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Rev. peru. biol. 15(2): 097- 102 (Febrero 2009)
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Versión *Online* ISSN 1727-9933

Differential gene expression of some lignocellulolytic enzymes in *Aspergillus niger* biofilms

Expresión diferencial de los genes de algunas enzimas lignocelulolíticas en biopelículas de *Aspergillus niger*

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Abstract

A preliminary evaluation of transcriptional gene expression in *Aspergillus niger* ATCC 10864 biofilms developed on polyester cloth was carried out. The expression analysis of genes encoding some lignocellulolytic enzymes and some regulatory genes by means of RT-PCR showed that *eng1*, *eglC*, *exo*, *eglA*, *eglB* and *xynB* genes are differentially expressed in biofilm fermentation either time-related or through the production of more than a transcript as compared to *A. niger* grown in submerged fermentation. Likewise, the regulatory genes *xlnR* and *creA* showed time-related expression patterns that were different in both fermentation systems. Results attained in this work contribute with an initial molecular evidence of differential gene expression as well as differential gene regulation patterns in fungal biofilms that may be related to cell adhesion.

Keywords: *Aspergillus niger*, biofilms, gene expression, cellulases, xylanases.

Resumen

Se realizó una evaluación génica preliminar a nivel transcripcional de biopelículas de *Aspergillus niger* ATCC 10864 desarrolladas sobre poliéster respecto a algunas enzimas lignocelulolíticas. El análisis de expresión de genes de enzimas lignocelulolíticas y genes reguladores mediante RT-PCR mostró que los genes *eng1*, *eglC*, *exo* y *eglA*, *eglB* y *xynB* son diferencialmente expresados ya sea temporalmente o mediante más de un transcripto en comparación con cultivos sumergidos. Asimismo, los genes reguladores *xlnR* y *creA* mostraron patrones temporales de expresión distintos en ambos sistemas. Los resultados obtenidos aportan la evidencia molecular inicial de expresión diferencial de genes en biopelículas así como patrones de regulación diferencial muy probablemente ligada a la adhesión celular.

Palabras clave: *Aspergillus niger*, biopelículas, expresión génica, celulasas, xilanasas.

Presentado: 09/06/2008
Aceptado: 19/08/2008
Publicado online: 26/02/2009

Introduction

Recently, a great deal of attention has been focused on the use of lignocellulose biomass to produce bioethanol and other useful metabolites by means of its hydrolysis with lignocellulolytic enzymes produced by various microorganisms (Bhat 2000; Gabel & Zacchi 2002; Kim & Dale 2004). Lignocellulose biomass consists of three main components: cellulose, hemicellulose and lignin, the first two being composed of chains of sugar molecules (Carpita 1996; Fry 2004). These sugar chains can be hydrolyzed to produced monomeric sugars, some of them can be fermented using ordinary producing microorganisms. However, the cost of obtaining sugars from lignocellulose biomass for fermentation is still high, mostly due to low enzyme yields of producing microorganisms (Gabel & Zacchi 2002). Submerged fermentation (SF) is the main process used for cellulase production but solid state fermentation (SSF) techniques are preferred in Asian countries. It is frequently considered that enzyme titers attain in SSF processes are higher than in SF as recently discussed by Viniegra-González et al. (2003). Also, it is considered that SSF resembles the natural way of living of many microorganisms, particularly fungi that grow attached to the surface of solid particles in the absence of free flowing water (Hölker et al. 2004).

Other fermentation techniques like fungal immobilization are also being considered for production of enzymes and other metabolites (Iqbal & Saeed 2005, Wu et al. 2005; Yang et al. 2005; Skowronek & Fiedurek 2006). Surface binding is commonly used to immobilize cells and involves cell attachment by either chemical bonding or cell adhesion. The latter is a simple and inexpensive procedure in which cells actively participate in the attachment process; thus, a distinction should be made between this type of immobilization and the other methods. Natural attached cells develop an active biofilm structure with changes in their physiology due to differential gene expression (Ghigo, 2003). Filamentous fungi are naturally adapted to growth on surfaces and in these conditions they show a particular physiological behavior which is different from that in SF; thus, they can be considered as biofilm forming organisms according to the above concept. Both SSF and fungal biofilm fermentation (BF) depend on surface adhesion and a new fermentation category named surface adhesion fermentation (SAF) was proposed by Gutiérrez-Correa and Villena (2003).

