Rev. peru. biol. 16(1): 101- 108 (Agosto 2009)

Versión *Online* ISSN 1727-9933

Initial intracellular proteome profile of *Aspergillus niger* biofilms

Perfil inicial del proteoma intracelular de biopelículas de *Aspergillus niger*

Gretty K. Villena[1], Lavanya Venkatesh[2], Akihiro Yamazaki[2], Shinji Tsuyumu[2] and Marcel Gutiérrez-Correa[1]

1 Laboratorio de Micología y Biotecnología, Universidad Nacional Agraria La Molina, Lima, Perú. Email Marcel Gutiérrez-Correa: mgclmb@lamolina.edu.pe

2 Institute for Molecular Biology and Biotechnology, Shizuoka University, Shizuoka, Japan.

Abstract

An initial profiling of the intracellular proteome of *Aspergillus niger* ATCC 10864 biofilm cultures developed on polyester cloth was carried out by using 2D-PAGE and MS-TOF analysis and it was compared to the proteome of conventionally grown free-living submerged cultures. A number of 2D-PAGE protein spots from both types of cultures were subjected to MS-TOF analysis and data interrogation of the NCBI nr database available for this species. Proteomic maps showed different expression patterns in both culture systems with differentially expressed proteins in each case. In biofilm cultures, 19% and 32% of the selected protein spots were over-expressed and differentially expressed, respectively. On the contrary, in free-living cultures, 44% and 7% of the selected protein spots were over-expressed and differentially expressed, respectively. Although preliminary, results presented in this paper show that there are significant differences between the proteomes of *A. niger* biofilm and free-living mycelia. It seems that cell adhesion is the most important stimulus responsible for biofilm development which is the basis of Surface Adhesion Fermentation.

Keywords: *Asperigillus niger*, biofilms, proteome, MS -TOF, 2D-PAGE.

Presentado: 02/03/2009
Aceptado: 30/07/2009
Publicado online: 28/08/2009

Resumen

Se realizó un perfil inicial del proteoma de biopelículas de *Aspergillus niger* ATCC 10864 desarrolladas sobre tela de poliéster mediante 2D-PAGE y análisis MS-TOF y comparado con el proteoma de cultivos sumergidos convencionales de micelio libre. De ambos tipos de cultivo se analizó un número de muestras proteicas de geles 2D-PAGE mediante MS-TOF y los resultados se compararon con la base de datos NCBI nr disponible para esta especie. Los mapas proteómicos mostraron patrones diferentes de expresión en cada caso. En cultivo de biopelículas, el 19% y el 32% de las muestras seleccionadas fueron sobre-expresadas y diferencialmente expresadas, respectivamente. Por el contrario, en cultivos sumergidos en micelio libre el 44% y el 7% de las muestras seleccionadas fueron sobre-expresadas y diferencialmente expresadas, respectivamente. Aunque preliminares, los resultados presentados en este trabajo muestran que existen diferencias significativas entre los proteomas de biopelículas y micelio libre de *A. niger*. Parece ser que la adhesión celular es el estímulo más importante para el desarrollo de biopelículas, las cuales son la base de la Fermentación por Adhesión a Superficies.

Palabras clave: *Asperigillus niger*, biopelículas, proteoma, MS –TOF, 2D-PAGE.

Introduction

Functional genomics is the new way for genome analysis which is the development and application of several procedures for the understanding of gene function at a global scale. Functional genomics includes transcriptomics, proteomics, metabolomics and, recently, fluxomics (Eisenberg et al. 2000, Fenyö 2000, Kurland et al. 2006, Stotz et al. 2006, Stoeckert 2005, Wittmann 2007). Functional genomics provides fundamental information for the new field of Systems Biology, which seeks to understand cell processes at systems level through the quantitative description of the interactions among all cell components allowing the development of complex computational models for predicting behavioral responses to diverse stimuli (Aggarwal & Lee 2003, Albeck et al. 2006, Janes & Yaffe, 2006, Jaqaman & Danuser 2006, Kersey & Apweiler 2006, Swedlow et al. 2006).

The genus *Aspergillus* includes several species of outstanding biotechnological importance (Meyer 2008, Ward et al. 2006). Several *Aspergillus* species are used for the commercial production of enzymes and other biochemical products and their genes are being gradually investigated as an effort to understand their molecular activity in the fungal cell and to expand current industrial applications (Abe et al. 2006, Gouka et al. 1997, Gilseman et al. 2004, van den Hombergh et al. 1997, Kim et al. 2008). The genomes of *A. nidulans*, *A. fumigatus, and A. oryzae* were the first to be available (Galagan et al. 2005a, Galagan et al. 2005b, Machida et al. 2005, Gilseman et al. 2004, Nierman et al. 2005). *Aspergillus niger* genome has been recently sequenced (Pel et al. 2007, Semova et al. 2006).

