \frac{J_{w,xs}}{D_f}\frac{r_m K_m}{D_f}\frac{1}{\left(K_m + C_s\right)^2} \tag{8}$$

By using the least-square method to fit the experimental data to a 3rd order polynomial equation, the experimental constants, *a*, *b*, and *c* as shown in Eq. 9, can be obtained to simplify Eq. 3.

$$C = C_s + a\left(x - x_s\right) + b\left(x - x_s\right)^2 + c\left(x - x_s\right)^3 \tag{9}$$

From Eq. 9, (representing 1st, 2nd and 3rd order derivatives) the following will be valid:

$$a = \frac{J_{w,xs}}{D_f} \tag{10}$$

$$b = \frac{1}{2}\frac{r_m}{D_f}\frac{C_s}{K_m + C_s} \tag{11}$$

$$c = \frac{1}{6}\frac{J_{w,xs}}{D_f}\frac{r_m K_m}{D_f}\frac{1}{\left(K_m + C_s\right)^2} \tag{12}$$

Eq. 10, 11 and 12 can then be solved by substituting numerical values C_s and $J_{w,xs}$ to determine the

parameters, D_f, r_m and K_m. As the shape of the DO profile above the biofilm surface (described as the nutrient-film layer) is exponential, it can be described by a function shown in Eq. 13.

$$\frac{C - C_s}{C_b - C_s} = 1 - \exp[-\beta(x - x_s)] \quad (13)$$

The nutrient-film coefficient, β, can be determined, when representing coordinates $\ln[1-(C-C_s)/(C_b-C_s)]$ and $(x - x_s)$. In addition, Eq. 10 was differentiated across the nutrient-film, to obtain Eq. 14.

$$(dC/dx)_{w,xs} = \beta(C_b - C_s) \quad (14)$$

$J_{w,xs}$ and $(dC/dx)_{w,xs}$ can be calculated by using the oxygen diffusivity coefficient, 1.161E-05 m^2/h, in water at 39°C with a salinity content of 3% (determined for our nutrient media using a hydrometer). The method assumed that the oxygen diffusion transfer was constant across the biofilm thickness. The flux as described in Eq. 1 with other mass transfer could therefore be calculated.

b) Diffusion Kinetics in Porous Surfaces [Frank-Kamenetskii, (1969)]

The overall rate of reaction on a porous material is the integral sum of the rates for the differential parts of the surface, which are characterised by different accessibilities with respect to diffusion. The overall rate depends on the shape, diameter, thickness and geometrical shape of the pores at different depths of the layer of material. To analyse the problem, regardless of the shape and diameter of the pores themselves, diffusion, within a mass of porous material, can be described with a diffusion coefficient (D_f) defined in such a way that the diffusion equation in the mass of material has the form shown on the R.H.S of Eq. 2. Provided that $r_m C/(K_m + C)$ is only a function of substrate concentration, C, the R.H.S of Eq. 2 can be integrated as a quadrate by using substitutions, obtaining Eq. 15 (Frank-Kamenetskii, 1969):

$$\frac{dC}{dx} = \sqrt{\frac{2}{D_f}\int \frac{r_m C}{K_m + C} dC} \quad (15)$$

The mass balance equation in Eq. 15 can then be transformed to Eq. 16 (Ntwampe, 2005).

$$\frac{dC}{dx} = -\sqrt{2\frac{r_m}{D_f}\left(C - K_m \mathrm{Ln}\left(\frac{K_m + C}{K_m}\right)\right)} \quad (16)$$

Using the parameters determined from Taylor's expansion, the values of (r_m / D_f) and K_m could be used in an ordinary differential equation solver to determine the first derivative of oxygen concentration distribution across the biofilm using Eq. 16. The modelled parabolas obtained were compared to the experimental data. This method was previously used to delineate substrate mass transfer in microbial biofilms (Nielsen *et al.*, 1990; Lewandowski & Beyenal, 2003a).

RESULTS AND DISCUSSION

Biofilm Development and DO Distribution in the Immobilised Biofilms

Biofilms were first noticed on the external surface of the membranes after 48 h of MGR operation. The biofilm thickness increased with time, as shown in Figure 5. The patterns of growth were similar to those determined in other studies of single capillary MGR (Ntwampe & Sheldon, 2006). Measurements of biofilm thickness obtained with the Carl Zeiss microscope were shown to be consistent across the three experiments. Biofilm thickness increased from an average of 912 µm after 72 h to 2246 µm after 264 h. The average diameter of *P. chrysosporium* pellets in submerged cultures was 2100 µm after 240 h in sealed shake-flasks (Michel *et al.*, 1992). The second decelerated growth phase was determined in the period 216 to 240 h (9 to 10 days). The secondary growth phase obtained by Kirk *et al.* (1978), occured after 10 days (mycelia cultured at 39°C, in a 125 ml flask with 21% O_2), which is in agreement with the results obtained in this study.

