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Physical and geometrical considerations on the growth of *Pichia pastoris* in polyurethane foam slabs

Consideraciones físicas y geométricas acerca del crecimiento de *Pichia pastoris* en tiras de espuma de poliuretano

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Abstract

This work introduces the use of polyurethane foam (PUF) slabs for solid-substrate fermentation. Attention is paid to the distribution and properties of the liquid broth within the slabs in relation to the growth parameters of Pichia pastoris. The experimental setup was made of thin slabs supported horizontally by screens within cylindrical chambers. This way, water loss due to compression or gravity drain was minimal and exposure to tangential air flow was maximal. The highest biomass was $X_{max} = 39.5 \pm 3.3\ gL^{-1}$ *with* $S_0 = 150\ gL^{-1}$ *of glucose. Biomass yield,* $Y_{X/S}$ *followed the correlation,* $Y_{X/S} = 0.525 - 0.0018S_0$ *(*$R^2 = 0.996$*), and the growth rate* $\mu = 0.24\ h^{-1}/(S_0/34.6)$*. The respiratory quotient, RQ, followed a biphasic pattern which is typical of many microbial batch cultures and corresponds to the various metabolic stages through the growth process. The use of horizontal PUF slabs with thickness of 0.7 cm seems to be a practical way to follow the metabolic activities of yeast grown as solid-state fermentation (SSF) under controlled air flow and temperature regimes. since a squared meter of such slabs could produce 83 g of yeast.*

Keywords: Polyurethane foam slabs, Solid-Substrate Fermentation, *Pichia pastoris*, respiratory quotient.

Resumen

Este trabajo introduce el uso de placas de espuma de poliuretano en la fermentación de sustrato sólido. Se presta atención a la distribución y propiedades del caldo líquido dentro de las losas en relación con los parámetros de crecimiento de Pichia pastoris. La configuración experimental se hizo con losas delgadas sostenidas horizontalmente por pantallas dentro de cámaras cilíndricas. De esta manera, la pérdida de agua debido a la compresión o el drenaje por gravedad fue mínima y la exposición al flujo de aire tangencial fue máxima. La biomasa más alta fue $X_{max} = 39.5 \pm 3.3\ gL^{-1}$ *con (So) de* $150\ gL^{-1}$ *de glucosa. El rendimiento de biomasa,* $Y_{X/S}$ *siguió la correlación,* $Y_{X/S} = 0.525\text{-}0.0018S_0$ *(*$R^2 = 0.996$*), y la tasa de crecimiento* $\mu = 0.24h^{-1}/(S_0/34.6)$*. El cociente respiratorio, RQ, siguió un patrón bifásico con un RQ máximo* ≈ 1 *cuando* $S_0 = 50\ gL^{-1}$*. Las micrografías de PUF mostraron que el líquido no se retuvo cuando la integridad de la red de poliuretano se rompió en los bordes cortantes de las losas con una profundidad aproximada de 0.025 cm, pero la fracción de PUF interrumpida sería pequeña para losas anchas y h = 0.7 cm. El uso de placas de PUF horizontales con un espesor de 0.7 cm parece ser una forma práctica para analizar las actividades metabólicas de levaduras crecidas sobre un soporte sólido. sujetas a regímenes controladas de flujo aéreo y temperatura.*

Palabras clave: Tiras de espuma de poliuretano, Fermentación sobre sustrato sólido, *Pichia pastoris*, Cociente respiratorio.