There are few reports on cellulase production by biofilm cultures of *Aspergillus niger*. In previous studies, it was showed that *A. niger* biofilms developed on polyester cloth produced 50—70% more cellulase activity than freely suspended mycelial cultures (Villena & Gutierrez-Correa 2003; Villena & Gutierrez-Correa, 2006; Villena & Gutierrez-Correa, 2007a). Other authors have also demonstrated that other species like *A. fumigatus* form biofilms and that this condition is most relevant in lung mycoses (Beauvais et al., 2007; Mowat et al., 2007; Mowat et al., 2008a). Although Chandrasekar and Manavathu (2008) discussed the potential ability of *A. fumigatus* growth on surfaces to satisfy key biofilm characteristics, all the research evidence recently available strongly supports that *Aspergillus* species actually forms biofilms when grown on surfaces and that some genes are differentially expressed in this condition (Mowat et al., 2008b).

Fungi can be considered as regular biofilm forming organisms with two inherent and fundamental processes: adhesion and subsequent differential gene expression to develop new and distinct phenotypes different from those of free living conditions (Wimpenny et al. 2000; Gutiérrez-Correa & Villena, 2003). From this point of view, the study of differential

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gene expression of biofilms developed by filamentous fungi may be the starting line of an analysis of differential physiological behavior as compared to submerged cultures, which is needed to establish the role of cell adhesion and the growth on surfaces on the productivity of submerged industrial processes. The aim of the present work was to study the gene expression patterns of some lignocellulolytic enzymes of *Aspergillus niger* during biofilm formation on polyester.

Materials and methods

Microorganism. *Aspergillus niger* ATCC 10864 maintained on potato dextrose agar slants was used throughout the study. Spores were washed from 5-day agar-slant cultures with 10 mL of 0,1% Tween 80 solution, counted in a Neubauer chamber and diluted to give 1 x 10^6 spores/mL. This suspension was used as inoculum at a proportion of 3% (v/v). Culture medium for both SF and BF was described elsewhere (Villena and Gutiérrez-Correa, 2006).

Submerged and Biofilm Fermentation. For both types of fermentation systems 250 mL flasks containing 70 mL culture medium were used. For BF each flask containing a polyester 100/1cloth square in 70 mL distilled water was inoculated with 2,1 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 70 mL of the culture medium. All flasks were incubated at 28 °C in a shaker bath at 175 rpm.

RT-PCR. At each time point 100 mg of either mycelial pellets (SF) or biofilm (BF) were ground in liquid nitrogen, and total RNA in the samples was isolated by RNeasy plant mini kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. Total RNA was used for reverse transcription by Omniscript RT kit (QIAGEN, Tokyo, Japan). Each reaction tube contained 12 µL total RNA (2 µg), 2 µL 10X RT buffer, 2 µL dNTP mix (5 mM each), 2 µL oligo dT-primer (10 µM), 1 µL RNase inhibitor (10U/mL) and 1 µL reverse transcriptase; the reaction was conducted at 37 °C for 1 h.