The genome of *Aspergillus* comprises 8 chromosomes being those of *A. niger* and *A. oryzae* of the highest size with 33,9 and 37,0 Mb, respectively. *A. niger* genome contains ca. 14165 genes, with 1572 bp average length per gen and 0,42 genes density (genes/Kb) (Pel et al. 2007), and about 87% of its genes containing introns (Archer & Dyer 2004).

Contrary to the fast genome sequencing, filamentous fungal proteomic studies are moving slowly specially referred to secreted proteins (Medina et al. 2005). Although protein analysis is improved by advances in mass spectrometry (MS) and the continuous update of genomic data bases, sequences are not completely available. Generally, protein identification implies obtaining of MS patterns to be compared with all possible proteins coded by a genome available in a data bank (Reinders et al. 2004). When sequences are available, data obtained by MALDI- TOF MS (matrix assisted laser desorption/ionization - time of flight mass spectrometry) or MS-TOF (mass spectrometry - time of flight) peptide fingerprint may facilitate protein identification (Carberry & Doyle 2007, Eisenberg et al. 2000, Fenyö 2000).

Proteomes of some *Aspergillus* species have been studied only in the last five years (Carberry & Doyle 2007, Kim et al. 2008). Thus, very few insights have been displayed on the proteomes of *A. nidulans* (Kim et al. 2008, Ström et al. 2005), *A. oryzae* (Oda et al. 2006, Zhu et al. 2004), *A. flavus* (Medina et al. 2004, Medina et al. 2005), *A. fumigatus* (Asif et al. 2006, Carberry et al. 2006), and *A. niger* (Wright et al. 2009).

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). However, the cost of obtaining sugars from lignocellulose biomass for fermentation is still high, mostly due to low enzyme yields of producing microorganisms (Gabel and Zacchi 2002). Submerged fermentation (SF) is the main process used for cellulase production but other fermentation techniques are being tested. Filamentous fungi are naturally adapted to growth on surfaces and in these conditions they show a particular physiological behavior due to differential gene expression which is different from that in SF; thus, they can be considered as biofilm forming organisms. Fungal biofilm fermentation (BF) depends on surface adhesion and a new fermentation category named surface adhesion fermentation (SAF) was proposed by Gutiérrez-Correa and Villena (2003).

We have recently showed that there is a differential gene expression in *A. niger* biofilms (Villena et al. 2009). From this point of view, the study of differential proteome 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 initiate the study of the intracellular proteome of *Aspergillus niger* during biofilm formation on polyester.

Material 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 & Gutiérrez-Correa 2006).

Submerged and Biofilm Fermentation

For both types of fermentation systems 250 mL flasks containing 70 mL culture medium were used. For SF each flask was inoculated with 2,1 mL of the above spore suspension. For BF each flask containing a polyester 100/1cloth square in 70 mL distilled water was also 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 for 72 h.

Mycelial preparation

Fungal biomass was determined by measuring its dry cell weight. For SF samples, the entire content of a flask was filtered through pre-weighed filter paper (Whatman N.° 1) under suction; the filter paper was dried at 80 °C for a constant weight. For BF samples, the liquid part was removed by decanting and then the same steps used for free suspension were followed. The biofilm was washed three times with distilled water and then dried as above. Samples were conserved at -70 °C.

Protein preparation

For intracellular protein extraction 2 g 72 h-old mycelial samples were ground in a mortar with liquid nitrogen. Powdered biomass was gently suspended in 5 mL of extraction buffer (10mM Tris HCL pH8; 1mM EDTA, 2% (w/v) polivinilpolipirolidona PVPP) containing protease inhibitors (1µg/mL chymostatin, 1µg/mL aprotinin, 1µg/mL leupeptin and 2mM PMSF), and centrifuged at 8000 rpm for 30 min at 4 °C; then, supernatant was collected.

Protein samples were precipitated with methanol-chloroform following the procedure of Wessel & Fluegge (1984). Protein samples were successively mixed with methanol (4 volumes), chloroform (1 volume), distilled water (3 volumes), and centrifuged at 12000 *g* for 1 min. The upper phase was carefully removed and 3 volumes of methanol were added followed by centrifugation at 9000 *g* for 2 min. The supernatant was discarded and the pellet was air-dried at room temperature. Protein concentration was estimated using either Bradford (1976) or Lowry et al. (1951) procedures.

Two-dimensional electrophoresis.