Oxygen penetration depth was determined from the measured DO profiles and the penetration ratio was calculated and compared to biofilm thickness as shown in Figure 6.

Oxygen penetration ratio decreased with an increase in biofilm thickness. Oxygen penetration depth was high when biofilm thickness was at 912.10 µm after 72 h of single capillary MGR

operation with the lowest penetration depth of 310 µm obtained after 120 h of single capillary MGR operation. The averaged DO penetration depth in the biofilms of three experiments was determined to be in the range of 306 to 530 µm. The penetration depth of oxygen obtained was similar to that of Lejeune and Baron (1997), which was in the range of 310 to 390 µm, where the growth of a filamentous fungus and substrate penetration depth were simulated in 3 dimensions (Lejeune & Baron, 1997). This represented a significant change in terms of DO distribution and penetration depth with biofilm age in cultures of *P. chrysosporium* immobilised in the MGR systems. Similar oxygen penetration depth in the range of 400 to 430 µm was seen for periods of 168 to 264 h. However, oxygen penetration depth of 400 µm in submerged mycelia pellets was achieved after 96 h.

In mycelia pellets, oxygen distribution at the external mycelia was differentiated with similar patterns of distribution obtained towards the centre of the pellets (Michel *et al.*, 1992). Patterns of oxygen penetration depth obtained in this study were not consistent with those observed in submerged cultures. In the MGR, the penetration depth was 530 µm after 72 h and was reduced to 310 µm after 120 h. The distribution of oxygen from the bulk phase, shell side, through the aerial mycelia (0 to 40 µm) was consistently similar across biofilms of different ages. This was not the case in submerged pellets. Differentiated DO distribution in the mycelia closest to the membrane surface (inner mycelia) was observed. This pattern was attributed to a different oxygen uptake rate as new mycelia were generated in the nutrient rich zones near the substratum. The distribution of oxygen in the cultures was consistent with the concept of a membrane gradostat. The mycelia close to the substratum were expected to be highly active as it grows in a nutrient rich zone. This obviously leads to increased use of DO, while the aerial mycelia are expected to be in a decelerated growth phase further away from the nutrient rich zone and limited usage of DO. The DO penetration ratio decreased from an average of 0.42 after 72 h to 0.12 after 264 h. This means that anaerobic areas closer to the membrane surface, where high glucose concentration is prevalent, increased with time, as shown in Figure 7. An oxygen penetration ratio of 0.22 was previously determined in submerged cultures after 96 h (Michel *et al.*, 1992); however, a similar penetration ratio in the MGR was achieved after 120 h of MGR operation.

Anaerobic zones increased from 602 µm after 72 h to 1940 µm after 264 h of single capillary MGR operation. The zones were closer to the substratum, where active mycelia are expected to grow. This was clearly identified as one of the limitations of the MGR system, as *P. chrysosporium* is known to produce ethanol under anaerobic conditions. Ethanol production was not determined during the course of this study. Initially when microorganisms establish themselves in a new environment, variation in morphological patterns occurs, thus resulting in variations of oxygen distribution parameters.

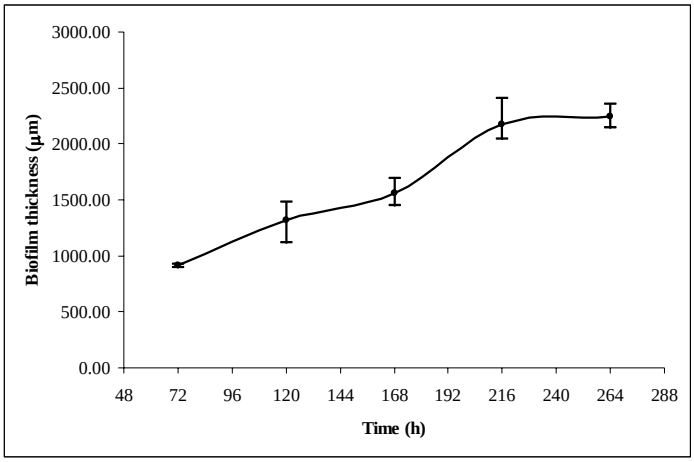


Figure 5: Biofilm thickness of *P. chrysosporium* grown in single capillary MGR systems over a period of 264 h. The thickness was determined at ~2 cm above the Teflon mould, from the bottom of the bioreactor systems, where DO profiles were measured.

