

Production of lignocellulolytic enzymes by *Aspergillus niger* biofilms at variable water activities

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Abbreviations:

a_w : water activity
BF: biofilm fermentation
ENG: endoglucanase
FPA: filter paper activity
IU: international unit
SAF: surface adhesion fermentation
SF: submerged fermentation
SSF: solid state fermentation
XYL: xylanase

Lignocellulolytic enzyme production by *Aspergillus niger* was compared both in submerged fermentation (SF) and biofilm fermentation (BF) at varying water activities. Maximal filter paper activity, endoglucanase and xylanase activities were much higher in BF (2.96, 4.7 and 4.61 IU ml⁻¹, respectively) than in SF cultures (1.71, 1.31 and 2.3 IU ml⁻¹, respectively) but biomass yields were lower in BF than in SF (0.338 g g⁻¹ and 0.431 g g⁻¹, respectively). In the presence of 20% ethylene glycol ($a_w = 0.942$) the enzyme activities decreased in both systems but BF still had higher levels (1.0, 1.0 and 2.6 IU ml⁻¹, respectively) than SF cultures (0.6, 0.7 and 1.5 IU ml⁻¹, respectively). An increase in xylanase specific activity of more than 2 fold (from 4.2 to 10.2 IU mg⁻¹ biomass) was observed in the presence of 20% ethylene glycol, suggesting differential regulatory mechanisms in biofilm fermentation related to cell adhesion.

Cellulases are increasingly used by several industries including fruit processing, feed production, textiles and

others (Bhat, 2000; Kirk et al. 2002). Cellulases and most industrial enzymes are produced by submerged fermentation (SF) but solid state fermentation (SSF) is used to a lesser extent. The main advantages of SSF are low technology and high volumetric productivity, thus reduced downstream processing costs (Hölker et al. 2004). For a long time it has been thought that the main advantages of SSF are due to water limitation of the system so that a higher product concentration is attained. An additional but less investigated advantage of SSF may be enhanced physiological processes in cell adhesion or biofilm formation that is characteristic for SSF.

Biofilm processes are used mainly for waste water treatment but they are also considered for metabolite and enzyme production (Freeman and Lilly, 1998; Fiedurek 2001; Iqbal and Saeed, 2005; Wu et al. 2005; Yang et al. 2005; Skowronek and Fiedurek, 2006). Although fungal biofilms are less known than bacterial biofilms, they can be used for cellulase production as it has been recently showed (Villena et al. 2001).

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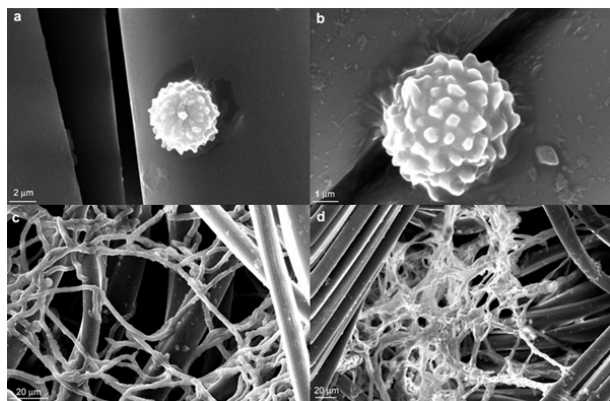


Figure 1. Cryo-scanning electron micrographs of *Aspergillus niger* spore adhesion and biofilm development on polyester cloth at 0%, $a_w = 0.976$ (a, c) and 20% ethylene glycol, $a_w = 0.942$ (b, d).

