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DIFFERENTIAL TOXICITY CAUSED BY METHANOL ON THE GROWTH OF *Pichia pastoris* CULTURED IN SOLID-STATE AND IN SUBMERGED FERMENTATION

TOXICIDAD DIFERENCIAL CAUSADAPOR METANOL EN ELCRECIMIENTO DE *Pichia pastoris* INCUBADA EN MEDIO SÓLIDO Y MEDIO LÍQUIDO

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Abstract

Growth kinetics of *P. pastoris* and the toxic effect produced by methanol in solid-state (SSF) and submerged fermentation (SmF) were compared. SmF cultures of P pastoris presented moderate lysis without methanol (MetOH) after 72 h. Addition of MetOH has negligible effect on SSF system at the same conditions. MetOH addition produced lysis in both types of cultures, but nearly three times more for SmF than for SSF. Maximal biomass levels were higher (52 gL^{-1}) for SSF as compared to SmF (42 gL^{-1}). Microscopic observations showed smaller yeast aggregates in SmF as compared to larger aggregates in SFF. This is the first work that shows lower yeast autophagy when cultured in large aggregates within the polyurethane foam as well as lower methanol toxicity as compared to shake flask cultures. Such results could be explained in terms of possible cell to cell communication and also in terms of diffusion barriers to methanol in large yeast aggregates. This work opens the possibility of using polyurethane foam support of SSF systems as a way to increase recombinant protein production using glycerol as starting substrate and methanol as a late inducer of specific operons.

Keywords: *Pichia pastoris*, methanol toxicity, solid-state fermentation (SSF), submerged fermentation (SmF).

Resumen

La cinética de crecimiento de *P. pastoris* y los efectos tóxicos derivados de la presencia de metanol en el medio, fueron comparados en medio sólido (SSF) y medio líquido (SmF). Los cultivos SmF presentaron lisis moderada a las 72h sin metanol, comparados con la ausencia de lisis que se observó en los cultivos SSF. La presencia de metanol causó lisis en SmF y SSF, sin embargo, fue tres veces mayor este efecto en SmF. La biomasa máxima producida en SSF fue 52 g/L, en comparación con la obtenida en SmF, 42 g/L. Las observaciones microscópicas mostraron agregados celulares más pequeños en SmF comparados con los encontrados en SSF. Este es el primer trabajo donde se muestra un menor efecto de la autofagia celular en cultivos en espuma de poliuretano a bajas concentraciones de metanol comparados con los cultivos en matraz agitado. Estos resultados pueden explicarse en términos de comunicación célula a célula y en diferentes condiciones en la difusión en los agregados celulares. Este trabajo abre la posibilidad para utilizar espuma de poliuretano para cultivos SSF con el objetivo de producir proteínas recombinantes utilizando glicerol como fuente de carbono y metanol como inductor para la expresión.

Palabras clave: *Pichia pastoris*, toxicidad de metanol, fermentación en medio sólido, (SSF), fermentación en medio sumergido (SmF).

1 Introduction

P. pastoris is a methylotrophic yeast widely used for the industrial production of heterologous proteins because it is easy to obtain in high density cultures due to the negative Crabtree metabolism, where fermentation is less inhibited by high substrate concentrations , as compared to strong inhibition of positive Crabtree metabolism of *Saccharomyces* species (Prielhofer *et al*., 2015). High density cultures, in turn, help to produce large amount of recombinant proteins that have better glycosylation patterns than other yeast and bacteria (Ahmad *et al*., 2014). Finally, the commercial availability of specific kits for the construction of recombinant strains that produce heterologous proteins (Cereghino and Cregg, 2000; Cregg *et al*., 1993; Tschopp *et al*., 1987) and the fast growth of this yeast to a high cell density when grown in defined minimal media (Brierley *et al*., 1990), has

