Brazilian Journal
of Chemical
Engineering

ISSN 0104-6632
Printed in Brazil
www.abeq.org.br/bjche

Vol. 33, No. 04, pp. 793 - 799, October - December, 2016
dx.doi.org/10.1590/0104-6632.20160334s20150262

OXYGEN TRANSFER IN THE SOLID-STATE CULTIVATION OF *D. monoceras* ON POLYURETHANE FOAM AS AN INERT SUPPORT

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(Submitted: April 25, 2015 ; Revised: July 24, 2015 ; Accepted: August 23, 2015)

Abstract - In recent years, there has been a resurgence of interest concerning solid-state cultivation processes due to a number of advantages over submerged cultivation. However, studies on oxygen transfer and the performance of bioreactors are lacking. Processes that use inert supports loaded with chemically-defined liquid media have several potential applications and offer numerous advantages, such as improved process control and monitoring and enhanced process consistency compared with cultivation on natural solid substrates. The aim of this research was to study the oxygen transfer for the production of allergenic proteins from a biomass of *Drechslera* (*Helminthosporium) monoceras* cultured in a packed-bed reactor filled with polyurethane foam. The results show that a high protein concentration can be produced on an inert support with a high overall oxygen transfer coefficient (K_La) during cultivation.
Keywords: Solid-state cultivation; *Drechslera* (*Helminthosporium*) *monoceras*; Oxygen transfer; K_La; Inert support; Polyurethane foam.

INTRODUCTION

Solid-state cultivation (SSC) has been defined as a process that involves a solid matrix and is carried out in the absence or near absence of free water, but with enough moisture to support the growth and metabolism of microorganisms (Pandey, 2003). Compared with submerged cultivation, higher biomass, higher enzyme production and lower protein breakdown contribute to the better productivity of enzymes by SSC (Viniegra-González *et al*., 2003).

SSC employs a support, which could be either a natural source of nutrients or simply an inert support impregnated with the nutrients required for the development of the microorganisms (Pandey, 2003). In our previous work, a packed bed column bioreactor, using wheat bran as natural support, was used for *Drechslera (Helminthosporium) monoceras* cultivation and protein production (Bastos *et al*., 2014). Besides natural supports, several inert materials have also been reported in the literature. Among them is polyurethane foam (PUF), which is cost-effective and has excellent thermal insulation, enhanced durability, flexible mechanical properties, versatility, light weight properties, recyclable, non-toxic, totally inert to most chemicals used in biological experiments and easy to manipulate (Banik *et al*., 2014). A biologically inert polyurethane foam was used as a supporting medium by Banik *et al*. (2014) for mass production of spores of *Beauveria bassiana*. These authors demonstrated that PUF can be a very useful support by producing a high number of spores per gram of medium in a short time of incubation, with ease spore extraction and reusability of the same

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mycelia. The use of an inert support loaded with a culture-specific liquid medium provides a constant physical structure throughout the process and facilitates reproducible and detailed physiological and kinetic studies in SSC, which will eventually be the basis for efficient process development, control strategies and reactor design (Gelmi *et al*., 2000; Ooijkaas *et al*., 2000). In addition, because the structure of the inert support cannot be altered by microorganisms or microbial metabolites, it can prevent agglomeration and limitations to heat and mass transfer (Chen, 2013).

Concerning the microorganisms used in SSC, filamentous fungus are the most important group of microorganisms used in SSC processes owing to their physiological, enzymological and biochemical properties. The hyphal mode of fungal growth and their good tolerance to low water activity (a_w) and high osmotic pressure conditions make fungi efficient and competitive in natural microflora for the bioconversion of solid substrates (Mienda *et al*., 2011). Filamentous fungi, beyond being the preferred source of industrial enzymes because of their excellent capacity for extracellular protein production (Jun *et al*., 2011), are associated with a number of allergic diseases in humans (Menezes *et al*., 2004). *D. monoceras* is a filamentous fungi soil saprophyte that produces allergenic proteins that cause respiratory allergies in atopic individuals (Menezes *et al*., 1995). Although there are few works in the literature concerning this fungi cultivation for the production of extracts for use in pathogenicity studies, their characteristics suggest that SSC could be a suitable fermentation process to produce allergenic extracts. Menezes *et al*. (1995) carried out studies to identify allergenic proteins from the crude extract of a *D. monoceras* biomass cultured by submerged fermentation. Hasan *et al*. (2003) produced allergenic extracts from a *D. monoceras* biomass cultured by SSC using wheat bran as the substrate. Bastos *et al*. (2014) studied oxygen transfer as a function of the initial moisture content in SSC with *D. monoceras* under controlled moisture conditions.