Protein separation by 2D-PAGE was as follows: Precipitated proteins (600—1000 µg) were resuspended in 7 M Urea, 2 M thiourea, 4% (w/v) CHAPS, IPG buffer 0,5% (v/v), 3 mg/mL DTT and loaded onto Immobiline Dry strips (IPG strip; Amersham) in the non lineal pH range 3—10. Gels underwent active rehydration at 30 V for 10 h, followed by a further 9 h focusing with a total of 19200 V applied. Following IEF, gels were equilibrated with 2,5 mL of reducing buffer (50 mM Tris–HCl, 6M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 0,002% (w/v) bromophenol blue) and 3 mg/mL DTT for 12 min followed by equilibration in alkylation buffer (50 mM Tris–HCl, 6M urea, 2% (w/v) SDS, 30% (v/v) glycerol, and 25 mg/mL iodoacetamide) for a further 5 min. The IPG strips (18 cm) were placed on homogeneous 12,5% SDS–PAGE gels. Electrophoresis was performed at constant current of 152 mA and 5V/gel for 16 hours until bromophenol blue dye migrated to the end of the gel using Ettan Daltsix Electrophoresis System (Amersham) with temperature maintained at 4 °C using a recirculating water bath. Mass spectrometry compatible silver staining was performed according with Blum et al. (1987).

Mass spectrometry

Mass spectrometry was carried out using an MS-TOF Autoflex mass spectrometer (Brukers Daltonics, Yokohama, Japan). Briefly, protein spots were manually excised, destained with equal volume mixture of 30 mM $K_3Fe(CN)_6$ and 100 mM $Na_2S_2O_3$, and were in-gel digested with 25 mM de NH_4HCO_3 and 5 ng/µL trypsin at 37 °C overnight. Digested protein peptides were extracted multiple times by sonication with 50% acetonitrile (ACN)/0,1% trifluoroacetic acid (TFA), concentrated and desalted using Zip-Tip C18 reverse phase peptide separation matrix (Zip Tip® Millipore Corporation), and deposited (1 µL) with 1 µL α-cyano-4-hydroxycinnaminic acid (4-HCCA; 5 mg/200 µL of 50% (v/v) acetonitrile in 0,1% (v/v) aqueous trifluoroacetic acid) onto mass spectrometry slides, and allowed to dry prior to delayed extraction, reflectron ToF analysis at 20 kV. Protein identification was carried out by m/z data interrogation of the NCBI nr database available for *Aspergillus* using MATRIX SCIENCE Mascot Search (http://www.matrixscience.com/search_forms_elect.htm).

Results and discussions

Intracellular proteomes of *Aspergillus niger* biofilm and submerged cultures were compared by using 2D-PAGE and MS-TOF. Proteomic maps showed different expression patterns in both culture systems with differentially expressed proteins in each case.

Figure 1 shows the intracellular proteome map of *A. niger* biofilms. Although protein concentration was somewhat low probably due to the low protein solubility in the IEF solubilization buffer, we found an adequate protein resolution. Forty eight protein spots were chosen among those showing either differential or higher expression levels. From the spots marked in the map 19% and 32% were over-expressed and differentially expressed proteins in biofilm cultures, respectively (Fig. 1). Highly stained spots were chosen for further MS-TOF analysis (56% of the 48 initial proteins), identifying 55% and 56% over-expressed and differentially expressed proteins, respectively.

All selected over-expressed proteins (spots 3B, 4B, 9B, 10B, 25B, and 26B) were hypothetic, i.e., proteins that their sequences do not match those of known sequence and function, yet they show shared domains with known proteins (Table 1). Thus, proteins 3B and 9B match an *A. nidulans* hypothetic protein that shares domains with an outer mitocondrial membrane protein porin of *N. fischeri* (E value= 5e-155). Protein 4B is strongly related to ubiquinol-cytochrome C reductase of *A. fumigates* (E= 0) and with hypothetical protein of *A. niger* (E value= 0) and *A. oryzae* (E value= 0). Protein 10B is related with both an *A. flavus and A. clavatus* cytrochorme b5 reductase (E= 6e-174 and 4e -171, respectively). Protein 25B shares domains with a hypothetical protein of *A. nidulans* (E= 3e-26) and with a putative β-xylosidase of *P. marneffei* (E= 1e-121). Finally, protein 26B is nearly related to a hypothetical protein of *A. niger* (E value= 0) and *A. oryzae* (E value= 0) and with AMP deaminases of both *A. fumigatus* and *A. terreus* (E= 0 in both cases).