Both SSF and biofilm fermentation (BF) depend on surface adhesion. A new fermentation category named surface adhesion fermentation (SAF) was first proposed by Gutiérrez-Correa and Villena (2003). The concept of a biofilm presumes either a population or a community of microorganisms living attached to a surface. Biofilms can be developed on either biotic or abiotic surfaces from a single species or as a community derived from several species (O'Toole et al. 2000; Fenchel, 2002). It should be noted that adhesion and subsequent differential gene expression to generate phenotypes distinct from those of free living organisms are two unifying processes of the biofilm concept (O'Toole et al. 2000; Ghigo, 2003). Filamentous fungi are naturally adapted to growth on surfaces and in these conditions they show a particular physiological behaviour which it is different to that in submerged culture; thus, they can be considered as biofilm forming organisms according to our former concept. The advantages of this form of growth have been industrially exploited by two culture systems: SSF and cell immobilization on inert surfaces.

Technology of cell immobilization was highly developed during the last two decades based on the operative advantages in the productive process instead of physiological issues (Groboillot et al. 1994). Natural adsorption on solid supports is an immobilization technique that it has been used with filamentous fungi thus neglecting its study as a way of biofilm formation. Actually, once spores are adsorbed to the support they grow attached to it thus forming a film. We prefer the term biofilm fermentation instead of cell immobilization because the microbe is an active and differential entity (Gutiérrez-Correa and Villena, 2003).

The importance of the water activity (a_w) in microbial physiological processes is well recognized. It is known that a_w is a critical factor affecting the growth and metabolism of fungi (Kredics et al. 2000; Parra et al. 2004) and,

especially in SSF it is also considered as a fundamental parameter for mass transfer (Gervais and Molin, 2003). Likewise, the production and secretion of enzymes could be affected by water activity (a_w) and by the nature of the a_w depressor (Acuña-Argüelles et al. 1994; Kredics et al. 2000; Gervais and Molin, 2003). Despite the importance of water activity in many enzyme production systems, its role in biofilm fermentation is not explored. This paper describes the effect of ethylene glycol as a water activity depressor on the lignocellulolytic enzyme production by *Aspergillus niger* biofilms.

MATERIALS AND METHODS

Fungal strain and inoculum formation

Aspergillus niger ATCC 10864 was used throughout the study and was maintained on potato dextrose agar slants. Spores were washed from 5-day agar-slant cultures with 10 ml of 0.1% (v/v) Tween 80 solution, counting in a Neubauer chamber and diluted to give 1×10^6 spores ml^{-1} . This suspension was used as the inoculum.

Culture medium

Duff (1988) medium was used in all experiments. The culture medium contained per liter: 2 g KH_2PO_4 ; 1.4 g $(\text{NH}_4)_2\text{SO}_4$; 0.3 g urea; 0.3 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 0.3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 1g peptone; 2 ml Tween 80; 5 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 1.6 mg $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$; 1.4 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 2mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$; and 10 g lactose. The initial pH was 5.5.

To test the effect of a_w , the same medium was used supplemented with ethylene glycol at the following final concentrations (% v/v): 5%, 10%, 15% and 20%.

Submerged fermentation

30 ml of the culture medium in 125 ml flasks was inoculated with 0.9 ml spore suspension to each flask. After inoculation the flasks were incubated at 28°C in a shaker bath at 175 rpm.

Biofilm fermentation

Polyester cloth 100/1 (65% denier and 35% textured polyester with circular stitch), was used as support for biofilm formation (Villena et al. 2001). 2 x 2 cm squares were thoroughly washed with distilled water and oven dried at 105°C. Each flask containing a cloth square in 30 ml distilled water was inoculated with 0.9 ml spore suspension, incubated for 15 min at 28°C in a shaker bath at 175 rpm to allow the attachment of spores. After this contact period, the squares were washed twice with distilled water under agitation at 175 rpm for 15 min; then they were transferred to flasks containing 30 ml of the culture medium and incubated at 28°C in a shaker bath at 175 rpm (Villena et al. 2001). Two flasks were prepared for each sampling time.