In aerobic fermentation, particularly in submerged culture, oxygen transfer from the gas phase to the liquid is undoubtedly the most important phenomenon to sustain microbial activity. The rate of oxygen transfer to the cells is often the limiting factor that determines the rate of biological conversion. Rajagopalan and Modak (1995) concluded, using a model developed for the growth of a unicellular organism in a biofilm of constant density, that oxygen is more likely to limit the growth within the film than lack of glucose, even under conditions where the outer edge of biofilm is high in oxygen concentration. In SSC, microorganisms grow within or on the surface of solid particles, which are surrounded by thin liquid films. Because of the relatively little amounts of liquid in the growth environment, microorganisms are in close contact with the gaseous oxygen that flows in the void space of the packed bed. The oxygen mass transfer to the microorganism in SSC is similar to that of a submerged cultivation. However, the overall oxygen transfer coefficient (K_La), used to quantify oxygen transfer to the fermentation medium, is not the same as that for submerged cultivation (Thibault *et al*., 2000).

Despite the great importance of studies on the oxygen transfer in SSC, many aspects pertaining to this phenomenon are yet to be studied in detail. In this context, the aim of this research was to extend the findings of previous studies (Bastos *et al*., 2014) by focusing on the influence of a packed-bed reactor filled with PUF on oxygen transfer in SSC for the production of allergenic proteins from a biomass of *D. monoceras*. In addition, this work included the study of moisture content throughout the fungal fermentation, not limited only to the analysis of the initial moisture, as well as an analysis of the influence of the liquid film thickness on the oxygen transfer rate.

MATERIALS AND METHODS

Solid Support

The inert solid support, PUF was washed several times with warm water, dried and cut into cylinders (30 mm in height and 30 mm in diameter). The apparent density and the maximum water absorption capacity were determined according to Lareo *et al*. (2005). The surface area and the porous volume of solids were determined by physisorption isotherms using the BET technique (Surface Area ASAP 2010). PUF images were obtained by microscopy (Leica model DM LM, England) and measurement of the liquid film thickness and the solid support thickness were made using the softwares LEICA Quips e LEICA QFAB.

Inoculum

The ICB-USP k-1-16, CBS15426 strain of *D. monoceras* used was a gift of the culture collection of the Laboratory of Microbiology, Biomedical Science Institute, University of São Paulo (USP), Brazil, and was maintained at 25 °C on potato dextrose agar

submerged in mineral oil. The subculture was prepared according to Hasan *et al*. (2003), in a 1-L flask containing slant agar and a modified Czapeck broth (CB): 2 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g KCl, 0.01 g $FeSO_4$, 15 g maltose, 15 g dextrose, and 10 g tryptone, balanced to a total of 1.0 L in distilled water and pH 6.8-7.0. The inoculum was obtained through suspensions in sterile water from 14 day-old colonies of biomasses from the agar surface. The biomass concentration in the inoculum was estimated by absorbance measurements at 550 nm using a standard curve relating absorbance and dry biomass weight (Menezes *et al*., 1995).

Experimental System

Figure 1 represents the experimental setup and oxygen transfer mechanism in the PUF. 12 glass column bioreactors (180-mm height and 30-mm diameter), like that represented in Figure 1 were used, which were attached to humidifiers immersed in a thermostatic bath at 25 °C for temperature control. Air was fed from an odontological compressor (1 HP power), and the flow rate was regulated by rotameters. A thermohygrometer (Digi-sense® Temperature/Humidity Logger) was placed at the bottom of the columns to monitor the relative humidity of the air. An oxygen analyzer (YSI Model 5300, Yellow Springs, USA) was used to measure the oxygen concentration at the outlet of the system.