Table 1. Over-expressed intracellular proteins in *Aspergillus niger* biofilms identified by MS-TOF analysis.

spot	Mr (Da)	pI	accession	NCBI Protein description	score	Related proteins (E value)	Searched/ matched peptides	Sequence coverage
3B	28891	9	gi | 40741129	Hypothetical protein AN4402.2 [*Aspergillus nidulans* FGSC A4]. 284 aa.	92	Outer mitochondrial membrane protein porin [*Neosartorya fischeri* NRRL 181] (5e-155) Outer mitochondrial membrane protein porin [*Ajellomyces dermatitidis* SLH14081] (2e-149)	19/8	20%
4B	47605	9	gi | 40739821	Hypothetical protein AN8273.2 [*Aspergillus nidulans* FGSC A4]. 458 aa.	38	Ubiquinol-cytochrome C reductase complex core protein 2, putative [*Aspergillus fumigatus* Af293] (0) Hypothetical protein An09g06650 [*Aspergillus niger*] (0). Hypothetical protein [*Aspergillus oryzae* RIB40] (0)	18/5	11%
9B	28891	9	gi | 40741129	Hypothetical protein AN4402.2 [*Aspergillus nidulans* FGSC A4]. 284 aa.	57	Outer mitochondrial membrane protein porin [*Neosartorya Fischer* NRRL 181] (5e-155) Outer mitochondrial membrane protein porin [*Ajellomycesdermatitidis* SLH14081] (2e-149) Hypothetical protein [*Aspergillus oryzae* RIB40] (1e-116)	12/5	17%
10B	51220	8.79	gi | 40739937	Hypothetical protein AN3862.2 [*Aspergillus nidulans* FGSC A4]. 468 aa.	36	Cytochrome b5 reductase, putative [*Aspergillus flavus* NRRL3357] (6e-174) Cytochrome b5 reductase, putative [*Aspergillus clavatus* NRRL 1] (4e-171)	9/4	11%
25B	12378	7.85	gi | 40743794	Hypothetical protein AN2633.2 [*Aspergillus nidulans* FGSC A4]. 110 aa.	44	Hypothetical protein AN7864.2 [*Aspergillus nidulans* FGSC A4] (3e-26) Beta-xylosidase, putative [*Penicillium marneffei* ATCC 18224] (1e-21)	8/3	26%
26B	99961	5.95	gi | 40744930	Hypothetical protein AN8872.2 [*Aspergillus nidulans* FGSC A4]. 878 aa	52	Hypothetical protein An03g06970 [*Aspergillus niger*] (0) AMP deaminase Amd1 [*Aspergillus fumigatus* Af293] (0) Hypothetical protein [*Aspergillus oryzae* RIB40] (0) AMP deaminase [*Aspergillus terreus* NIH2624] (0)	5/5	6%

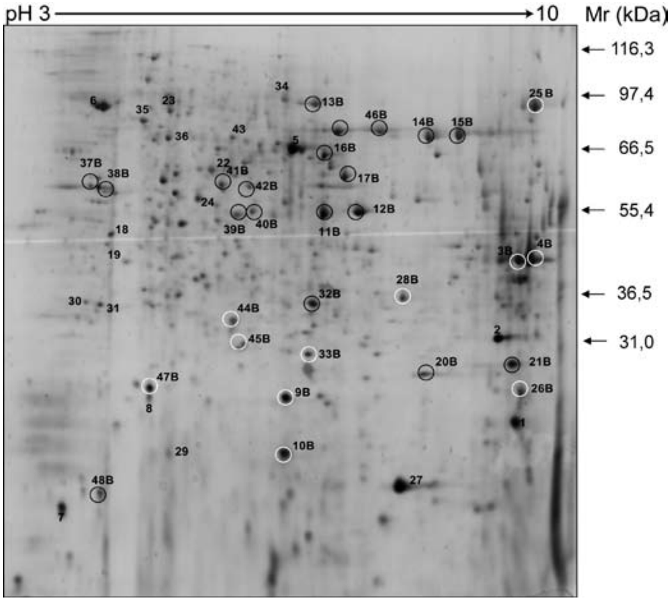

Figure 1. Silver-stained 2-DE gels of intracellular *Aspergillus niger* biofilm proteins. White circles correspond to over-expressed proteins and black circles to differentially expressed proteins that were analyzed by MS-TOF. Uncircled spots were not analyzed.