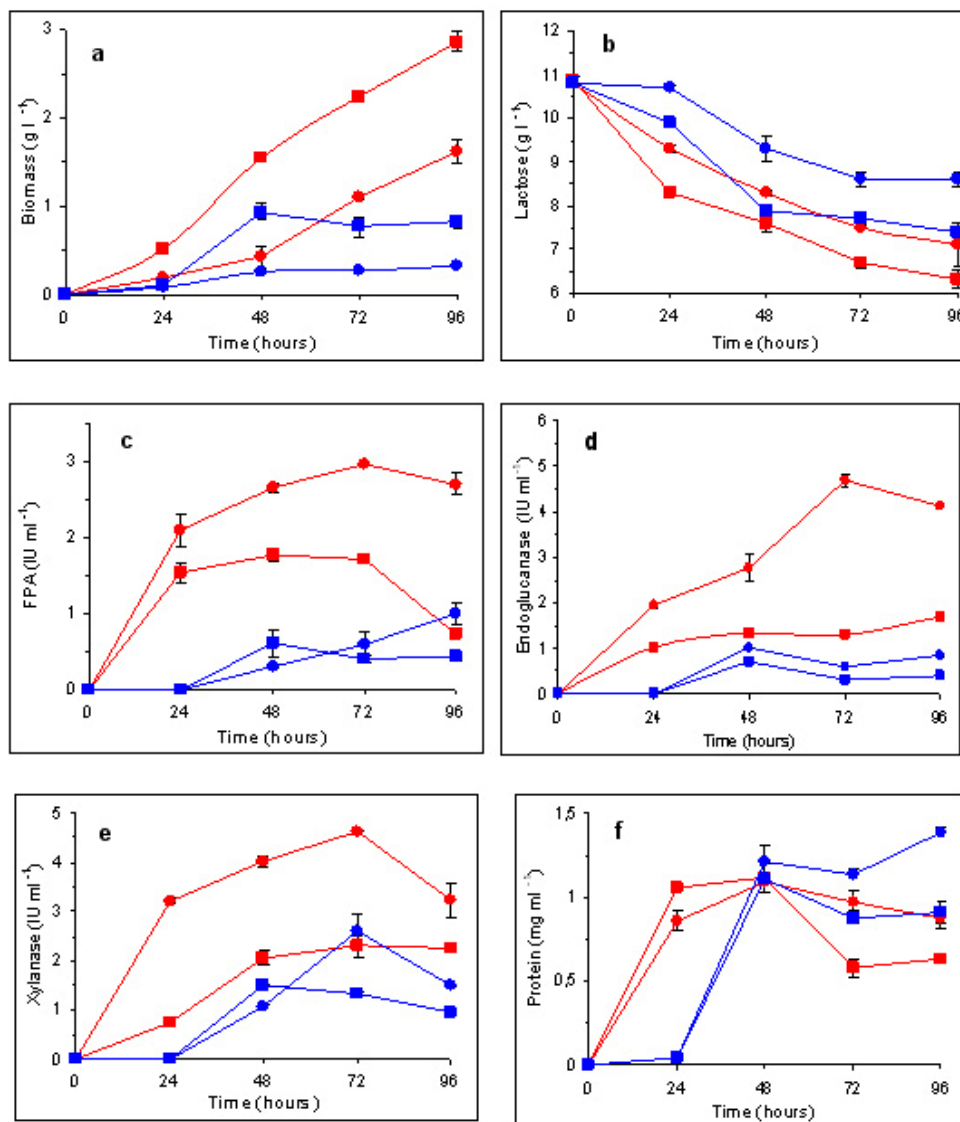


Figure 2. Time course profiles of fermentation variables of *Aspergillus niger* biofilm (●,●) and freely suspended mycelial (■,■) cultures at 0% (red symbols) and 20% ethylene glycol (blue symbols). The error bars on the graphs represent two replicate samples.