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1 Introduction

Yeast metabolism is widely exploited in many biotechnological processes, i.e., in the production of alcohol, wine, beer, and bread (Boekhout and Robert, 2003; De Winde 2003; Corona-González *et al* 2020). On the other hand, *Pichia pastoris* is a methyltrophic yeast, used in the production of heterologous proteins (Zahrl *et al*., 2017; López-Pérez and Viniegra-González 2019) because it has a negative Crabtree metabolism that is not inhibited when the substrate concentration is very high and can be genetically transformed to produce high yields of recombinant proteins. There are numerous examples in the literature, such as the production of phytase (Hang *et al*., 2009) and the production of xylanase (Cayetano-Cruz *et al*., 2016), or production of synthetic proteins (Bartolo-Aguilar *et al*., 2021). Most studies and industrial applications of this yeast are carried out under SmF (submerged fermentation) (González-Hernández *et al* 2012). However, high cell density cultures have a very large biological oxygen demand (BOD) and show significant autolysis (Tschopp *et al*., 1987; Yinliang *et al*., 1997; Li and Xia, 2018). Solid-state fermentation has become interesting for researchers for several reasons, some of the most relevant are its low energy requirements and the little generation of wastewater that is produced, as has e shown I recent studies (Trujillo-Ramírez *et al*., 2022). On the other hand, it is interesting to indicate that both fungi and yeasts evolved under conditions of limitation by water, which allows organisms to grow optimally. The use of polyurethane foam as solid support in Solid-state fermentation (SSF), has a very interesting advantage since using highly porous material with a high specific area, such as polyurethane foam (PUF), which has a high level of passive oxygen transfer (López-Pérez and Viniegra, 2015), shows less autolysis and tolerates high levels of methanol (Pandey and Mitchell, 2000). The use of PUF has as a fundamental element that it allows increasing the area-volume ratio (up to 300 cm-1) with respect to submerged medium (1cm-1), characteristically this physical property facilitates a greater dispersion of the culture, maximizing the air transfer with respect to liquid medium (López-Pérez and Viniegra, 2015). In this sense, most reports on *P. pastoris* have been based on the conventional submerged fermentation (SmF), either in shake flasks or in stirred fermenters (Cayetano-Cruz *et al*., 2016; Shang *et al*., 2017). Under such conditions, bakery yeast (Saccaromyces cerevisiae), would exhibit metabolic changes associated to the Crabtree effect (De Deken, 1966; Petrikm Käppeli and Fiechte, 1983; Van Urk *et al*., 1988; Vasilakou, 2016; Dashko, 2014). This effect occurs as a response to high glucose concentrations in the fermentation culture, where ATP production becomes more dependent on the glycolytic pathway than on oxidative phosphorylation (Thomson *et al*., 2005). However, *P. pastoris* has been classified as Crabtree negative (Veiga *et al*., 2003) and for that matter the yeast controls glucose entry by a H^+ symport system, preventing metabolic overflow (Van Urk *et al*., 1989). López-Pérez and Viniegra-González (2015) suggested that PUF might be useful for the production of recombinant proteins by high cell density cultures of *P. pastoris*, using high initial glycerol concentrations in PUF cubes with length of edges of 1 cm (López-Pérez *et al*., 2010), but these are difficult to make and leak the broth when they are piled up in more than two layers. because gravity overcomes the capillary forces holding liquid to the polymer honeycomb. This makes difficult to study the physiology under well controlled mass and heat transfer conditions because it is difficult to analyze de air flow through various layers of PUF cubes placed in a bottom of a flask. Here, the geometry of thin PUF slabs is proposed using a horizontal reactor where the slabs are held within horizontal screens and the air flows tangentially to the surface of the slabs. The characterization of this system involves the distribution of the liquid broth within the PUF matrix and on-line monitoring of respiratory activity. Here it is shown that such a system yields reproducible physiological results.

2 Materials and methods

2.1 Strains, media composition, and chemicals

The yeast strain used in this work was *P. pastoris* X33 (Mut+) acquired from Invitrogen Thermo Fisher Scientific Inc. (Waltham, MA, USA). It was cultured in the medium proposed by Hang *et al*. (2009), supplemented with citrate-phosphate buffer to maintain pH values higher than 4. The liquid load was 15 mL of broth per gram of PUF below maximum loading capacity.

2.2 Solid-state fermentation

SSF was done in PUF slabs with a thickness, H = 0.3, 0.5, and 0.7 cm and a PUF dry density of 17 kg m^{-3} (Figure 1). PUF slabs were previously washed with hot running water (60 ºC) followed by a wash out of distilled water, at room temperature, then dried at 60 °C and kept away from light. The slabs were arranged within horizontal glass cylinders, supported by sheaths of plastic mesh, which will allow tangential air flow (Figure 1). For the inoculation, *P. pastoris* cells were used after 3 days of activation in shake flasks. After this time, cells were isolated for their incorporation into the PUF with three glucose concentrations 50, 100 y 150 gL^{-1}. In the column, 3 polyurethane foam plates of constant length and width were introduced, but of variable thickness between 3 and 7mm. Each one of the plates was separated from the others by means of plastic meshes with the aim that they did not remain adhered, and that aeration was facilitated to the culture medium embedded

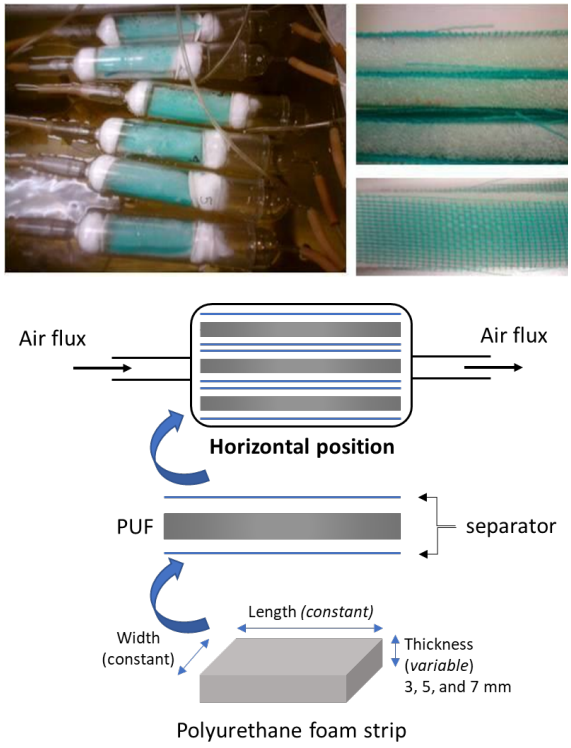

Figure 1. Columns filled with polyurethane foam used in fermentation processes.

in the polyurethane between the plates. The used air stream supplies oxygen to the culture. This air is saturated with water so that the medium does not dehydrate.