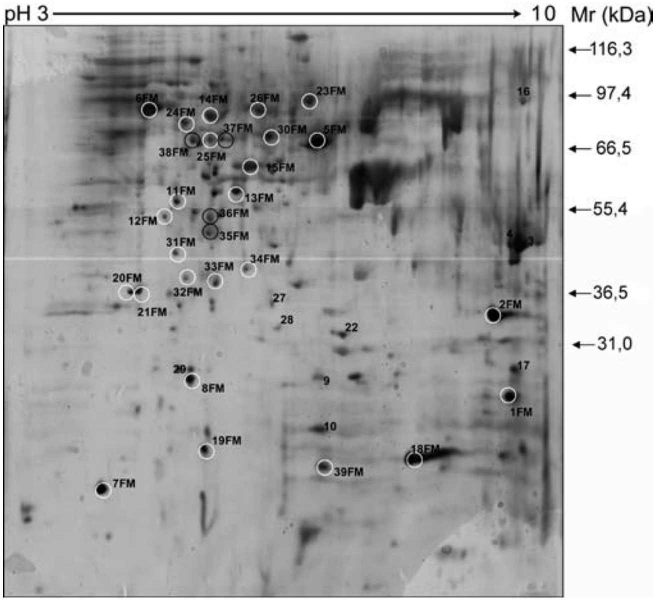

Figure 2. Silver-stained 2-DE gels of intracellular proteins from *Aspergillus niger* free-living mycelial submerged cultures. White circles correspond to over-expressed proteins and black circles to differentially expressed proteins that were analyzed by MS-TOF. Uncircled spots were not analyzed.

Differentially expressed proteins in biofilm cultures are presented in Table 2. As above, most of the spots were hypothetic proteins. Protein 16B matches putative calcium P-type ATPase similar to that of *A. fumigates and A. flavus* (E= 0 in both cases), while protein 21B matches α-sarcin – a type of fungal RNAse (E= 9e-101). Other spots were of hypothetic proteins (see Table 2 for details).

Intracellular proteomic map of *A. niger* conventionally grown as free-living mycelium in SF is presented in Fig. 2. From the spots marked in the map, 44% were over-expressed and only 7% were differentially expressed proteins. Unfortunately, none of differentially expressed protein spots could be analyzed due to the low concentration of them. However, 42% of the over-expressed proteins were MS-TOF analyzed. Most of the over-expressed proteins were identified and only 3 of them were hypothetic (see Table 3 for details). Spot 2FM is a hypothetical protein sharing domains with ribosomal proteins and it is closely related to both *A. fumigatus* L19e 60S ribosomal protein (E= 0) and an unnamed protein product of *A. niger* (E= 0). Spot 11FM did not share domains with other proteins and it showed similarity to a conserved hypothetical protein of *A. fumigatus* (E= 2e-48).

Since protein expression levels depend on regulatory systems, proteomes are highly dynamic but this allows comparative studies under different conditions (Carberry & Doyle 2007). As it has been described above, intracellular proteome analysis of *A. niger* grown under biofilm and submerged fermentation conditions demonstrated that there are different protein expression patterns in both fermentation systems. Although most of the proteins found in BF are hypothetic, they showed shared domains with known proteins which, in turn, may help to assign their functions. Protein identification through MS patterns mainly depends on the quality of the annotated gene sequences available in the database banks. In this sense, as significant fungal expressed sequence tags (EST) data is lacking, particularly for *A. niger* and other *Aspergillus* species, gene prediction strongly depends on *de novo* prediction (Galagan et al. 2005a). Semova et al. (2006) could sequence and identify only 650 out of 4856 new genes from 12820 ESTs obtained from 15052 transcripts of *Aspergillus niger* N402, FGSC#4732. In this work, we have used the NCBI database with *A. niger* sequences released by April 2005 and *A. nidulans* orthologues, since this species has 64% protein homology with it whereas 20% of the genes do not have homology (Semova et al. 2006). However, only 7,5% ORFs of *A. nidulans* have an assigned function (Mogensen et al. 2006) from which a complete identification is less probable. This also explains some low aligning scores obtained in our MS TOF analysis. Although all over- or differentially expressed proteins could not be completely identified there are interesting differences between both fermentation systems. Thus, *A. niger* biofilms differentially expressed a putative calcium P-type ATPase which is important both in the homeostatic maintenance of calcium concentration in the endoplasmic reticulum and in cation-dependant functions of Golgi apparatus (Vashit et al. 2002); this protein is probably involved in cAMP-mediated signaling (Bencina et al. 2005). Also, another differentially expressed protein found in biofilms is an *A. giganteus* α-sarcin – a cytosolic basic protein with ribonucleolytic activity – with biotechnological potential as anti-tumor agent (Moreno et al. 2006, Olmo et al. 2001).