Water activity determination

Water potential for each ethylene glycol concentration in the culture medium was measured using a WESCOR HR-33T Dew Point Microvoltmeter model 5103 with C-52 sample chamber. Water potential and water activity are related by $\Psi V_m = RT \ln(a_w)$, where Ψ is the water potential (Pa), V_m is the molar volume of water (mol m^{-3}), $R = 8.314$ is the gas constant ($\text{J mol}^{-1} \text{K}^{-1}$), T is the temperature (K) and a_w is the water activity. Under the conditions used the water activity of the cultured medium containing 0%, 5%, 10%, 15% and 20% of ethylene glycol were 0.976 ($\Psi = -3.35$ MPa), 0.971 ($\Psi = -4.05$ MPa), 0.964 ($\Psi = -5.03$ MPa), 0.954 ($\Psi = -6.5$ MPa) and 0.942 ($\Psi = -8.25$ MPa), respectively.

Cryo-SEM

Biofilm samples were immersed in 10% glycerol for 2 hrs at 4°C. Then, they were thoroughly washed with 0.05 M phosphate buffer (pH 7.4) and frozen by plunging them into liquid nitrogen. Surface water was removed by sublimation at -65°C for 10 min and specimens were sputter-coated with gold (Ma et al. 2005). Samples were then examined at -80°C with a LEO 1420 PV variable pressure scanning electron microscope.

Assays

Biomass from BF was determined by removing polyester squares from the fermentation broth at different time

intervals, washing them three times by shaking in 30 ml 50 mM citrate buffer, pH 4.8, for 10 min each wash at 28°C and 175 rpm, dried overnight and weighed. Biomass from SF was determined by filtering the fermentation broth through pre-weighed filter paper, drying at 105°C overnight and weighing.

For intracellular activity measurement, biofilm or mycelial biomass was washed three times with citrate buffer 50 mM, pH 4.5, and kept frozen before milling in a mortar with liquid nitrogen. Powdered biomass was resuspended in buffer acetate 50 mM, pH 4.8, and centrifuged at 10000 rpm for 5 min. The supernatant was collected and kept frozen until analysis.

Cellulase as filter paper activity (FPA), endoglucanase (ENG) and xylanase (XYL) were measured from the fermentation broth as previously reported (Dueñas et al. 1995). One international unit (IU) of enzyme activity was defined as the amount of enzyme that releases 1 μ mol product per min (glucose equivalents for FPA and ENG and xylose equivalents for XYL). Soluble protein and lactose in the fermentation broth were determined by the standard

Lowry and 3, 5-dinitrosalicylic acid methods, respectively.

RESULTS AND DISCUSSION

The effect of water activity on *Aspergillus niger* biofilm fermentation and enzyme production was studied by using ethylene glycol as water activity depressor. The spore adhesion process was not altered by 20% ethylene glycol ($a_w = 0.942$) as it can be seen in Figure 1. However, both biofilm and free submerged growth were depressed at high ethylene glycol concentrations. Gervais et al. (1988) found that spore germination is strongly affected by the nature of the water depressor. Also, at high ethylene glycol concentration hyphal turgor is negatively affected and gummy materials are secreted (Figure 1). Low spore germination may explain the low biomass attained at low a_w (see below).

Comparative time course profiles of fermentation under normal and water stress conditions are depicted in Figure 2. Due to the inoculation process used for biofilm cultures neither freely floating mycelium nor sloughing were observed (Papagianni et al. 2002; Papagianni and Matthey,