2.3 *Biomass, CO_2, and moisture content determination*

Biomass concentration was determined by turbidimetry. A standard curve was previously obtained by measuring optical densities (OD) of *P. pastoris* suspensions. Samples were diluted with a physiological solution and the OD was measured at 600 nm in a Shimadzu UV-1800 spectrophotometer (Japan). Biomass was determined in triplicate at each point of the kinetic experiments. Carbon dioxide concentration was measured on-line with a respirometer (Pliego-Sandoval *et al.*, 2012). Moisture content was determined with a thermobalance (Ohaus, Parsippany, NJ, USA).

2.4 *Kinetic and yield parameters*

Kinetic parameters were estimated using biomass data. Biomass to substrate yield, $Y_{X/S}$ was calculated by the following expression:

$$Y_{x/s} = \frac{X_f - X_0}{S_0 - S_f} \tag{1}$$

Where, X_0 and X_f are the initial and final biomass concentrations. Likewise, S_0 and S_f are the respective initial and final values of substrate concentration. Equation (1) can be simplified to be, $Y_{X/S} \approx X_f/S_0$, because the inoculum, X_0, was 100 times smaller than the final biomass concentration, X_f. and final substrate concentration was negligible ($S_f \ll S_0$). The specific CO_2 production rate was calculated by the logistic equation from the data of CO_2 production versus time, as previously described (Viniegra *et al.*, 2003).

$$\frac{dCO_2}{dt} = \mu_m \left[1 - \frac{CO_2}{CO_{2m}}\right] CO_2 \tag{2}$$

Where, CO_2 is carbon dioxide concentration at a given time (gL^{-1} or g kg^{-1}), μ_m is the maximum specific growth rate (h^{-1}), and CO_{2m} is the asymptotic level of CO_2, for which, $dCO_2/dt = 0$ assuming $CO_2 > 0$. Subsequently, the Solver program was used to estimate μ_m and CO_{2m}. Lag time was measured as the intersection of the linear part of log (CO_2) to the horizontal axis. Finally, the respiratory quotient, RQ, was calculated by the ratio of CO_2 production rate over the O_2 uptake rate at a given fermentation time, as indicated in equation (3).

$$RQ = \frac{\delta CO_2}{\delta O_2} \tag{3}$$

2.5 *Glucose determination and HPLC analysis*

The glucose concentration was assayed according to the specifications of the Spinreact kit, (Girona, Spain) based on the formation of hydrogen peroxide by the action of glucose oxidase, with the concomitant oxidation of phenol in the presence of a peroxidase. Additionally, glucose concentration in the cell-free culture medium was measured by high-performance liquid chromatography (HPLC), using a Perkin-Elmer chromatograph equipped with an LC 250 pump, an LC-30 refractive index detector (Perkin Elmer, Waltham, MA, USA), fit with an ion exclusion column (AminexHPX-87H; Bio-Rad Laboratories, Hercules, CA, USA). The eluent was an isocratic 5 mM H_2SO_4 solution with a flow rate of 0.6 $mLmin^{-1}$ at 65 °C. All samples were passed through 0.45-μ_m Durapore filters (Millipore, Milford, MA, U.S.A), and 20 μL of each sample were injected into the HPLC column.

3 Results

3.1 *Effect of the geometrical dimensions of PUF slabs on their water retention capacity*

Polyurethane is hydrophobic but polyurethane foam has a high-water holding capacity because the liquid is dispersed

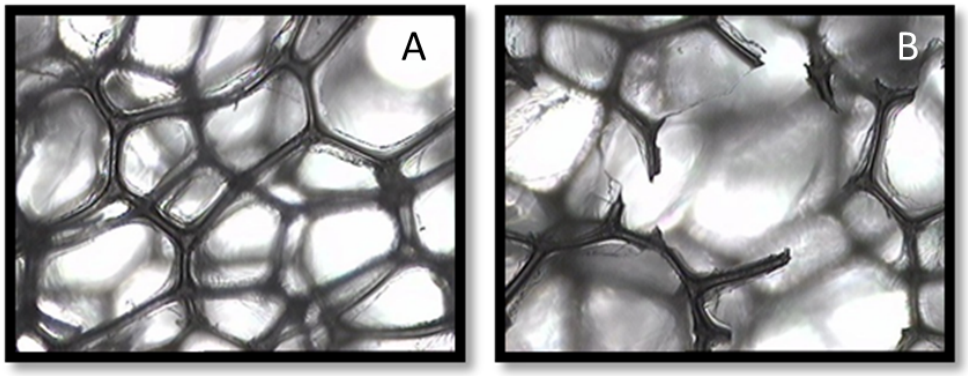

Figure 2. Polyurethane foam micrographs. A: Structure of the intact polyurethane matrix. The effects of polyurethane foam cutting are shown in B, revealing that the formed geometry hinders the formation of water menisci.