Although we could not identify any of the differentially expressed proteins in *A. niger* SF due to their very low concentrations, some of the over-expressed proteins were related to stress conditions. Thus, cyclophilin-like peptidyl prolyl cis transisomerase is related to endoplasmic reticulum stress (Damveld et al. 2005) and 6 beta-hydroxyhyoscyamine epoxidase is related to secondary metabolism. A peroxisomal like protein of unknown function was also over-expressed (Aign & Hoheisel 2003). Finally, a subunit of pyruvate dehydrogenase E1 (Table 3, spot 14FM) was over-expressed as it has been recently found in *A. nidulans* under hypoxic conditions (Shimizu et al. 2009).

Table 2. Differentially expressed intracellular proteins in *Aspergillus niger* biofilms identified by MS-TOF analysis.

Spot	Mr (Da)	pI	Accession	NCBI Protein description	Score	Related proteins (E value)	Searched/ matched peptides	Sequence coverage
11B	24160	4.73	gi | 49525279	Unnamed protein product [*Candida glabrata*]	27	Mitochondrial Ribosomal protein MRP8 (2e-70)	30/4	16%
12B	50549	8.93	gi | 40738989	Hypothetical protein AN6650.2 [*Aspergillus nidulans* FGSC A4]. 460 aa.	38	Hypothetical protein [*Aspergillus oryzae* RIB40] (0) Citrate synthase Cit1, putative [*Aspergillus flavus* NRRL3357] (0) 2-methylcitrate synthase, mitochondrial precursor [*Aspergillus terreus* NIH2624] (0)	10/4	8%
13B	43391	6.91	gi | 40743549	Hypothetical protein AN5646.2 [*Aspergillus nidulans* FGSC A4]. 417 aa.	59	Hypothetical protein An04g05720 [*Aspergillus niger*] (0) Hypothetical protein [*Aspergillus oryzae* RIB40] (0) 3-ketoacyl-coA thiolase peroxisomal A precursor [*Aspergillus flavus* NRRL3357] (0)	19/7	16%
14B	43391	6.91	gi | 40743549	Hypothetical protein AN5646.2 [*Aspergillus nidulans* FGSC A4]. 417 aa.		Hypothetical protein An04g05720 [*Aspergillus niger*] (0) Hypothetical protein [*Aspergillus oryzae* RIB40] (0) 3-ketoacyl-coA thiolase peroxisomal A precursor [*Aspergillus flavus* NRRL3357] (0)	17/7	21%
15B	38975	8.63	gi | 40743825	Hypothetical protein AN2713.2 [*Aspergillus nidulans* FGSC A4]. 345 aa.	44	Hypothetical protein AN3471.2 [*Aspergillus nidulans* FGSC A4](0) Hypothetical protein AN6966.2 [*Aspergillus nidulans* FGSC A4](0)	24/6	16%
16B	126249	6.65	gi | 6688831	Hypothetical protein NCU05154 [*Neurospora crassa* OR74A]. 1152 aa.	51	P-type calcium ATPase [*Aspergillus fumigatus* Af293](0) P-type calcium ATPase, putative [*Aspergillus flavus* NRRL3357](0)	19/9	8%
17B	60416	8.13	gi | 40745893	Hypothetical protein AN1884.2 [*Aspergillus nidulans* FGSC A4]. 544 aa.	42	Cytochrome P450 monooxygenase, putative [*Aspergillus fumigatus* A1163](0) Conserved hypothetical protein [*Aspergillus terreus* NIH2624](0) Hypothetical protein An11g02990 [*Aspergillus niger*](0)	12/5	7%
20B	78048	8.83	gi | 40739380	Hypothetical protein AN6752.2 [*Aspergillus nidulans* FGSC A4]. 696 aa.	37	Hypothetical protein [*Aspergillus oryzae* RIB40](0) Fatty-acyl coenzyme A oxidase (Pox1), putative [*Aspergillus fumigatus* A1163](0) Conserved hypothetical protein [*Aspergillus terreus* NIH2624](0)	14/6	9%
21B	19712	9.26	gi | 2311	Ribonuclease alpha-sarcin.177 aa.	42	a-sarcin precursor [*Penicillium daleae*](9e-101) Gigantin [*Aspergillus giganteus*] (4e-96)	33/6	30%

Table 3. Over-expressed intracellular proteins in *Aspergillus niger* free-mycelium (mycelia pellets) identified by MS-TOF analysis.