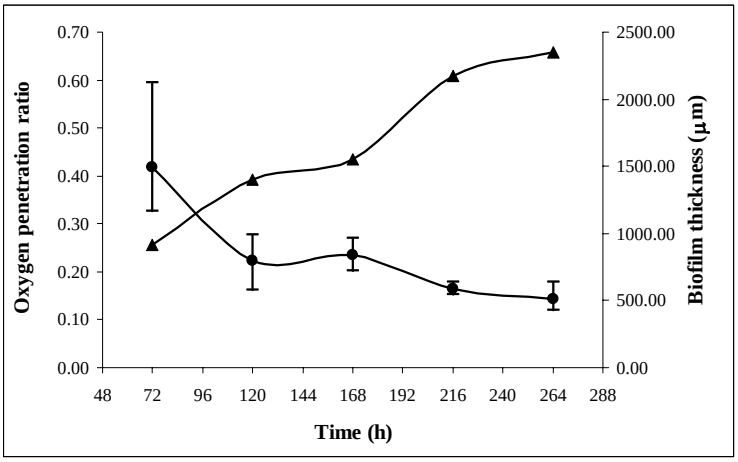


Figure 6: Oxygen penetration ratio determined as the ratio between DO penetration depth and biofilm thickness. Penetration ratio (-●-) over the duration of the study compared to average biofilm thickness (-▲-).

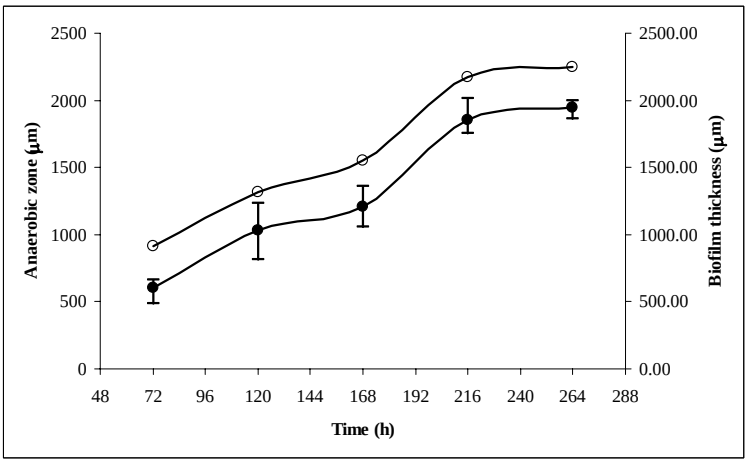


Figure 7: The anaerobic zones (-●-) in immobilised biofilms of *P. chrysosporium* quantified over the 264 h period in the MGR systems as biofilm thickness (-○-) increased.

Dissolved Oxygen Parameters

The parameters, D_f, r_m and K_m, were determined using the Taylor's expansion series. A 3rd order polynomial fit was used to determine experimental constants, shown in Eq. 9. The averaged oxygen profiles from the MGR systems were used to quantify these parameters. From a graph represented by coordinates: 1) experimental DO concentration (C) and 2) oxygen penetration depth $(x - x_s)$, a 3rd order polynomial fit was used to determine experimental constants (a, b, c) in Eq. 9. Values of the experimental constants and a correlation coefficient (R^2) of 0.99 obtained from the fit are shown in Table 3.

The values obtained were then used to quantify DO mass transfer parameters using Eq. 10, 11 and 12. To reproduce the average DO profiles using Eq. 16, the following parameters were considered in the model; 1) the concentration of oxygen used in the model at the biofilm surface, C_s, 2) the Monod saturation constant, K_m, and 3) the ratio between the substrate consumption and oxygen diffusivity coefficient in the biofilm, r_m / D_f. The parameters calculated from the experimental constants are listed in Table 4. The parameters were used to reproduce the averaged DO profiles obtained at different times from this study. Examples of simulated profiles are shown in Figure 8.

Table 3: Experimental coefficients obtained from the 3rd order polynomial fit, using the least square method with correlation coefficients obtained from the fit.