and retained in thin lamellae formed, by capillarity within the polymer honeycomb (Fig. 2A) but they cannot be formed if such honeycomb structure is broken (Fig. 2B) as it happens by the shearing of the material during the cutting process. Marín-Cervantes *et al.* (2008) observed that porosity decreased in minced PUF compared to small PUF cubes (1 cm). Direct microscopic observation of PUF slabs impregnated with a methylene blue solution showed a disrupted polymer zone with depth h = 0.025 cm. (Figure 2) Such observations, supported a geometrical model for slab of length, L; width- W, and depth, H, to estimate the relative water retention as a function of the slab geometry where the total volume, V, is given by equation (4)

$$V_T = LWH \tag{4}$$

Assuming a uniform depth h = 0.025 cm of the broken PUF (Fig. 2B) the effective volume V_E, by equation (5) where, h is counted twice because of the slab symmetry.

$$V_E = (L - 2h_c)(W - 2h_c)(H - 2h_c) \tag{5}$$

Defining, $R = V_E/V$; and assuming fixed values of L and W, equation (6) is obtained as a growing function of H

$$R = A\left[1 - \frac{2h_C)}{H}\right] \tag{6}$$

Where, A = (1-2h/L) (1-2h/H). Since water retention proportional to the effective volume with intact polymer honeycomb, the measurement of water retention per unit volume of a set of slabs with different values of H can be followed to estimate parameters A and h. Data for such estimates is shown in Fig. 3 where a growing function of R is obtained for values H(cm) = 0.3, 0.5 and 0.7. Such changes were consistent with h = 0.025. This result can be applied to different PUF geometries. For example, for a cube with edge = c, the effective volume will be given by equation (7)

$$R = (1 - 2h/c)^3 \tag{7}$$

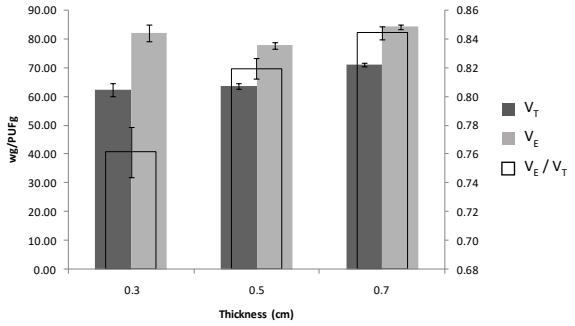

Figure 3. Water retention capacity of PUF. V_T (water retention capacity total). W_E (water retention capability intact). Wg (water gram) PUFg (polyurethane foam gram).

For shallow ($H \approx 1$ cm), but very wide ($W \gg 2h$) and long slabs (L = 2h), the parameters of equation (6) would be, $A \approx 1$, and $R \approx 0.95$, the corresponding cubic size from equation (7) would be $c = 2.95$ cm. This shows that geometry of PUB is an important consideration, both from the water retention capacity and the dimensions of the PUB structure that surely affect mass and heat transfer across the porous structure as will be noted in the Discussion.

3.2 *Effect of the PUF slab thickness and glucose concentration on P. pastoris' mass balance and kinetic growth parameters*

Initial glucose concentrations, S|, (gL^{-1}) from 50 to 150, were chosen to ascertain the effect of PUF slab thickness on *P. pastoris* mass balance, growth and metabolism. Table 1 shows the general effect of hypertonic broths ($S_0 > 50$ gL^{-1}) decreasing biomass yield, $Y_{X/S}$, growth rate, $Y_{X/S}$, and metabolic activity, q, and increasing lag time, as a reflection of overall substrate inhibition. However, there are some differences in the carbon dioxide yield ($Y_{CO2/S}$) that are not easy to explain given the limited number of point data

Table 1. Most relevant values of kinetic and growth parameters found in the fermentation.