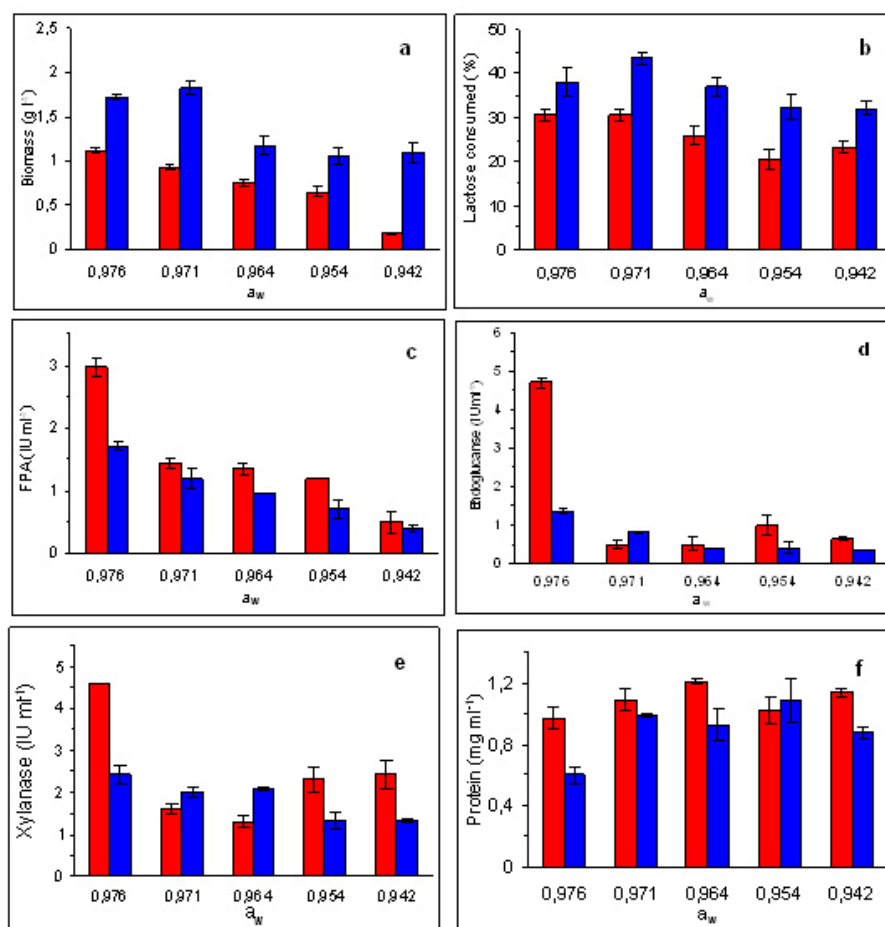


Figure 3. Comparison of fermentation variables of *Aspergillus niger* biofilm culture (red bars) and submerged culture (blue bars) at different water activity levels. The error bars on the graphs represent two replicate samples.

2004). Under normal water activity, SF produced more biomass (3.05 g l^{-1}) than BF (1.6 g l^{-1}) as previously found (Gutiérrez-Correa and Villena, 2003) (Figure 2a, b). On the other hand, depression of water activity by ethylene glycol negatively affected the growth of both culture systems, being BF the most (1.1 g l^{-1} and 0.18 g l^{-1} for SF and BF, respectively). Low water activity levels decrease growth due to several mechanisms, among them mass transfer of water and solutes across the cell membranes may be of special importance since it would increase the maintenance energy expenditure by the fungus (Francis et al. 2002) as well as the decrease of turgor pressure in the hyphae, which has a great importance on fungal growth (Gervais and Molin, 2003).

Maximal FPA, ENG and XYL production activities were much higher in BF (2.96 , 4.7 and 4.61 IU ml^{-1} , respectively) than in SF (1.71 , 1.34 and 2.45 IU ml^{-1} , respectively) (Figure 2 c, d, e), which it is consistent with production yields reported for most of the surface adhesion fermentation processes (Gutiérrez-Correa and Villena, 2003; Viniegra-González et al. 2003; Hölker et al. 2004). This difference cannot be ascribed to the biomass generated in both systems as it was concluded by Díaz-Godínez et al.