Solid-State Cultivation

SSC was carried out in the packed-bed columns, and the experimental setup was adapted from Hasan *et al*. (2003). The PUF was loaded with CB nutrients. The support was sterilized for 15 min at 121 °C and adjusted to a final moisture content with deionized water. The flow rate of the inlet gas was monitored by rotameters at 0.4 and 1.0 L/min.

Crude extracts were obtained after the solids had been separated from the support according to Hasan *et al*. (2003). Briefly, deionized water (1:15 w/v ratio) was added and the solution was adjusted to pH 9.0 and incubated in a shaker at 30 °C for 16 h. The suspension was filtered and centrifuged at 10,000 g for 10 min. In this extract, the protein concentration was estimated by the Bradford assay method (Bradford, 1976), and the glucose level was assessed by an enzymatic method using a commercial kit (Laborlab, Brazil). The moisture content of the PUF during the experiments was determined by drying the solid for approximately 24 h at 105 °C until a constant weight (Shojaosadati and Babaeipour, 2002). All glucose, protein and moisture analyses were performed in duplicate and the standard deviation was calculated.

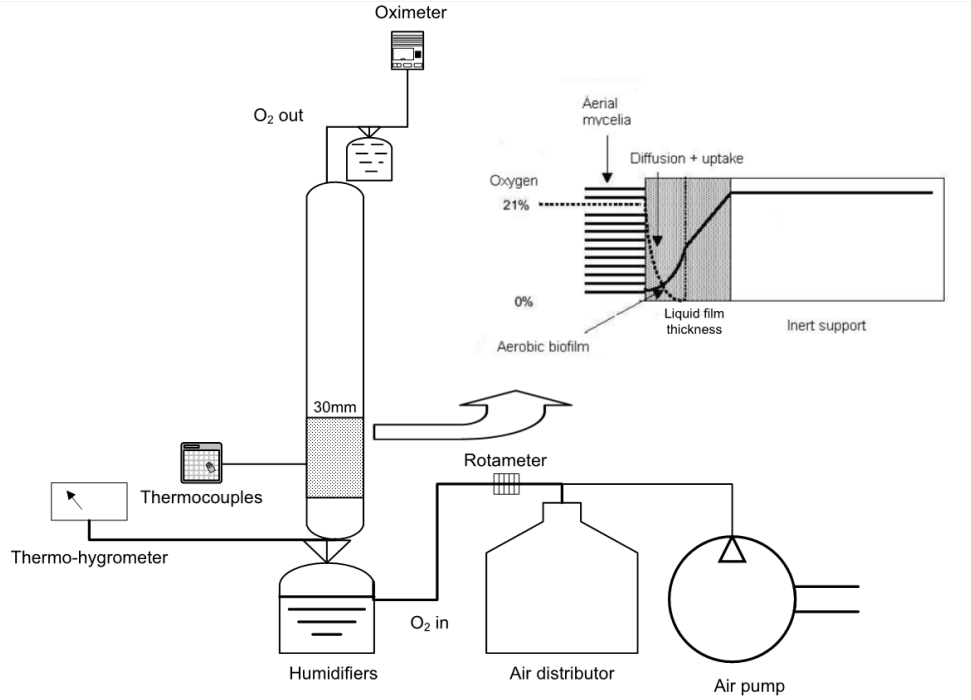

Figure 1: Experimental setup and oxygen transfer mechanisms in SSC on an inert support.

Estimation of the Overall Oxygen Transfer Coefficient (K_La)

The mechanism of oxygen transfer from the gas phase to the microorganism was determined by the static liquid film mass transfer resistance as described by Thibault *et al*. (2000). The overall oxygen transfer rate (N) can be stated by Equation (1):

$$N = \frac{1}{RV_S}\left[\left(\frac{Fp_{O_2}}{T}\right)_{in} - \left(\frac{Fp_{O_2}}{T}\right)_{out}\right] \quad (1)$$

where R is the ideal gas constant; V_S is the solid support volume in the bed; F is the gas flow rate; p_{O2} is the partial pressure of oxygen in the gas; and T is the absolute temperature. The total air pressure was assumed to be standard atmospheric pressure, i.e., 760 mmHg. An oxygen mass balance allows the calculation of the average rate of oxygen consumption in the liquid film (Equation (2)):