Spot	Mr (Da)	pI	Accession	NCBI Protein description	Score	Related proteins (E value)	Searched/ matched peptides	Sequence coverage
1FM	18861	8.87	gi | 4322946	Cyclophilin-like peptidyl prolyl cis-trans isomerase cypA- *Aspergillus niger*. 174 aa.	170	Peptidyl-prolyl cis-trans isomerase [*Aspergillus flavus* NRRL3357] (6e-81) Peptidyl-prolyl cis-trans isomerase [*Aspergillus clavatus NRRL* 1] (7e-81)	10/9	51%
2FM	305669	6.58	gi | 40739159	Hypothetical protein AN5840.2 [*Aspergillus nidulans* FGSC A4]. 2788 aa.	39	60S ribosomal protein L19 [*Aspergillus terreus* NIH2624] (0) Unnamed protein product [*Aspergillus niger*] (0) Hypothetical protein [*Aspergillus oryzae* RIB40] (0)	11/8	3%
5FM	54818	5.17	gi | 40745270	ATPB_NEUCR ATP synthase beta chain, mitochondrial precursor [*Aspergillus nidulans FGSC A4*]. 513 aa.	131	ATP synthase F1, beta subunit [*Aspergillus fumigatus* Af293] (0) ATP synthase F1, beta subunit, putative [*Neosartorya fischeri* NRRL181] (0) Hypothetical protein [*Aspergillus oryzae* RIB40] (0)	18/12	31%
6FM	34370	8.64	gi |40746425	RL5_NEUCR 60S ribosomal protein L5 (CPR4) [*Aspergillus nidulans* FGSC A4]. 301 aa.	16	60S ribosomal protein L5, putative [*Neosartorya fischeri* NRRL 181] (1e-172) 60S ribosomal protein L5 [*Aspergillus terreus* NIH2624] (1e-154) Hypothetical protein An08g05730 [*Aspergillus niger*] (4e-151)	10/2	6%
7FM	18441	5.36	gi | 2769700	Allergen Asp F3 [*Aspergillus fumigatus* Af293]. 168 aa.	48	Allergen Asp F3 [*Neosartorya fischeri* NRRL 181] (1e-92) Allergen Asp F3 [*Aspergillus clavatus* NRRL 1] (1e-89) Hypothetical protein An12g08570 [*Aspergillus niger*] (5e-86)	13/4	31%
8FM	22840	7.21	gi | 3869086	CAP59 [*Cryptococcus bacillisporus*]. 199 aa.	58	Hypothetical protein An04g04630 [*Aspergillus niger*] (1e-11) Hypothetical protein An18g00730 [*Aspergillus niger*] (5e-10) Polysaccharide export protein (CAP59) [*Aspergillus fumigatus* Af293] (1e-09)	7/4	16%
11FM	16626	5.72	gi | 40741469	Hypothetical protein AN8625.2 [*Aspergillus nidulans* FGSC A4]. 150 aa.	35	Conserved hypothetical protein [*Aspergillus fumigatus Af293*] (5e-48) Hypothetical protein NFIA_047840 [*Neosartorya fischeri* NRRL 181] (6e-48) Conserved hypothetical protein [*Aspergillus clavatus* NRRL 1] (3e-47)	12/3	13%
13FM	47377	5.46	gi | 2118302	6 beta-hydroxyhyoscyamine epoxidase [*Aspergillus oryzae* RIB40]. 438 aa.	80	Hypothetical protein [*Aspergillus oryzae* RIB40] (0) Enolase/allergen Asp F 22 [*Aspergillus clavatus* NRRL 1] (0) Hypothetical protein An18g06250 [*Aspergillus niger*] (0)	14/8	28%

Continúa ...

14FM	40378	7.57	gi | 45771900	Pyruvate dehydrogenase E1 B-subunit [*Aspergillus niger*]. 374 aa.	75	Hypothetical protein An01g00100 [*Aspergillus niger*] (0) Pyruvate dehydrogenase E1 beta subunit PdbA, putative [*Neosartorya fischeri* NRRL 181] (0) Pyruvate dehydrogenase E1 component beta subunit, mitocondrial precursor [*Aspergillus terreus* NIH2624] (0)	13/7	20%
15FM	50762	9.21	gi | 40740127	EF1A_ASPOR Elongation factor 1-alpha (EF-1-alpha) [*Aspergillus nidulans* FGSC A4]. 470 aa.	56	Translation elongation factor EF-1 alpha subunit , putative [*Neosartorya fischeri* NRRL 181] (0) Hypothetical protein An18g04840 [*Aspergillus niger*] (0) Elongation factor 1-alpha [*Aspergillus terreus* NIH2624] (0)	13/6	14%

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 there are significant differences between the proteomes of *A. niger* biofilm and free-living mycelia. This is in agreement with our previous results on transcriptomics analysis in the same culture conditions (Villena et al. 2009). New insights will be obtained with the new available genomes of *A. niger* CBS 513.88 (Pel et al. 2007) and ATCC1015 (draft version in: http://genome.jgi-psf.org/Aspni5/Aspni5.home.html), and the recent attempt to use proteomic data for *A. niger* genome annotation (Wright et al. 2009). 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, Perú), CONCYTEC (Ministry of Education, Perú), and the Institute of Molecular Biology and Biotechnology (Shizuoka Univesity, Japan).