	GROWTH PARAMETERS				KINETIC PARAMETERS	
S_0 (g L^{-1})	X_F (g L^{-1})	$Y_{X/S}$ X_F / S_0	$Y_{CO2/}$ (C_M/S_0)	Lag phase (h)	q (mg CO_2/ h g PUF)	μ h^{-1}
			Thickness 0.3 cm			
50	22.27±1.51^{a}	0.458±0.018^{h}	0.1781±0.009^{m}	10.48±0.51^{t}	0.0914	0.338±0.0049$^{c'}$
100	32.44±4.85^{b,c}	0.341±0.025^{i}	0.1868±0.025^{m}	17.52±0.12^{u}	0.0644	0.221±0.0091$^{d'}$
150	30.38±5.15^{b,c}	0.222±0.024^{j}	0.1104±0.027^{n}	27.98±0.25^{v}	0.0231	0.156±0.0125$^{e'}$
			Thickness 0.5 cm			
50	21.87±1.33^{a}	0.455±0.012^{h}	0.2339±0.009^{o}	13.01±0.09^{w}	0.1027	0.319±0.0125$^{c',f'}$
100	29.38±4.88^{b,c,d}	0.308±0.041^{i,j}	0.2881±0.015^{p}	17.78±0.08^{x}	0.0668	0.178±0.0041$^{g'}$
150	31.46±2.31^{b,c,d}	0.226±0.019^{k}	0.1891±0.009^{q,m}	26.76±2.01^{v,z}	0.0340	0.133±0.0091$^{h'}$
			Thickness 0.7 cm			
50	23.75±1.11^{a,e}	0.489±0.025^{k,l}	0.3112±0.0001^{p,r}	12.92±0.31$^{w,a'}$	0.1209	0.291±0.0125$^{i'}$
100	36.16±1.65^{b,c,f}	0.379±0.021^{h,i}	0.3238±0.0251^{p,r}	18.06±0.02$^{b'}$	0.0701	0.161±0.0035$^{e'}$
150	39.48±3.31^{b,f,g}	0.277±0.031^{j,i}	0.2721±0.0231^{s,p}	25.29±1.51$^{v,z,b'}$	0.0422	0.115±0.0001$^{j'}$

S_0 ; initial glucose concentration: X_f; final biomass concentration; $Y_{X/S}$; biomass yield; $Y_{CO2/S}$; carbon dioxide yield; q; specific respiration rate; μ = specific growth rate. q: specific rate production of CO_2; Statistical comparisons of parameters were made using the conventional analysis of variance (ANOVA) technique and Tukey's multiple comparisons, using $P < 0.05$ as the set point of significance. Different letters superscripts (a to j'), indicate significant differences for equivalent columns having the same parameter($P<0.05$)

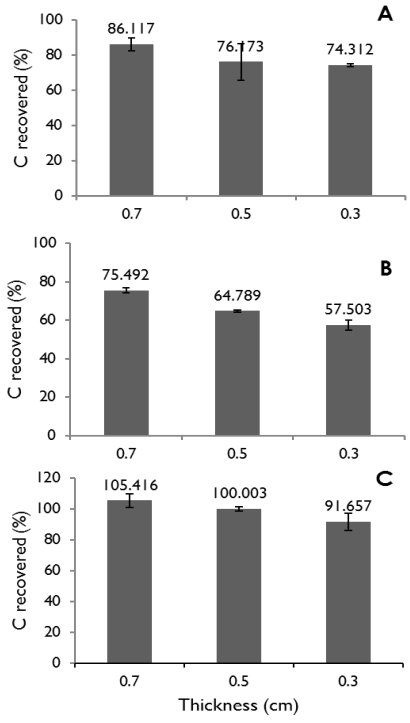

Figure 4. Carbon balance in the kinetics determined with different initial glucose concentrations and different polyurethane foam thickness [A] 150 g L^{-1}, B) 100 g L^{-1}, and C] 50 g L^{-1}].

for each experiment but could be related to the low carbon recovery observed with high glucose levels, shown in Fig. 4. On the other hand, Figure 4 shows the carbon balances made with the same conditions described in Table 1. To calculate the partial balances of carbon, ethanol production was omitted, because it could not be recovered due to the lack of a condenser system. It was noted, however, that using the measurement of three parameters: a) biomass, b) CO_2, and c) citric acid, which, at the end of the kinetic balance, resulted in 100% of sheets approaching S_0 = 50 gL^{-1}; being lower for S_0 = 100 and 150 gL^{-1}. In the figure it can be observed independently for the three initial concentrations of substrate, the thickness of 7mm yielded the best recovery data, which corresponds to the data presented in the table, also under these experimental conditions the higher data in the growth parameters, which may be due to its greater thickness, since a greater volume of polyurethane foam allows greater water retention compared to the other two conditions. On the other hand, the figure also shows a notable decrease in recovered carbon as the concentration of glucose in the medium increases. Going from recovery values of 100% for a concentration of 50 gL^{-1}, to values of 75 and 86% respectively at concentrations of 100 and 150 gL^{-1} for 7mm of thickness. For the 5mm thickness, a similar effect is observed, going from recovery values of 100% for 50gL^{-1} to a decrease of 64.7 and 76.1 respectively for concentrations of 100 and 150gL^{-1} respectively. Effect that has the same trend, but with smaller recovery values for the 3mm thickness.