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helped to introduce *Pichia* pp. as a popular host for the expression of recombinant proteins. However, *P. pastoris* high cell density cultures demand plenty of oxygen than can be met using atmospheres enriched which increase the cost of the fermentation process (Yinliang *et al*., 1997). On the other hand, biomass production in solid state fermentation using supports with specific area higher than 100 cm^{-1} reduces the problems of oxygen transfer, and has already been employed in the production of recombinant proteins in filamentous fungi such as *Aspergillus niger* and *Penicillium* (Téllez *et al*., 2006; Álvarez-Cervantes *et al*., 2016; Ramos-Ibarra *et al*., 2017) and others metabolites using yeast (López-Flores *et al*., 2016), showing very encouraging results. Currently there is only a report that describes the production of a recombinant protein using *P. pastoris* on solid support (López *et al*., 2010). On the other hand, it is known that *P. pastoris* can use methanol as the sole carbon source (Egli *et al*., 1980; Veenhuis *et al*., 1983) but cannot tolerate very high methanol concentrations, due to the accumulation of formaldehyde and hydrogen peroxide inside the cells, both of which are the oxidation products of methanol by the action of alcohol oxidase (Couderc and Baratti, 1980; Cregg and Madden 1988; Van der Klei *et al*., 1990). Previous work on this yeast grown in submerged fermentation (SmF) in glycerol and then exposed to methanol, showed that biomass decayed exponentially with methanol concentration (Santoso *et al*., 2012). This work is focused on the physiological comparison between cultures of *P. pastoris*, previously grown in broths containing glycerol and ammonium tartrate, as carbon sources, but using, either solid-state (SSF) or submerged (SmF) fermentation techniques, and then supplied with different methanol concentrations. Such comparison was done in relation to, extrapolated final biomass density (X_{max}, g of DW biomass per liter), and the rate of biomass decay κ (h^{-1}). The aim of this comparison is to show that *P. pastoris* grown in polyurethane foams (PUF) shows less autophagy, either with or without late methanol addition.

2 Materials and methods

Regeneration dextrose base (RDB), yeast extract-peptone-dextrose (YPD), buffered minimal glycerol (BMG) and buffered minimal methanol (BMM) media were prepared according to the manual for the *Pichia* Expression Kit (Invitrogen). Both BMG and BMM media were supplemented with ammonium tartrate (AmT), having a fixed glycerol/tartrate ratio of 2.9 to have a C/N ratio of 9, and with 200 μmol L^{-1} of $CuSO_4$. Yeast nitrogen base medium without amino acids was obtained from Difco (Detroit, MI, USA). All chemicals were analytical grade and purchased from Sigma-Aldrich (St Louis, MO).

2.1 *P. pastoris cultures by SSF and SmF*

SSF experiments were carried out in PUF with a dry density of 17 g L^{-1}, cut in cubes of 0.5 cm, and treated as previously described (López *et al*., 2010). Buffered minimal glycerol (BMG) medium, with different glycerol and AmT concentrations, was used as indicated in Table 1, in a ratio of 15 mL g^{-1} of PUF into 250 mL Erlenmeyer flasks. Incubations were performed at 30 °C for 48, 72 or 168 h in a shake turntable to ensure uniform temperature with gentle stirring to prevent the leakage and stratification of solids and broth. The broth was found to be spread within PUF as lamellae or menisci shown in Figure 1.

Table 1. BOD: biochemical oxygen demand. AmT: ammonium tartrate; TG: elapsed culture time in glycerol and time for the addition of methanol

BODgL^{-1}	**AmT gL-1**	**Glycerol gL^{-1}**	**MetOH gL^{-1}**	**TG (h)**	**carbon (gL^{-1})**
13.1	3.07	12.6	0	48	5.73
21	3.07	12.6	7.9	48	8.68
44.7	3.07	12.6	31.6	48	17.56
52.6	12.22	50.4	0	72	22.89
60.5	12.22	50.4	7.9	72	25.85
84.2	12.22	50.4	31.6	72	34.72
131.5	30.78	90.4	0	168	43.36
139.4	30.78	90.4	7.9	168	46.32
163.1	30.78	90.4	31.6	168	55.2

Methanol was spread within the flask using an aerosol, after glycerol was depleted and a further incubation was carried out at 30 °C for 48 h. SmF experiments were performed in 250 mL Erlenmeyer flasks (Cregg and Madden 1988) having 15 mL of BMG medium with the same glycerol and AmT concentrations as SSF, incubated at 30 °C and 250 rpm for 48, 72 or 168 h. Afterwards, methanol was added at the same final concentrations as for SSF and a further incubation was carried out at 30 °C for 48 h.