(2001) who suggested that increases in exopectinase production by SSF system were related to better fungal growth but not to higher productivity of the enzyme. Also, there was not a significant difference in soluble protein production between both culture systems (Figure 2f). Addition of ethylene glycol decreased maximum FPA, ENG and XYL activities in both SF (0.6 , 0.7 and 1.5 IU ml^{-1} , respectively) and BF (1.2 , 1.1 and 2.4 IU ml^{-1} , respectively) but it increased soluble protein production, being higher in biofilm cultures. The reason for the increase in soluble protein production found in low water activity cultures is not understood and the opposite was found for ethylene glycol depressed SSF cultures of *A. niger* for exopectinase production (Acuña-Argüelles et al. 1994).

Comparison of fermentation variables at 72 hrs of fermentation between SF and biofilm cultures at different ethylene glycol levels are depicted in Figure 3. Biomass and lactose consumption were continuously decreased as ethylene glycol concentration was increased. As stated above, it seems that lactose consumption was hampered by mass transfer limitations due to a decrease in solute diffusion (Gervais and Molin, 2003). Thus growth could be negatively affected due to low carbon-energy availability

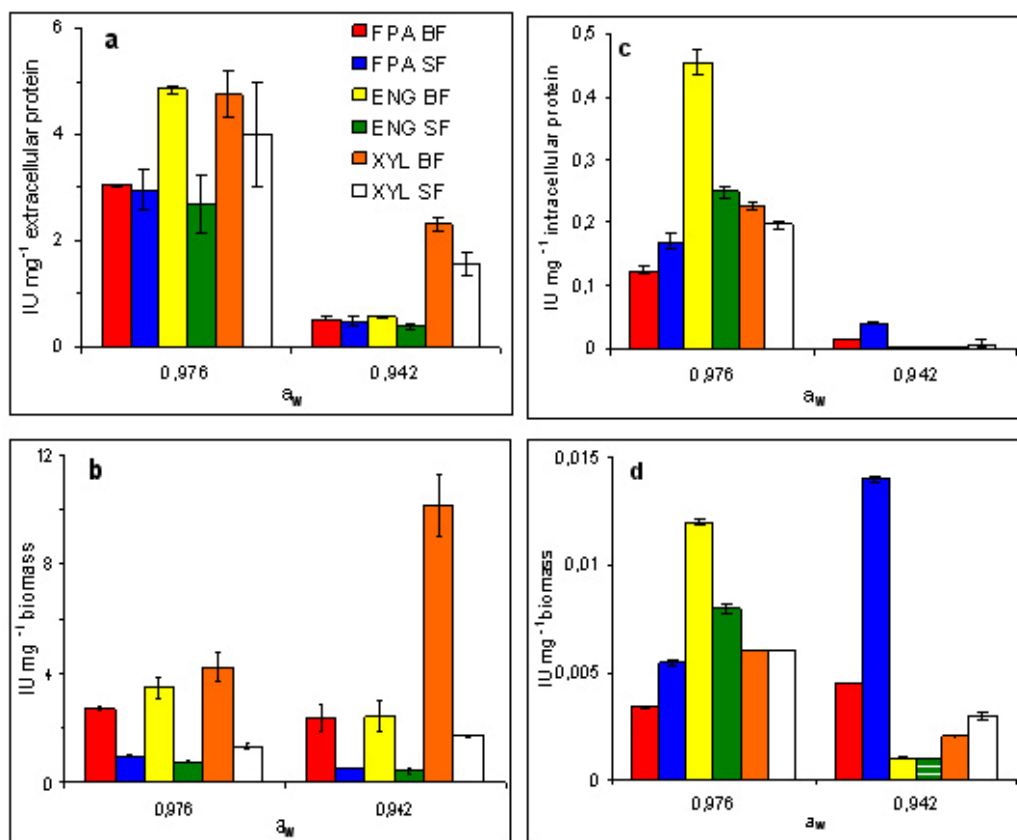


Figure 4. Comparison of extracellular (a, b) and intracellular (c, d) cellulolytic enzyme specific activities attained by *Aspergillus niger* biofilm (BF) and submerged (SF) cultures at 0% ($a_w = 0.976$) and 20% ($a_w = 0.942$) ethylene glycol. The error bars on the graphs represent two replicate samples.