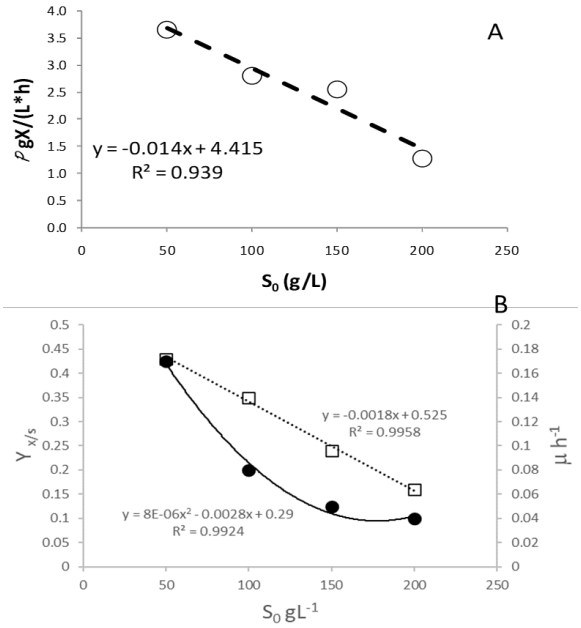

Figure 5. A. Glucose, initial concentration effect (S0) on the biomass productivity calculated as $P = Y_{X/S} S_0 \mu_{\max}$. B. Correlation between $Y_{x/s}$ (■) and specific growth rate (♦) μh^{-1} with respect to initial glucose concentration.

3.3 *Effect of the initial glucose concentration (S_0) on productivity*

Data reveal a glucose consumption greater than 99% for the 50, 100, and 150 gL^{-1} initial concentrations and only 65% for 200 gL^{-1}. The increase of the substrate's initial concentration ($S_0 \geq 50$ gL^{-1}) affected the yield ($Y_{X/S}$) and the rate of growth ($\mu_{\max}$), and reduced productivity, as shown in Figure 5A. The negative linear correlation concerning S_0 showed a maximum value (extrapolated) of $Y_{X/S} \approx 0.5$, which would be expected if the culture was adequately oxygenated. These results indicate that an excess of substrate reduces the biosynthesis efficiency. In other words, there are limitations of oxygen transfer to the interior of the polyurethane foam matrix, limitations that are suppressed or attenuated significantly as a result of reducing the biological oxygen demand (BOD) that is proportional to the S_0. Also, it can be observed that the growth of *P. pastoris* is inhibited in hypertonic media ($S_0 > 50$ g/L). Additionally in figure 5B represents the variation of the averaged value for the three yield thicknesses $Y_{x/s}$ with respect to the specific growth rate, finding that for low concentrations of substrate 50gL^{-1}, the yield values and specific growth rate are maximum, and as the amount of substrate in the medium increases, both values decrease dramatically, reaching values of 0.1 biomass gram per glucose gram and 0.05 h^{-1}, respectively. Additionally, it is pertinent to emphasize that the values for yield decrease following a linear model, while the specific growth rate decreases following a quadratic model, producing the greatest decrease when glucose is exceeded 90gL^{-1}, subsequently it continues to decrease in a lesser proportion. until reaching values around 0.04 h^{-1} with 200 gL^{-1} of glucose.

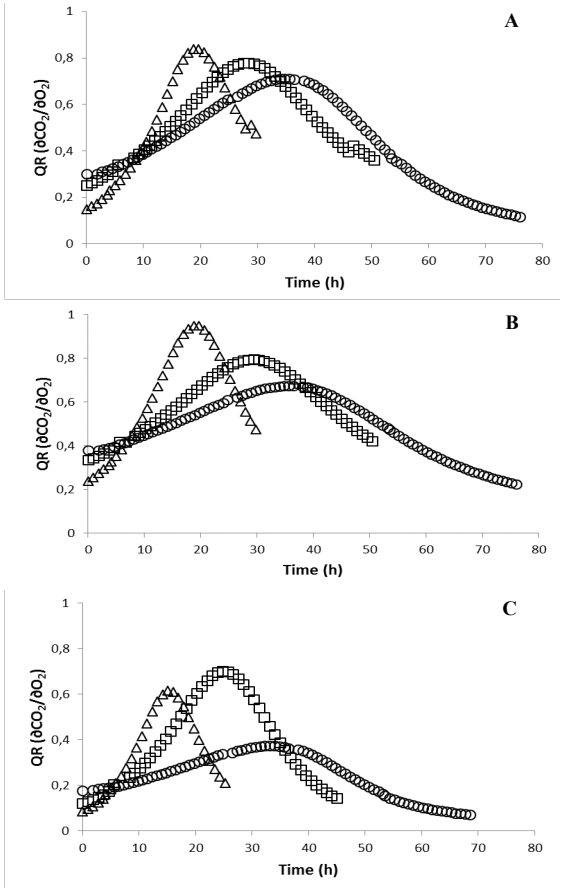

Figure 6. Respiratory quotient profiles. Data are presented for each of the thicknesses. A, 0.7 cm; B, 0.5 cm; and C, 0.3 cm. In each of the thicknesses measured, the respiratory quotient was determined for three glucose concentrations. Δ: 50 gL^{-1}, □: 100 gL^{-1}; o: 150 gL^{-1}.