$$R_{O_2} = \frac{\left((FC_G)_{in} - (FC_G)_{out}\right)}{V_L} \quad (2)$$

where R_{O2} is the average oxygen consumption rate per biofilm volume; and V_L is the liquid volume in the packed-bed. To calculate the dissolved oxygen concentration in the liquid film, Equation (3) was used to calculate the radial oxygen concentration profile assuming a zero order consumption reaction:

$$\left(\frac{C_L(r)}{C^*}\right) = 1 + \frac{1}{6}\left(\frac{R_{O_2} R_2}{D_{O_2,L} C^*}\right) \left[\left(\left(\frac{r}{R_2}\right)^2 - 1\right) + 2\left(\frac{R_1}{R_2}\right)^3 \left(\left(\frac{R_2}{r}\right) - 1\right)\right] \quad (3)$$

where $C_L(r)$ is the oxygen concentration in the liquid film at the "r" position; r is the radial position coordinate; C^* is the equilibrium oxygen concentration; $D_{O_2,L}$ is the oxygen diffusivity in the liquid, based on Fick's law of diffusivity (Doran, 1995); R_1 is the support thickness; and R_2 is the combined thickness of the solid support and the liquid film. To estimate C_L, the $D_{O_2,L}$ value (at 25 °C) was assumed to be 2.5 x 10^{-9} m^2/s. Thus, K_La was determined according to Equation (4):

$$N = K_La(C^* - C_L) \quad (4)$$

where C_L is the dissolved oxygen concentration in the liquid film as calculated in Equation (3) for different biofilm thicknesses in the range of 5 to 45 μm. The liquid film thickness was calculated by subtracting the solid support thickness (R_1), measured from dried columns as control, from the thickness of solid support and liquid film (R_2).

RESULTS AND DISCUSSION

Solid Support Characterization

The PUF used in the experiments had a 0.0275 g/cm^3 density, 67% porosity, superficial area 3.49 m^2/g and 0.07 cm^3/g porous volume. Table 1 presents the liquid film thickness for different moisture contents. According to Figure 2, a high liquid film thickness was observed, which enables considerable fungal growth. Moreover, the observed void space of the bed also leads to a development of aerial mycelia. These findings indicate that the fungus could grow in the liquid film and the aerial phase of the PUF, suggesting that PUF could be used as a solid matrix for the SSC of filamentous fungi.

Table 1: Physical properties of the PUF.

Moisture (g/100 g)	Liquid film thickness (μm)	Standard deviation (μm)
25	14.45	2.65
35	17.85	2.60
45	18.04	2.95
94 (water saturation)	24.10	2.98

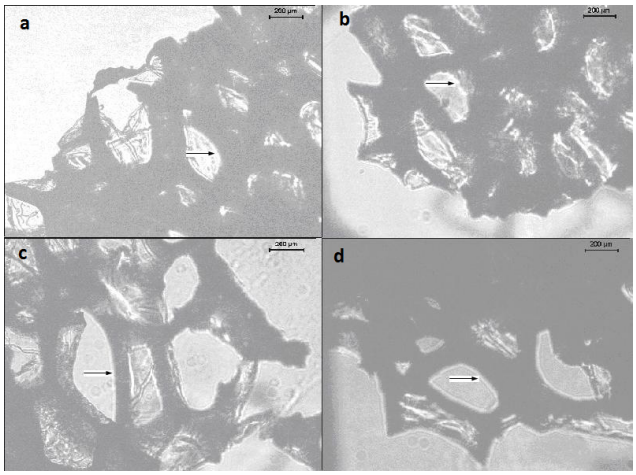

Figure 2: Micrographs of PUF at different moisture contents: 25% (a), 35% (b), 45% (c) and saturation (d). Arrows indicate liquid film on the porous superficies.

Solid-State Cultivation on an Inert Support

Figure 3 shows the solid medium moisture contents for 0.4 and 1.0 L/min air flow rates. For both air flow rates, there was an increase in the moisture content, as found by Bastos *et al*. (2014) and Motta and Santana (2014), who used saturated air in the column inlet. The results indicate that the moisture content increased more considerably at 0.4 L/min, leading to high microbial activities and poor water evaporation rates. Thus, there exists an equilibrium between the water produced by microbial respiration and the water evaporation from the bed at 1.0 L/min, which maintained a moisture level close to the optimal and initial values.