2.2 Microscopic observations

Samples of *P. pastoris* strain grown by SmF and SSF techniques for 96 h using BMG medium with 90.40 and 30.78 g L^{-1} of glycerol and AmT, respectively, were examined as fresh slides by light microscopy (at 10× and 40×) using a BOECO BM^{-1}80microscope (Boeckel & Co., Hamburg, Germany), equipped with a digital camera, and Image-Pro Plus image processing software (Media Cybernetics Inc., Bethesda, MD, USA).

2.3 Measurement and characterization of cell growth

Cell growth was followed by measuring the dry cell weight from culture media filtered through 0.45 μm HNWP nylon membranes (Millipore, Billerica, MA, USA). For SSF experiments, culture medium was previously obtained by pressing PUF cubes in a 50 mL syringe. Additionally, 30 mL of distilled water were used to rinse off any medium component still adhering to the PUF. Evolution of dry cell weight was followed by the Velhurst-Pearl logistic equation as previously described (Viniegra-González *et al*., 2003).

$$\frac{dX}{dt} = \mu\left(1 - \frac{X}{X_{max}}\right)X \tag{1}$$

Where, X_{max} is the maximum value of biomass concentration X(g L^{-1}), and $\mu(h^{-1})$ is the specific growth rate. After integration equation 1 between the initial value X_0 and the final value $X_{(t)}$, the following expression is obtained:

$$X_{(t)} = \frac{X_{max}}{[1 + (X_{max} - X_0)/X_0 * \exp(-\mu t)]} \tag{2}$$

The parameters, X_0, X_{max} and μ, were estimated by minimizing the sum of least squares of residual errors between the above integrated equation [2] and the observed values of $X(t)$ for each fermentation run, providing that X(t) was increasing function of time and R^2 >0.95, with at least nine different duplicate or triplicate values corresponding to nine or more incubation times. The minimization program was Solver, and is derived from the Marquardt algorithm, as presented in an Excel spreadsheet. 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. The statistical analyses (analysis of variance, regression analysis and graphical optimization) were carried out using the Statistical software (Version 7.0, Minneapolis, MN, USA).

2.4 Estimation of cell lysis

For decaying values of $X(t)$ there was no attempt to analyze the trend by an exponential function, because there were too few data points, instead an approximate value of final lysis was calculated by equation (3)

$$L = 100\frac{X_{max} - X_{final}}{X_{max}} \tag{3}$$

Where, X_{max}, was the estimated value in equation (2) and X_{final}, the average endpoint of each given experimental condition.

2.5 Determination of glycerol and tartrate

Glycerol and tartrate concentrations in cell-free each culture were measured by high performance liquid chromatography (HPLC), using a Perkin-Elmer chromatograph equipped with a LC 250 pump, an LC-30 refractive index detector (Perkin Elmer, Waltham, MA, USA) and an ion exclusion column (AminexHPX-87H; Bio-Rad Laboratories, Hercules, CA), performing an isocratic elution with 5 mmol L^{-1} H_2SO_4 at a flow rate of 0.5 mL min^{-1} at 50 °C. All samples were filtered through 0.45 μm Durapore filters (Millipore, Milford, MA) and 20 μL of each sample were injected into the column. Carbon yields ($Y_{x/c}$) values were calculated, using the expression [4].

$$Y_{x/c} = \frac{X_{max}}{[(Gly_{(igc)} + Amt_{(igc)} + MetOH_{(igc)}) - (Gly_{(rgc)} + Amt_{(rgc)} + MetOH_{(rgc)})]} \tag{4}$$