PCR. Synthesized cDNA was used for PCR. Primer combinations for the amplification of some lignocellulolytic genes were derived from the *A. niger* data bank at the National Center of Biotechnology Information (www.ncbi.nlm.nih.gov; as available in 2005) and are presented in Table 1. The semi-quantitative PCR was carried out for the exponential phase after cycle optimization. Each tube contained 1,5 µL 10X buffer; 1,2 µL dNTP mix (5 mM each); 0,3 µL forward primer (10 µM); 0,3 µL reverse primer (10 µM); 0,08 µL Gen Taq polymerase (5U/µL; WaKo); 1,5 µL cDNA sample and 10,12 µL ultra-pure water. Reaction process was as follows: cycle 1 (1x) 95 °C (4 min); cycle 2 (40x), step 1 95 °C (30 s), step 2 55 °C (30 s), step 3 72 °C (30 s); cycle 3 (1x) 72 °C (5 min); and cycle 4 (1x) 4 °C until use. PCR products were separated by agarose electrophoresis containing trace amounts of ethidium bromide and visualized with UV transilluminator and analized in a Fluor S Multimager (BIORAD).

Assays. Cellulase as filter paper activity (FPA) 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 µmol product per min (glucose equivalents for FPA and xylose equivalents for XYL).

Table 1. Primer sequences for some lignocellulolytic enzyme genes of *Aspergillus niger*.

Genes	Forward	Reverse
Cellobiohydrolases		
cbhA	5′ tgaaggtcgagttgatggga 3′	5′ gacaccggaatccaagatgac 3′
cbhB	5′ gcaagtgtacccactcacaca 3′	5′ aagcggttgtacgtgcaaga 3′
exo	5′ tgtgctctcgttgccctcttg 3′	5′ agtgcattggcgccttcctc 3′
Endoglucanases		
eglA	5′ tccccgtgtcacttgctatg 3′	5′ cagttcatagtcgccgctaga 3′
eglB	5′ atctcaaccaagcagccatt 3′	5′ ccaggatatccagcataccc 3′
eglC	5′ tggtgttaccggtctcttcaaaaccga 3′	5′ gctataccagggatagacttacactgcgaa 3′
eng1	5′ cgacttggtcagtttgatacc 3′	5′ atacccgtgtaagcagttcc 3′
β-glucosidases		
bgl1	5′ tacggcatgaaccactacac 3′	5′ agcatccctttgcaggtatc 3′
bgl2	5′ agctacactctgaacagctg 3′	5′ aagcactcgtttgagatgg 3′
Xylanases		
xyn B	5′ agcggatcatgggaaaccga 3′	5′ gtgtaatctatgaatgcctatagcgggtaa 3′
Cellulase represor		
creA	5′ ctcccagcaagccaatgacc 3′	5′ gacggcaaatgcagatcgcgct 3′
Cellulase activator		
xlnR	5′ acgacatcctccgaccctcatc 3′	5′ tccaagatttctgccgctgcttc 3′
Hydrofobin		
hydrophobin	5′ agtgcattggcgccttcctc 3′	5′ tgtgtctcgttgccctcttg 3′
Elongation factor		
EF	5′ gaaccatctacacctaccccaaca 3′	5′ tgctgccgaccaattgacct 3′

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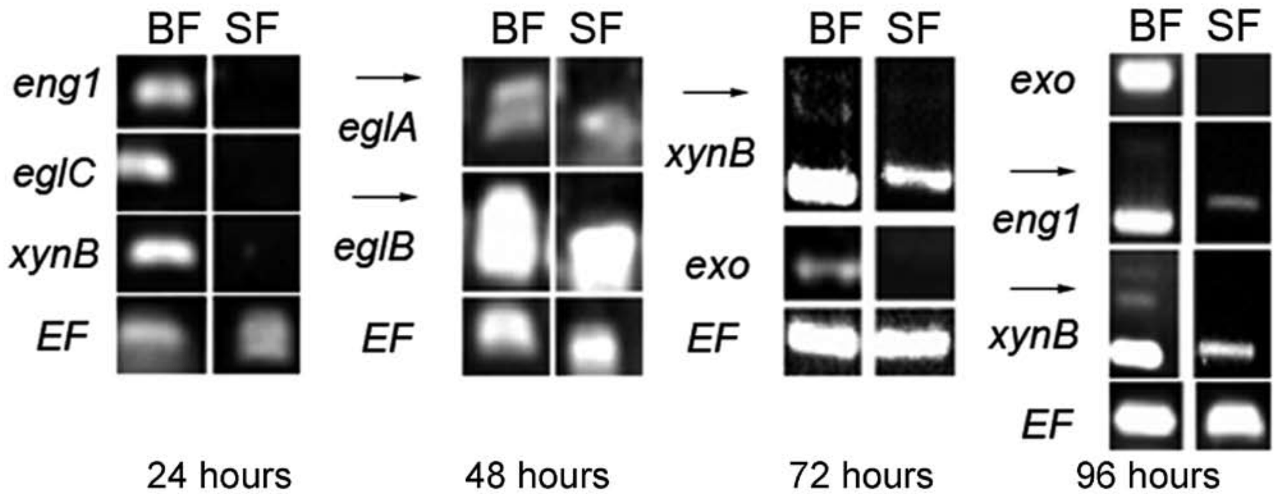