Time (h)	a x 10^4	b x 10^7	c x10^{10}	R^2
72	3.19	5.93	3.76	0.99
120	4.01	6.85	0.271	0.99
168	3.25	4.61	0.518	0.99
216	3.20	5.07	1.51	0.99
264	3.64	7.01	3.91	0.99

Table 4: Analysis of modelled profiles from averaged DO profiles using parameters determined using an ordinary differential equation solver.

Time (h)	C_s (g/m^3)	$2r_m/D_f$ x 10^8 (g/m^5)	K_m (g/m^3)
72	5.863	3.03	0.999
120	5.950	2.77	0.041
168	5.880	1.92	0.164
216	5.699	2.25	0.398
264	5.935	3.27	0.512

Parameters obtained in submerged fungal fermentations are still used to predict model parameters in bigger and alternative bioreactor systems. The values of 0.5 ± 0.3 g/m^3 for the Monod's saturation constant and 0.76 ± 0.1 g/m^3 for oxygen uptake rate were obtained in mycelia pellets of a submerged system (Michel *et al.*, 1992). Saturation constants in the range of 0.041 to 0.999 g/m^3 were obtained in the MGR systems over 264 h. There was no clear indication of an increase or a decrease in the saturation constants obtained from the averaged DO results. The oxygen uptake rate obtained in the MGR was in the magnitude of 10^2 to 10^3 g/m^3 and was much higher than that obtained in submerged cultures. The oxygen uptake rate was determined to be in the range of 894.53 to 2739.70 g/(m^3.h) (see Table 5). Higher oxygen uptake values, above 1000 g/(m^3.h) were determined at 72, 120 and 264 h, while lower than 1000 g/(m^3.h) were obtained at 168 and 216 h. The high oxygen uptake rate was attributed to microbial activity associated with growth. The hypotheses that submerged cultures can be used to model substrate transport in biofilms immobilised in the MGR system was determined to be misleading as the patterns of substrate distribution in aerial mycelia and inner mycelia (near the membrane), as well as the substrate consumption values, were found to be different to those obtained in MGR systems. Similarities were found in the oxygen penetration depth, penetration ratio, mycelia growth rate and the Monod saturation constant. Table 5 lists the following set of mass transfer parameters: 1) oxygen uptake rate; 2) oxygen diffusion in the biofilm; and 3) oxygen flux across the biofilm surface. Oxygen diffusion coefficients in the range of 7.94E-06 to 1.750E-05 m^2/h were obtained. The calculated effective oxygen diffusivity coefficients in the biofilms were higher than that of water (7.2E-06 m^2/h) and that calculated for the nutrient feed at 39°C (1.161E-05 m^2/h). A distinctive pattern was established from the DO effective diffusion coefficients. During decelerated growth phases (72 h and 264 h) in the MGR, higher diffusion coefficients were obtained as described by Ntwampe and Sheldon (2006). During other periods, oxygen diffusion coefficients obtained were much closer to 1.161E-05 m^2/h.

As the biofilms of *P. chrysosporium* were placed in direct contact with the gaseous phase, it was assumed that the active mycelia in the nutrient rich zones experienced decelerated growth at certain times during the MGR operation, resulting in reduced oxygen uptake rate in the biofilms. This was concluded to be the reason for higher effective diffusion coefficients. Theoretically, aerial mycelia in the MGR system were in the decelerated or stationary growth phase compared to the mycelia in the nutrient rich zones. This means that they provided negligible growth in terms of biofilm thickness achieved, when compared to the active mycelia. Further evidence was seen in the differentiated distribution of DO in the inner mycelia, when compared to DO distribution in the aerial mycelia. Oxygen flux in the range 0.27 to 0.7 g/(m^2.h) was determined in the MGR systems over the operation period of 264 h.

The mass transfer parameters determined in Tables 4 and 5 were validated by using them to reproduce experimental profiles using Eq. 16, as shown in Figure 8.

Table 5: Mass transfer parameters determined from averaged oxygen profiles.