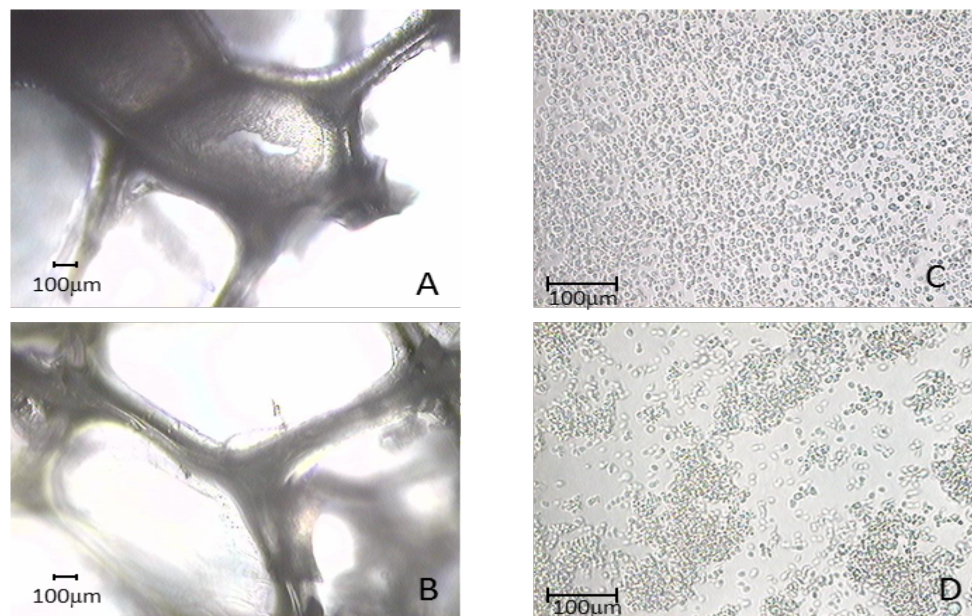

Fig. 1. Morphology differences between cellular aggregates in SSF and SmF. Micrographs of cultures at 96h. (A) Characteristic cellular aggregates on PUF. (B) Polyurethane foam without cel (C) Structure of cell aggregates grown in SmF. (D) Morphology of cell aggregates recovered from SSF by pressing PUF cubes.

Where *Gly* is glycerol, *Amt* is Ammonium tartrate, and MetOH is methanol, igc is the initial gram of carbon and *rgc* is the final gram of remnant carbon.

3 Results

3.1 Morphology differences between cellular aggregates in SSF and SmF

Figure 1, A and D, show the microscopic appearance of the yeast culture grown in PUF. The broth was found to be evenly dispersed in fine lamellae or menisci retained by a framework of PUF mesh. The thickness of the polymer branches was smaller than 0.01 cm and is consistent with reported meniscus thickness around 6×10^{-3} cm. This thickness corresponds to an average specific area (A/V) $\approx$ 333 cm^{-1}, taking into account that each meniscus is exposed to air in two different interphases. It should be note that 15 mL of liquid in an Erlenmeyer 250 mL size has a diameter of 8 cm with a corresponding specific area of 200 cm^2 (A/V ratio $\approx$ 13,4 cm^{-1}). Figure 1D shows yeast aggregates grown within the meniscus and spread on microscope slide with hundreds of cells per aggregate. Fig. 1A and D show *in situ* aggregates of thousands of cells each one close to the other. Fig. 1C shows the appearance of yeast suspensions grown in shake flask having less coalescences and forming aggregates with dozens of cells. The average yeast diameter was around 10^{-3} cm. The polymer cells have an average diameter of the order or 0.1 cm. It should be noticed that yeast aggregates can span whole polymer cells at the final stages of SSF process. Biofilms have two significant dimensions, the length from the center to the air interface of the biofilm, around 3×10^{-3} cm, and the average polymer cell (0.1 cm). Thus, the diffusiontal time ratio is proportional to

$$R = \left(\frac{0.1}{.003}\right)^2 = 1.1 \times 10^3 \tag{5}$$

3.2 Effect of initial BOD on the specific growth rate (μ) in SmF and SSF

Figure 2 shows a decreasing trend (μ) of vs (BOD), both for SmF open the comparative analysis of the specific rate of growth (μ), this is consistent with substrate inhibition effect due to increasing osmolarity and concomitant decrease of water activity in the culture medium. It should be noted that logistic equation was fitted only during the initial activity