Figure 1. Differential expression analysis of genes coding for some lignocellulolytic enzymes (see Table 1) in *Aspergillus niger* grown in biofilm fermentation (BF) and submerged fermentation (BF). Arrows show additional transcript bands.

Results and discussions

A transcriptomic evaluation of *A. niger* under biofilm and submerged conditions was carried out by using RT-PCR for the expression of some lignocellulolytic enzyme genes. Celobiohydrolase A (*cbhA*) and β-glucosidase 1 (*bgl1*) genes were never amplified in neither fermentation system while hydrophobin gene was poorly expressed only at 48 h growth in BF.

Figure 1 shows differentially expressed genes in both fermentation systems. At 24 h growth biofilms differentially expressed *eng1*, *eglC* and *xylB* while at 48 h growth biofilm genes *eglA* and *eglC* showed additional bands. At 72 and 96 h growth when biofilm cellulolytic enzyme activity was maximal and higher than SF, exoglucanase gene (*exo*) was differentially expressed while *eng1* and *xynB* showed also additional bands. Although several genes were expressed in both fermentation systems, their expression levels were different as shown in Figure 2. At all sampling times transcription of either cellulase or xylanase genes was higher in BF. In SF very low transcription level was found for *bgl2* (24, 72, and 96 h growth), *eglB* (24 h growth), *eglC* (24 and 96 h growth), and *xynB* (48 h growth). At 96 h and 120 h growth expression level of *eglA* and *eglB* was not notoriously higher in BF unlike *eglC, cbhB* and *bgl2* genes. Considering that maximal cellulase and xylanase occurred at 72 h and 96 h growth, respectively, it is possible that differentially expressed Exo and XynB enzymes in those moments may be the main contributors to the lignocellulolytic activity.

It has been stated that in fungi differential gene expression is related to pH, nutrient type and availability, thermal shock, and culture conditions (Denison 2000; Aro et al. 2005; Ward et al. 2006). From this point of view, higher cellulase and xylanase production attained by *A. niger* biofilms could be associated to differential gene expression mechanisms. As was mentioned above, RT-PCR analysis shows different moments of gene expression and, in general, expression levels are always higher in biofilms. Two groups of genes are differentially expressed: the first is comprised by *eng1*, *eglC* and *exo*, and the second by *eglB* and *xynB*. The former is principally expressed at the beginning of the fermentation period and the latter showed more that one band in the gels in biofilm samples. Among biofilm overexpressed genes, other than those mentioned above, there is the *bgl2* gene that codes for a β-glucosidase with exoglucohydrolase activity (Witte & Wartenberg 2004).