Time (h)	r_m (g/(m^3.h))	D_f x 10^5 (m^2/h)	J_f (g/(m^2.h))
72	1298.68	0.858	0.27
120	2423.41	1.75	0.7
168	958.78	0.998	0.32
216	894.53	0.794	0.25
264	2739.70	1.68	0.61

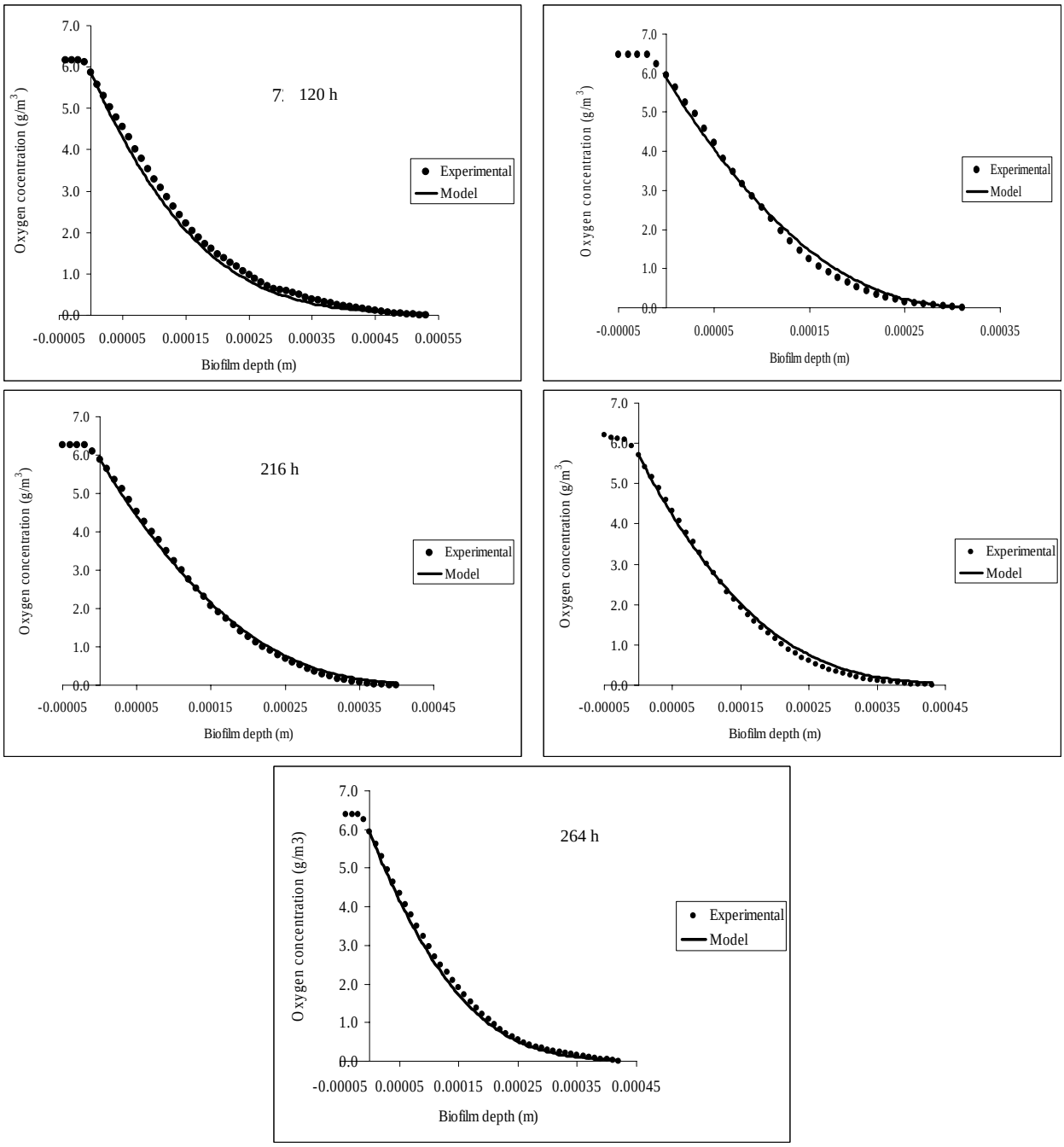


Figure 8: A comparison between experimental averaged DO profiles and modelled profiles using parameters in Tables 4 and 5.

In this study, oxygen diffusivity coefficients of up to 150% of that of oxygen in water were determined. Siegrist and Gujer (1985) found higher effective diffusivities, which varied from a low 40% up to 140% of the corresponding value in pure water. They determined that high effective diffusivity: 1) increased with biofilm thickness and 2) was higher for biofilms grown on synthetic media than on industrial effluent. Also their biofilms grew on a permeable membrane and the substrate diffused across the biofilm thickness as in this study. They concluded that an increase in substrate diffusion could be described by *eddy diffusion* in the inner mycelia due to microbial activity. This means that eddy currents occur due to microbial activity and growth, as the biofilms try to use limited substrates. Emanuelsson and Livingston (2004), including Larsen and Harremoes (1994) also reported substrate diffusion coefficients greater that 100% using synthetic media and this was attributed to eddy flow or currents in the biofilms.