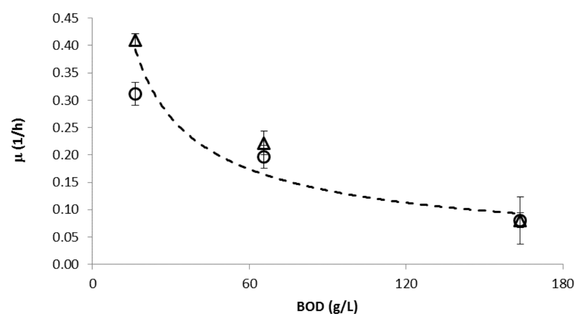

Fig. 2. Effect of BOD (gL^{-1}) on the specific growth rate, μ (h^{-1}) of *Pichia pastoris* estimated by logistic equation with data points before lysis. Dotted line was fitted by $y = 2.2651x^{-0.63}$, with $R^2 = 0.9064$. Comparison between SmF (Δ) and SSF (□) was not statistical significant.

before the presence of yeast decays or any other perturbation related to the addition of methanol.

3.3 Effect of initial BOD on the extrapolate maximal biomass concentration (X_{max}) in SmF and SSF

In a similar fashion to previous section, the initial growth data points of six independent fermentations of *P. pastoris*, before the addition of methanol, were fitted by the logistic equation in order to estimate the maximal extrapolated values of biomass concentration (X_{max}). It is worth noticing that observed maximal values were higher than 95% of extrapolated X_{max} values. Thus, this parameter is a good indication of the biomass level achieved by the use of glycerol as the main carbon source and before adding methanol. Figure 3 shows an increasing quadratic trend of X_{max} *vs* BOD with a remarkable higher level of X_{max} in SSF around 50 g L^{-1} vs 40 g L^{-1}(SmF) when initial BOD was around 164 g L^{-1}. This result is similar to that obtained by López *et al*., (2010) with the same fermentation system or Romero *et al*., (2000) with *A. niger* grown in PUF and has been assumed to be to a better oxygenation in the thin layers of broth shown in Fig. 1. The quadratic trend seems to show a decreasing biomass yield perhaps due to cell density interference with oxygen mass transfer.

3.4 Cellular lysis in SmF and SSF without or with methanol addition

Autophagy is well documented phenomenon of high cell density cultures (Levine and Klionsky, 2004;

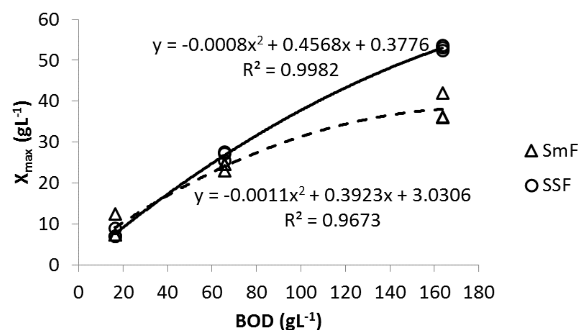

Fig. 3. Effect of BOD (gL^{-1}) on the extrapolated values of X_{max}, (gL^{-1}) of *Pichia pastoris* estimated by logistic equation with data points before lysis. Trends were fitted by the following quadratic equations, $y = -.0011x^2 + 0.3923x + 3.0306$ with $R^2 = 0.9673$ for SmF (△) and $y = -.0008x^2 + 0.4568x + 0.3776$ with $R^2 = 0.9982$ for SSF (o). Each trend was fitted with six data points. The negative quadratic term shows that the quotient X_{max}/BOD is decreasing with BOD and lower for SmF as compared to SSF when BOD = 170 gL^{-1} but not with BOD smaller than 70 gL^{-1} suggesting better oxygen supply in PUF than in shake flasks as commented in Discussion and Conclusions.