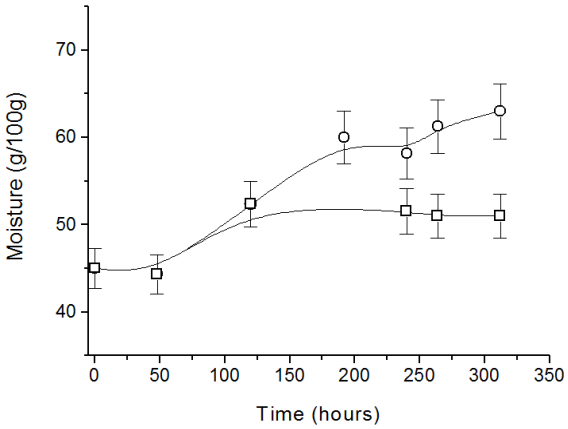

Figure 3: Moisture profile at 0.4 (o) and 1.0 L/min (□) air flow rates.

According to the behavior of the solid medium moisture, the minimum K_La values for 0.4 and 1.0 L/min were similar and were both approximately 20 s^{-1} (Figure 4). However, a greater change was observed in the K_La value with respect to liquid film thickness variations at 0.4 L/min. Thus, high air flow rates in SSC lead to high values of K_La for film liquid and a nearly constant moisture content. These values were higher than the values found in experiments using natural supports, such as in the work of Gowthaman *et al*. (1993), that used wheat bran and *A. niger* for the production of the enzyme amyloglucosidase at various air flow rates, and in the work of Bastos *et al*. (2014), where *D. monoceras* was cultured on wheat bran in a packed bed column bioreactor.

Figure 5 presents the protein content extracted from the fungi. The results indicated that a higher protein content was obtained than that from *D. monoceras* on wheat bran supports in packed bed columns (Hasan *et al*., 2003). These values may be attributed to the growth of *D. monoceras* in the liquid and aerial phases. The fungal growth in the liquid film formed on the outer surfaces of the PUF and the development of aerial mycelia within the pores contributed to the protein content measured in the Bradford assays (Bradford, 1976). In cultures of *Aspergillus niger* on a wheat flour model substrate, the aerial hyphae contributed up to 75% of the fungal oxygen uptake rate (Rahardjo *et al*., 2002). It is suggested that the high respiration rates and the fast development of biofilm and aerial hyphae were obtained by the cultivation of fungus on inert supports having high void space. These growing conditions may explain the high values of protein content found in our study. These observations were confirmed when analyzing the data presented in Figure 6, i.e., fast fungal growth occurred on the PUF, which led to glucose depletion in approximately 150 h for 0.4 and 1.0 L/min air flow rates. The physical characteristics of the inert support allowed efficient oxygen transfer and the development of a biofilm and an aerial phase, which resulted in a high protein content and faster glucose depletion. The ability of the fungus to form an aerial phase and the diffusion of glucose by capillary action are more significant factors than oxygen transfer rates in determining a successful fungal culture. This is because the mass transfer resistance in the gas can be neglected (Rahardjo *et al*., 2006). These findings correlated with the high values of K_La obtained for 0.4 and 1.0 L/min air flow rates.

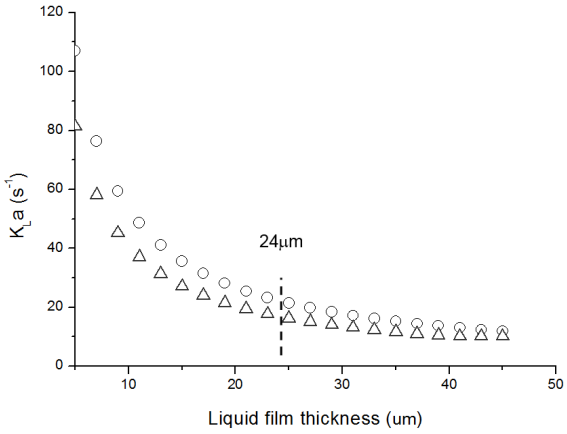

Figure 4: K_La profile at 0.4 (o) and 1.0 L/min (Δ) ait flow rates, indicating maximum film liquid thickness.