3.4 *Effect of the polyurethane foam slabs thickness and glucose concentration on the respiratory quotient*

Figure 6 presents the evolution of the respiratory quotient (RQ) as a function of time at different initial glucose concentrations and different thicknesses of PUF. The respiratory quotient (RQ) variation was analyzed depending on two parameters: a) different initial glucose concentrations and b) different PUF thicknesses. It is interesting to emphasize that all the experiments corresponded to diphasic curves of RQ on time. For all experimental conditions, at the beginning of the fermentation, RQ < 0.4 grew progressively

until reaching a maximum value that was never greater than the unit, and then decreased. Particularly, it is important to note that for conditions with a 50 gL^{-1} as initial glucose concentration and a thickness of 0.5 cm, (Fig 6B) the respiratory metabolism of glucose was complete (RQ > 0.9) and the RQ decline is due to glucose exhaustion. For conditions where glucose concentrations were greater than 50 gL^{-1} and PUF thickness of 0.3 and 0.5 cm, (Fig 6B and C) the RQ value was less than 0.9. Finally, the maximum values of the respiratory quotient, $0.9 \leq RQ \leq 1$ corresponded to S_0 = 50 gL^{-1}; in all slabs with S_0 = 150 gL^{-1}, the respiratory quotient was in the range: $0.4 \leq RQ \leq 0.7$. All the curves were biphasic but the width of such curves increased with S_0. This is consistent with a general inhibition of glucose uptake and metabolic activities by hypertonic sugar solutions. Lower RQ values were with smaller H values suggest possible metabolic deviations related to slab thickness.

Discussion and Conclusions

The metabolism of *P. pastoris* has been studied in depth. Although polyurethane foam slabs have been used in other studies, such as wastewater treatment (Henry and Thomson., 1993), the use of glucose on an inert carrier, such as polyurethane foam in slabs configuration, to assess *P. pastoris*' growth physiology has not been done previously. The main purpose of this work was to show the feasibility and practical advantages of using thin horizontal PUF slabs (Fig. 1) for studying the physiology of *Pichia pastoris* in an experimental set-up with well defined geometry and air flow conditions by means of online analysis of the respiratory rates. This is justified because *P. pastoris* it is a common industrial organism for the production of transgenic properties when grown in PUF (López-Pérez, *et al*, 2010; López-Pérez and Viniegra-González, 2015). The use of horizontal slabs is also justified because liquid broths leach out in conventional packed bed, vertical columns. Also, current use of few layers of PUF small cubes, do not seem amenable for studying the effect of mass transfer phenomena of yeast cultures. The effect of mass and heat transfer on the kinetics of *Aspergillus niger* grown on shallow beds of perlite has been studied by Figueroa-Montero *et al*. (2011). But, to the best of our knowledge, this is the first report using PUF as inert support. It is worth noticing that small pieces of PUF are currently used as a model support for SSF because in PUF it is easy to recover biomass and products at the end of each fermentation experiment (López-Pérez and Viniegra-González, 2015). However, little attention has been paid to the distribution of liquid broths within the web polymer structure. In this work it is described and analyzed such distribution attributed by others (Romero-Gómez *et al*., 2000; López-Pérez *et al*. 2010, Lima-Pérez *et al*., 2018) to the formation of small lamellae of liquid broth in the regions where the polymer honeycomb is intact, but little attention has been paid to liquid runoff in the regions where PUF honeycomb is broken by shearing the material when it is been cut in small pieces. This subject matter was studied here, by direct microscopy and also by measuring water retention in PUF slabs of same width and length but different depth this justified a simple model related to a disruption depth h = 0.025 cm, where relative water retention, R. increases with the slab macroscopic depth, H. Extrapolation of slab to cubic geometry showed that the equivalent cubic dimension (c, cubic edge), with similar, R, was almost thrice the slice depth, ($c \approx 2.95$ H).

From this result it appeared the question whether differences in slab depth, H, would produce significant physiological changes for the yeast culture. In this work it is shown that such differences exist when the differences in slab thickness are in the range 0.3 cm < H < 0.7 cm, in physiological features such as, metabolic balances and respiratory quotient, linked to the fate of used up glucose. But there are of minor consequences in relation to overall parameters such as, biomass yield, growth rate or lag time.

The use of conventional packed bed PUF vertical columns is not practical because gravity force produces liquid runoffs. But the use of thin horizontal slabs, as shown in Fig. 1 is amenable for respiratory and physiological online measurements without liquid run-offs.