Santoso *et al*., 2012) and is shown in Fig. 4 where extrapolated values of the logistic model are compared to final biomass concentrations. Biomass decay was higher in SmF as compared to SSF and such lysis was increased with higher values of X_{max}. From Fig. 4A lysis in SmF was found to be 15% and 21% for 7.4 and 42 (gL^{-1}) of X_{max}, respectively. Whereas for SSF the experimental lysis shown in Fig. 4B was found to be 2% and 6% for 7 and 52 (g L^{-1}) of X_{max}, respectively. Methanol is a toxic compound than can increase cell lysis as shown in Fig 5, but such lytic effect was found stronger in SmF than in SSF. Figure 4A shows the that addition of methanol with concentrations higher than 1% v/v led to a final lysis around 50% of the extrapolated X_{max} value (40 gL^{-1}), whereas in Fig 4B the corresponding values were of only 20% of the extrapolated X_{max} value (50 gL^{-1}).

Discussion and Conclusions

The physiological differences between, SmF and SSF, observed in this work can be associated to mass transfer phenomena of two separate processes: a) oxygen mass transfer between air and the fermentation broth and b) substrate mass transfer between the broth

Table 2. BOD: biochemical oxygen demand. $Y_{x/c}$: gram of biomass per gram of carbon. Different letters indicate significant differences ($P < 0.05$). Each parameter were statistically compared between SSF and SmF. Different letters superscripts (a to k), indicate significant differences for equivalent columns having the same parameter (P <0.05).Statistical analysis were realized individualized by treatment $Y_{x/c}$).

BOD	$Y_{x/c}$(SSF)	$Y_{x/c}$ (SmF)
13.1	1.38±0.080a,b,c	1.25±0.073a,b,c
21	0.97±0.014d,e	0.85±0.006e,f
44.7	0.53±0.002k	0.71±0.078h,i,j
52.6	1.09±0.041d,e	0.97±0.062d,e,f
60.5	0.91±0.074d,e,f	0.86±0.051d,e,f
84.2	0.74±0.045h,g	0.63±0.041h,i,j
131.5	1.26±0.076a,b,c	0.81±0.013f,g
139.4	1.01±0.025d,e	0.78±0.049f,g,h
163.1	0.86±0.018e,f	0.62±0.061j,i

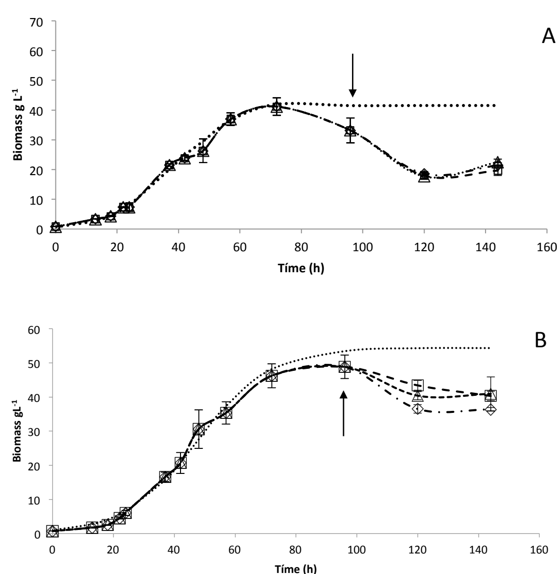

Fig. 4. Methanol effect on cellular lysis in SmF (A) and SSF (B). □: 1.5% v/v MetOH; Δ: 4% v/vMetOH; ◊ 0.75% v/v MetOH and (· · · ·) estimated logistic model without MetOH. The arrow indicates the time where the methanol was added.