The appearance of more than one band for a specific gene is usual in transcriptional studies by RT-PCR (Donzelli and Harman 2001; Dai et al. 2004; Damveld et al. 2005) and it can be explained in part due to the presence of similar enzyme-encoding

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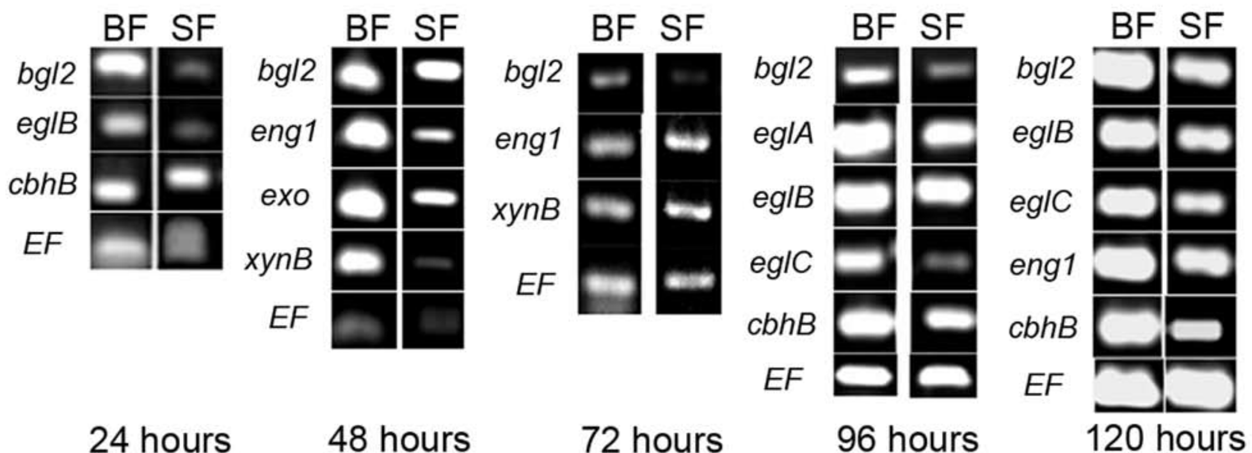

Figure 2. Analysis of expression level of genes coding for some lignocellulolytic enzymes (see table 1) in *Aspergillus niger* grown in biofilm fermentation (BF) and submerged fermentation (BF).

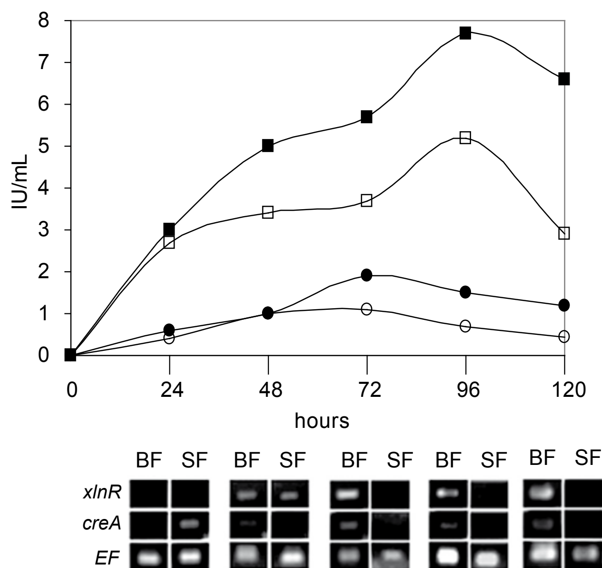

Figure 3. Expression of cellulase (FPA, circles) and xylanase (squares) regulatory genes as related to enzyme production kinetics in *Aspergillus niger* grown in biofilm fermentation (BF, closed symbols) and submerged fermentation (SF, open symbols).

genes with a high degree of homology (Kinoshita et al., 1995), gene duplication events (Constanzo et al. 2006), the presence of genomic DNA in the reaction sample for which competitive RT-PCR is recommended (Damveld et al. 2005), but also it can be due to alternative splicing during mRNA processing as it has been reported for several genes (Birch et al.,1995; Lodato et al.,2003; Yadav et al. 2003; Baba et al., 2005). As long as the test conditions and amplified genes were equal in each fermentation system as well as the same strain and the absence of contaminating genomic DNA, the appearance of other bands due to either homology or gene duplication is little probable; thus a possible explanation for the additional bands found for *eglA*, *eglB* and *xynB* genes may be alternative splicing. This process can regulate the expression through an unusual processing of the same gene creating different reading frames (Galagan et al. 2005; Stotz et al. 2006), and it is possible that the alternative splicing may be responsible for cellulase gene diversity (Baba et al., 2005). A Northern blot analysis together with the sequencing of transcribed bands could confirm the presence of variants of the genes studied in this paper.