Practical Use of the Findings

Parameters determined during the study can be used to monitor and estimate DO distribution during the MGR operation as the MGR is used to immobilised aerobic microbes, to produce low-volume high-value bio-products. Anaerobic zones were evident in the immobilised biofilms; other aeration devices, like the use of technical grade oxygen (~100%) or oxygen carriers, need to be implemented to reduce these zones in the biofilms. Production of unwanted by-products in the MGR, which deactivates high value bio-products needs to be investigated in future studies.

CONCLUSION

The methods used in this study enabled us to obtain a clearer understanding of oxygen mass transfer in *P. chrysosporium* biofilms grown in continuous vertical single capillary MGR's over a period of 264 h. Oxygen transfer parameters in the MGR biofilms were found to differ from those obtained in submerged pellets. It was found that:

- Biofilm thickness of immobilised *P. chrysosporium* increased from 912 µm after 72 h of bioreactor operation to 2246 µm after 264 h in the single capillary MGR systems.
- The average oxygen penetration depth of the biofilms was higher at 72 h and lower at 120 h of bioreactor operation.
- DO penetration ratio decreased from 0.42 to 0.12 with anaerobic zones in the biofilms increasing from 602 µm after 72 h to 1940 µm after 264 h of single capillary MGR operation.
- The Monod saturation constants used to model experimental profiles were in the range of 0.041 to 0.999 g/m^3 and the uptake rate of oxygen in the biofilm was in the 894.53 to 2739.70 $g/(m^3.h)$ range.
- Oxygen distribution in aerial mycelia was determined to be similar, with varying distribution in the mycelia closer to the substratum of the bioreactor system due to oxygen uptake rate and active transport of DO to sustain inner mycelia growth.
- Results of biofilm thickness, oxygen penetration, oxygen penetration ratio and the Monod saturation constants were found to be similar to those obtained in submerged mycelia pellets of *P. chrysosporium*, with higher oxygen uptake values achieved in cultures in the MGR.
- Oxygen diffusion coefficient in the range of 7.94E-06 to 1.750E-05 m^2/h and DO flux of 0.27 to 0.7 $g/(m^2.h)$ were determined during the operation of the single capillary MGR systems.

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NOMENCLATURE

Symbols

a, b, c	Experimental coefficients for Taylor's expansion method	(-)
β	Nutrient film coefficient	m^{-1}
β_a	Experimental coefficient in the aerial mycelia	m^{-1}

C	Substrate (oxygen) concentration	g/m^3
C_b	Oxygen concentration in air	g/m^3
C_s	Oxygen concentration at the biofilm surface	g/m^3
$D_{a,f}$	Oxygen diffusion coefficient in the aerial mycelia	m^2/h
D_f	Oxygen diffusion coefficient in the biofilm	m^2/h
D_w	Oxygen diffusion coefficient in water at incubation temperature	m^2/h
J	Substrate flux	$g/(m^2.h)$
$J_{a,m}$	Averaged oxygen flux in the aerial mycelia	$g/(m^2.h)$
$J_{f,xs}$	Oxygen flux at the biofilm surface	$g/(m^2.h)$
$J_{w,xs}$	Oxygen flux to the biofilm surface from the nutrient film layer	$g/(m^2.h)$
K_m	Half-saturation coefficient	g/m^3
r_m	Maximum uptake rate of oxygen	$g/(m^3.h)$
x	Biofilm thickness	m
x_s	Biofilm surface	m
$(x - x_s)$	Biofilm penetration depth	m

Abbreviations

DO	Dissolved oxygen
ECS	Extracapillary space
LiP	Lignin Peroxidase
MBR(s)	Membrane bioreactor
MGR(s)	Membrane gradostat reactor
MnP	Manganese Peroxidase
P. chrysosporium	*Phanerochaete chrysosporium*
R^2	Correlation coefficient between experimental and modelled data
R.H.S	Right hand side

Subscripts

f, x_s	Biofilm surface
a, f, x_s	Aerial mycelia region
w, x_s	Nutrient film layer

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