Despite the fact that we did not analyze the expression profiles of the genes involved in this metabolism activation, the large differences obtained under these experimental conditions suggest that there is a differential growth physiology for low and high initial concentrations of glucose (Bauer and Pretorius, 2000). Considering this Crabtree negative condition with the biomass production data, it is important to point out that these types of yeast have higher affinity systems for glucose uptake than yeasts like *Saccharomyces cerevisiae* (Does and Bisson, 1989; Ozcan and Johnston, 1999). In addition, decreased yield values and net values of biomass produced, as shown in Table 1, allow inferring that *P. pastoris* inhibits high affinity receptors, preventing the entry of glucose. The fact that *P. pastoris* is Crab negative supports the use of high substrate concentrations because its metabolism is not inhibited by them. However, the mass balance expressed recovery was found to be an inverse function of slab thickness. This could be related to higher losses of volatile compounds such as ethanol that was not measured int this work. For such reasons the thickness H = 0.7 cm was chosen for the rest of the physiological experiments.

Pichia pastoris is a common model for expression and production of transgenic proteins and has been shown (López *et al*., 2010) that such process can be done in PUF cubes, with low levels of autolysis, significant resistance to methanol and high yields of transgenic laccase but there is a need to link the respiratory physiology to the biosynthetic activity and for this reason it was necessary to explore the use of PUF thin slabs together with online

respiratory measurements, including the mass balance of the fermentation process with was found satisfactory as shown in Fig. 4, This shows the presence of a complex and heterogeneous metabolism involving aerobic and anaerobic pathways, possibly in different regions of each slab which is characteristic of solid-state fermentations. However, the mass balance expressed as carbon recovery was found to be an inverse function of slab thickness. This could be related to higher losses of volatile compounds such as ethanol that was not measured int this work. Figure 5A shows biomass productivity in terms of the liquid broth volume, since this is the significant variable related to product recovery that is known to be the major cost in a fermentation process. The results show a strong linear decrease that was explained by a decrease of biomass yield ($Y_{X/S}$) and specific growth rate, μ. Based on these investigations and the results obtained in terms of biomass production and yield values, it is possible to infer that *P. pastoris* can use glucose at the same time by the aerobic and anaerobic pathways and, therefore, produce biomass and fermentation products with yields that are going to vary depending on the glucose concentration and the oxygenation degree of the culture, which, in this case, is continuous, diffusive, and depends on the PUF slabs' thickness. In addition, we found low yield levels, although there was no oxygen limitation; in this sense, Kern *et al*., 2007, concluded that a possible cause for the low yields found with *P. pastoris*, when grown in the presence of glucose, is the strong induction of an alternative oxidase, whose catalytic site has a lower thermodynamic efficiency, producing more heat in detriment of the chemical energy in terms of ATP, which, consequently, affects the production of biomass (Kern *et al*., 2007). The respiratory quotient (RQ) is a physiological indication of the type of net carbon mass balance per unit of oxygen uptake. A value RQ = 1 shows a complete oxidation to carbon dioxide, lower values suggest the accumulation of collateral products. Figure 5 shows the effect of substrate concentration S_0 and slab thickness H. From the analysis of the RQ curves a general pattern is shown, the dispersion (width) and height of RQ curves is larger with higher values of S_0 which is consistent with the inhibition effect of high sugar concentration on growth rate. But the RQ pattern seems to be similar of thickness H = 0.5 cm and 0.7 cm. For H = 0.3 the patter was modified with lower RQ values. Perhaps this could be related to a critical specific area of the liquid broth which will increase with lower H. Nevertheless, those results show the feasibility of doing reproducible respiratory measurements which was one of the major goals of this work, wince it is known that changes in RQ are related to different phases of the growth cycle and are related to significant changes in the regulatory network of microbial cells. For example, different blocks of enzymes and proteins are produced in each phase of the RQ cycle. This respiratory pattern indicates that, most of the time, a significant part of the metabolism is different from the classic aerobic metabolism with RQ = 1 and cannot be explained only by the ethanolic fermentation. However, it could be explained by the formation of some partially oxidized products, such as citric acid, consuming oxygen, without release of CO_2. These results are confirmed by a previous study by Hang *et al*., 2009, who identified that the full respiratory metabolism of glucose is established with values of QR $\geq$ 0.9 (Hang *et al*., 2009). As a conclusion we have shown that, thin horizontal PUF slabs are a practical set up for future physiological and mass transfer studies of SSF, opening the way for correlations between, metabolomics, genomics and physiology of important industrial microorganisms such as *Pichia pastoris* when grown on solid support. This is part of a long-term research project on the use of solid-state fermentations for the production of high value products such as transgenic proteins or biopesticides.

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Nomenclature

RQ	Respiratory quotient
PUF	Polyurethane foam
SSF	Solid-state fermentation
HPLC	High-performance liquid chromatography
V_T	Total capacity water retention
V_E	Intact capacity water retention
SRP	Specific rate CO_2 production (Mg CO_2/ h g PUF)
μ	Specific rate growth (h^{-1})
BOD	Biological oxygen demand
AOX	Alternative oxidases
AOD	Alternative oxidases
A_w	Water activity

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