and yeast cells. The values of oxygen mass transfer coefficient in SSF have been measured by Thibault *et al*. (2000) to be 10 times higher (≈ 1,000h^{-1}) than the kLa values in shake flasks (≈ 100h^{-1}) as indicated by by Maier and Buchs (2001). This can be related to a much larger specific area (≈ 300 cm^{-1}) for the thin layers of broth dispersed in the PUF as compared to lower specific area (≈ 14 cm^{-1}) in shake flasks since the mass transfer in SSF has been attributed mainly to the thickness of the fermentation film (Thibault *et al*., 2000).Therefore, shake flasks are more prone to be oxygen limited than PUF cultures when BOD values are higher than 10 gL^{-1} and this explains why the extrapolated value, X_{max} = 50 gL^{-1} for SSF is higher than X_{max} = 40 gL^{-1} in SmF when the initial BOD value was around 160 gL^{-1}. It should be noted that such extrapolated, X_{max}, values were calculated from growth curves before the addition of methanol and are only related to the use of glycerol and citrate as carbon sources. According to Santoso *et al*. (2012) SmF cultures of *P. pastoris* with densities above 10gL^{-1} (14×10^7 cells per mL) show autolysis and this phenomenon is enhanced by the addition of methanol at concentrations as high as 20%. Yeast autolysis has been the subject of much work and is reviewed by Levine & Klionsky (2004). Essentially autophagy is a physiological response to cell starvation and physiological stress. Faster yeast lysis in SmF as compared to SSF can be related to cell to cell communication and cooperation in cell aggregates that were larger in SSF than SmF. Furthermore, substrate diffusion across large cell aggregates in SSF would be much slower than substrate diffusion in the planktonic culture (SmF), mainly because the SSF system is not stirred as compared to SmF. This in turn has two effects on SSF system. On the one hand, cell aggregates have longer tangential diffusion paths spanning around 0.1 cm in each biofilm cell. On the

other hand, oxygen diffusion path is transversal to the biofilm with diffusion distances lower than 0.003 cm, as shown in Fig. 1. Therefore, yeast cells will not be limited by oxygen supply but by substrate supply in a similar way to macroscopic fed-batch cultures since the average diffusional time for oxygen will be one thousand times shorter than the average diffusional time for substrates because the diffusional time is proportional to the square value of the diffusional path length. This may account for a more equilibrated flux of oxygen as compared to the local BOD around the yeast cells and with lower maintenance coefficients that are known to increase with osmotic stress in SmF systems as reported for SmF cultures of *S. cerevisiae* (Gustafsson *et al*., 1993) was observed. This is an important consideration because it is known that high cell density cultures have important problems of oxygen mass transfer (Siegel and Brierley, 1989; Crowley *et al*., 2005). In the SSF system, cell aggregates would work as small fed batch cultures where local substrate concentrations are maintained much lower than the bulk value and they also affect the membrane permeability avoiding a metabolic stress by substrate excess, as has already been reported in other studies (Hohmann, 2002; Steltenkamp *et al*., 2006). Those considerations may help to explain the lower toxic effect of methanol in SSF as compared to SmF (López *et al*., 2010; Stapleton *et al*., 2004; Otterbein *et al*., 2000) and is in line with the resistance to tannic acid by *Aspergillus niger* grown in PUF reported by Aguilar *et al*., (2001). Resistance to toxic compounds seems to be a general property of biofilm systems (Mah *et al*., 2001). The diffusional nature of the barriers to methanol transport within the yeast aggregates was shown by a higher threshold of methanol as inducer of the expression of recombinant laccase in PUF cultures of *P. pastoris* as compared to SmF system (López *et al*., 2010). In addition, SSF cutures of other organisms, such as filamentous fungi, have been observed with nearly absence of carbon catabolic repression phenomenon even with glucose concentrations higher than 100 grams per liter (Viniegra-González *et al*., 2003). It is worth noticing that wild yeast often grow on the surface of decaying materials, capturing the nutrients present in solid media with low water activity as compared to artificial SmF cultures grown as planktonic suspensions with diluted substrates.

As a major conclusion of this work, drawn from present results, the use of SSF *Pichia pastoris* cultures within PUF seems to have a clear advantage over SmF planktonic cultures because they reach higher cell densities with lower autophagy and lesser toxic effect of methanol, preserving the cell density for the later use as genetic vectors of recombinant proteins induced by methanol as previously shown by López *et al*. (2010). This way cellular lysis will be minimal with higher levels of active biomass.

Nomenclature

SSF	Solid-State Fermentation
SmF	Submerged Fermentation
MetOH	Methanol
DW	Dry weight gL^{-1}
κ	Rate of Biomass Decay (h^{-1})
PUF	Poly-Urethane Foam
RDB	Regeneration Dextrose Base
YPD	Yeast Extract-Peptone-Dextrose
BMG	Buffered Minimal Glycerol Medium
BMM	Buffered Minimal Methanol Medium
AmT	Ammonium Tartrate
BOD	Biochemical Oxygen Demand

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