since the fungus had to spend more energy for membrane transport, and synthesis of compatible solutes (Ruijter et al. 2004). As a weakly chaotropic compound, ethylene glycol can freely traverse the cell membrane and it may not affect hyphal turgor at low concentrations (5% to 10%) but at higher concentrations (above 10%) it will cause a water stress with general adverse effects on cellular macromolecules (Hallsworth et al. 2003). Also, biomass yields ($Y_{x/s}$) in both SF and BF systems decreased linearly almost at the same rate as ethylene glycol concentration increased ($y = -0.0408x + 0.4675$, $R^2 = 0.923$, and $y = -0.039x + 0.3839$, $R^2 = 0.554$, respectively). The low coefficient of correlation between biofilm $Y_{x/s}$ and ethylene glycol concentration may indicate that water stress in this type of culture is stronger than in submerged cultures, possible due to the participation of matrix potential in addition to osmotic potential in the former culture because of the presence of cloth and the biofilm structure itself (Gervais and Molin, 2003).

Enzyme production related to ethylene glycol concentration by SF and BF are presented in Figure 3. As it can be seen, all tested enzyme activities strongly decreased in both culture systems although biofilm cultures generally produced more. In submerged cultures the decrease of FPA, ENG and XYL activities was linearly correlated with the increase of water depressor concentration ($R^2 = 0.978$, 0.831 and 0.841 , respectively), indicating a clear direct negative effect of water stress on fungal physiology (Acuña-Argüelles et al. 1994; Díaz-Godínez et al. 2001). However, in biofilm cultures more complex phenomena may be implicated since the decrease of FPA, ENG and XYL activities due to increasing amounts of ethylene glycol follows different patterns. On the other hand, extracellular protein production increased at high water depressor concentrations in both culture systems contrary to the results obtained for exopectinase production in SSF and SF cultures (Acuña-Argüelles et al. 1994; Díaz-Godínez et al. 2001). The reason for this finding is not clear but it may be related to some type of defence mechanism. Hallsworth et al. (2003) have found that induced water stress resulted mostly in the upregulation of proteins involved in stabilization of biological macromolecules and membrane structure. Extracellular and intracellular enzyme specific activities expressed as both IU per mg extracellular or intracellular protein and IU per mg biomass are depicted in Figure 4. Although all specific activities dropped at high ethylene glycol concentration, those related to biomass had the lowest decrease. It is worth mentioning that biofilm XYL extracellular specific activity per biomass increased more than two fold (from 4.2 to 10.2 IU mg⁻¹ biomass). It has been considered that in *A. niger* both cellulases and xylanases are members of the same regulatory pathway under the control of the transcriptional activator XlnR (van Peij et al. 1998). However, this does not seem to be the case in biofilm cultures under severe water stress and other molecular mechanisms may be involved. On the other hand, the intracellular activity of all enzymes evaluated did not contribute significantly to the overall enzyme activities

(Figure 4 c, d) in all a_w levels tested. Although Kredics et al. (2000) found that enzymatic activities of *Trichoderma harzianum* and the amount of enzyme secretion (expressed as relative activity) depend on the water potential (or water activity), it is not clear whether the decrease of relative enzymatic activities is directly related to a secretion limitation. According to our results it seems possible that the low water activity affects enzyme biosynthesis rather than their secretion.

In summary, it has been found that biofilm fermentation produces higher cellulolytic enzyme yields than submerged fermentation at lower biomass yields suggesting differential gene expression mechanisms related to cell adhesion (Gutiérrez-Correa and Villena, 2003). Contrary to the findings on SSF, biofilm fermentation can better resist water stress and a differential regulation of xylanase is evident under this condition. Further work is being conducted to clarify some common molecular mechanisms involved in surface adhesion fermentation.

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