The microbial production of water in SSC can be controlled with high air flow rates on an inert support that enable fungal growth in the biofilm and aerial phases. Oxygen transfer problems in SSC can be overcome using PUF with high porosity and water absorptivity. These results clarify the role of the biofilm and aerial phases on oxygen transfer.

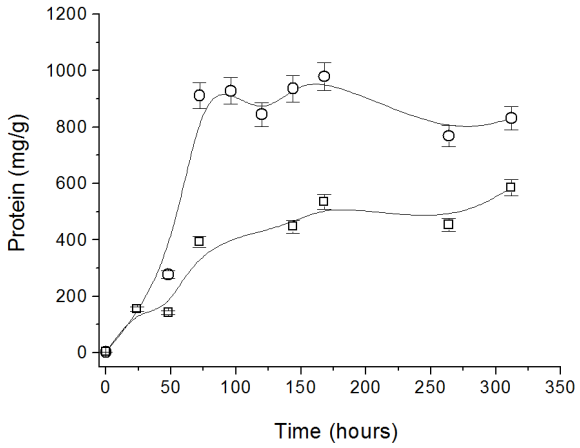

Figure 5: Protein content profile in the fungal extract at 0.4 (o) and 1.0 L/min (□) air flow rates.

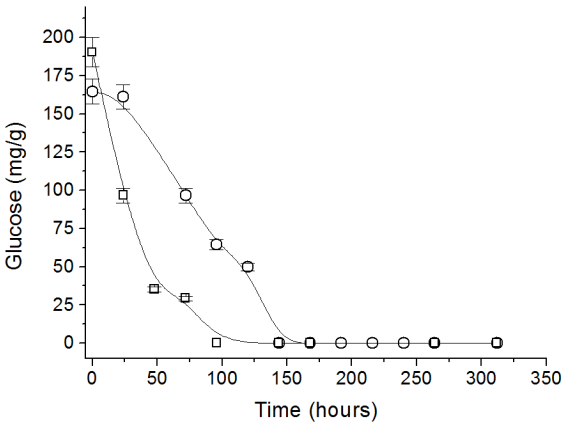

Figure 6: Glucose content profile in the fungal extract at 0.4 (o) and 1.0 L/min (□) air flow rates.

CONCLUSIONS

Moisture content, protein content, glucose concentration and K_La profiles were obtained using an inert PUF support. According to the results, the physical structure of the inert support did not change during culture and maintained a high porosity and nearly constant liquid film thickness. These conditions promoted the development of microorganisms in the biofilm and aerial phases and also led to high values of K_La. The lower air flow rate used promoted higher moisture content and film liquid thickness, which allowed a higher protein production by *D. monoceras*. According to these results, the use of nutritionally inert materials for SSC is promising for control processes, scale-up strategies and the study of oxygen transfer.

ACKNOWLEDGEMENT

The authors gratefully acknowledge Prof. Walderez Gamballe for the donation of the fungal strain and the Brazilian agencies CNPq, CAPES and the University of Campinas, Brazil.

NOTATION

C^*	equilibrium oxygen concentration [mmol/L]
C_G	oxygen concentration in the gas phase [mmol/L]
$C_L(r)$	oxygen concentration on the liquid film at the "r" position [mmol/L]
C_L	dissolved oxygen concentration in the liquid film [mmol/L]
$D_{O_2,L}$	oxygen diffusivity in the liquid phase [m/s^2]
F	air flow rate [L/min]
K_La	overall oxygen transfer coefficient [s^{-1}]
N	overall oxygen transfer rate [mmol/s.L]
p_{O2}	partial pressure of oxygen in the gas [atm]
R	ideal gas constant [atm. L/(gmol.K)]
R_1	solid support thickness [mm]
R_2	thickness of solid support and liquid film [mm]
R_{O2}	oxygen consumption rate per biofilm volume [mmol/s.L]
V_L	liquid volume in the packed-bed [L]
V_S	volume of solid medium [L]

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