Aspergillus niger cellulase and xylanase regulatory genes *xlnR* and *creA* were also studied. The former codes for a transcriptional activator and the latter codes for a cellulase repressor protein that acts principally through catabolite repression. Biofilm cultures expressed *xlnR* with increasing intensities from 48 h growth up to the end of the fermentation and *creA* from 72 h up to the end of the fermentation. On the contrary, submerged cultures expressed *creA* at 24 h growth and *xlnR* at 48 h and 96 h with very little intensity (Fig. 3). This may explain that higher enzyme activities found in BF could be related to transcriptional activation by XlnR protein; however, the lack of XlnR activation in SF is not clear but it may suggest different regulatory mechanism and probably synergistic with XlnR in biofilms. A differential transcription analysis using *xlnR* and *creA* mutants in SF and BF could clarify such regulatory mechanisms (Mogensen et al. 2006).

It is known that *A. niger eglA*, *eglB*, *eglC*, *cbhA*, *cbhB*, *xlnB*, *xlnC* and *xlnD* genes contain binding sequences (GGCTAAA) to XlnR protein as well as binding sequences to CreA, a repressor protein acting in the presence of monomeric sugars (i.e., glucose) as a self-regulating mechanism (Gielkens et al. 1999; de Vries 2001; Hasper et al. 2002; Aro et al. 2005). However, there is no information about binding sequences to XlnR and CreA proteins in *exo*, *eng1* and *xynB* genes.

Iwashita (2002) has suggested that high enzyme production in SSF may be due to a specific solid-state signal responsive to a_w and to an unidentified *trans* activator that would cause both morphological and physiological changes and trigger a differential gene expression. According to our former hypothesis (Gutiérrez-Correa & Villena, 2003) this specific signal for BF could be stimulated by cell adhesion since temperature and a_w are similar in SF and BF, and a_w has not showed any positive influence in BF (Villena & Gutiérrez-Correa, 2007b).

Other important factor in the regulation of gene expression is related to mRNA stability. The expression level of a gene transcript is a function of both its synthesis rate and its degradation rate, the latter being important in controlling the cell adaptative capability. Thus, mRNA recycling gives cells flexibility to perform rapid changes in response to regulatory signals (Caddick et al., 2006). This mechanism could be efficiently coordinated in biofilms and it would explain in part their higher transcriptional levels of cellulase and xylanase genes as it is showed in Figure 2.

Conclusions

It seems that cell adhesion is the most important stimulus responsible for biofilm development and its particular morphogenetic and physiological responses derived from this biological process in accordance to our former hypothesis, which is the basis of Surface Adhesion Fermentation. Although preliminary, results presented in this paper show that, at least for several enzyme-encoding genes, a differential gene expression is actually active when *A. niger* grows as a biofilm. Also, it seems that the regulatory mechanism for cellulase and xylanase synthesis in *A. niger* biofilms is quite different from that prevailing in *A. niger* when growing in submerged fermentation. Most of the studies done on enzyme synthesis and regulation in fungi have been conducted in submerged cultivation without considering the natural tendency of filamentous fungi to grow adhered to surfaces as it occurs in nature. We are conducting a global transcriptomic and proteomic analysis of *A. niger* biofilms to clarify the process of cell adhesion as related to biofilm fermentation.

Acknowledgments

This work was supported by INCAGRO (Ministry of Agriculture, Peru), CONCYTEC (Ministry of Education, Peru), and the Institute of Molecular Biology and Biotechnology (Shizuoka University, Japan).

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