Literature cited

Abe K., K. Gomi, F. Hasegawa & M. Machida. 2006. Impact of Aspergillus oryzae genomics on industrial production of metabolites. Mycopathologia 162: 143–153.

Aign V. & J.D. Hoheisel. 2003. Analysis of nutrient-dependent transcript variations in Neurospora crassa. Fungal Genet. Biol. 40: 225–233.

Aggarwal A. & H. Lee. 2003. Functional genomics and proteomics as a foundation for systems biology. Brief. Funct. Genom. Proteom. 2: 175–184.

Albeck J.G., G. MacBeath, F.M. White, P.K. Sorger, D.A. Lauffenburger & S. Gaudet. 2006. Collecting and organizing systematic sets of protein data. Nat. Cell Biol. 8: 803-812.

Archer D.B. & P.S. Dyer. 2004. From genomics to postgenomics in Aspergillus. Curr. Opin. Microbiol. 7: 499-504.

Asif A.R., M. Oellerich, V.W. Amstrong, B. Riemenschneider, M. Monod & U. Reichard. 2006. Proteome of Conidial Surface Associated Proteins of Aspergillus fumigatus Reflecting Potential Vaccine Candidates and Allergens. J. Proteome Res. 5: 954-962.

Bencina M., M. Legi & N.D. Read. 2005. Cross-talk between cAMP and calcium signalling in Aspergillus niger. Mol. Microbiol. 56: 268–281.

Bhat M.K. 2000. Cellulases and other related enzymes in biotechnology. Biotechnol. Adv. 18: 355-383.

Blum H.; H. Beier & H.J. Gross. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8: 93-99.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal. Biochem. 72: 248-254.

Carberry S., C.M. Neville, K.A. Kavanagh & S. Doyle. 2006. Analysis of major intracellular proteins of Aspergillus fumigatus by MALDI mass spectrometry: Identification and characterization of an elongation factor 1B protein with glutathione transferase activity. Biochem. Biophys. Res. Comm. 341: 1096–1104.

Carberry S. & S. Doyle. 2007. Proteomic studies in biomedically and industrially relevant fungi. Cytotechnol. 53: 95–100.

Damveld R.A., P.A. vanKuyk, M. Arentshorst, F.M. Klis, C.A.M.J.J. van den Hondel, A.F.J. Ram. 2005. Expression of agsA, one of the 1,3-β-D-glucan synthase-encoding genes in Aspergillus niger, is induced in response to cell wall stress. Fungal Genet. Biol. 42: 165–177.

Eisenberg D. E.D. Marcotte, J. Xenarios & T. Yeates. 2000. Protein function in the post-genomic era. Nature 405: 823-826.

Fenyö D. 2000. Identifying the proteome: software tools. Curr. Opin. Biotechnol. 11: 391-395.

Galagan J.E., M.R. Henn, L.J. Ma, C.A. Cuomo & B. Bruce. 2005a. Genomics of the fungal kingdom: Insights into eukaryotic biology. Genome Res. 15: 1620-1631.

Galagan J.E., S.E. Calvo, C. Cuomo, et al. 2005b. Sequencing of Aspergillus nidulans and comparative analysis with A. fumigatus and A. oryzae. Nature 438: 1105-1115.

Galbe M. & G. Zacchi. 2002. A review of the production of ethanol from softwood. Appl. Microbiol. Biotechnol. 59: 618-628.

Gilseman J.E.M., M.J. Anderson, P.F. Giles, et al. 2004. CADRE: the Central Aspergillus Data Repository. Nucleic Acids Res. 32: Database issue D401-D405.

Gouka R.J., P.J. Punt & C.A.M.J.J. van den Hondel. 1997. Efficient production of Secreted proteins by Aspergillus: Progress, limitations and prospects. Appl. Microbiol. Biotechnol. 47: 1–11.

Gutiérrez-Correa M. & G.K. Villena. 2003. Surface adhesion fermentation: a new fermentation category. Rev. peru. biol. 10: 113-124.

Jaqaman K. & G. Danuser. 2006. Linking data to models: data regression. Nat. Cell Biol. 8: 813-819.

Janes K.A. & M.B. Yaffe. 2006. Data-driven modeling of signal-transduction networks. Nat. Cell Biol. 8: 820-828.

Kersey P. & R. Apweiler. 2006. Linking publication, gene and protein data. Nat. Cell Biol. 8: 1183-1189.

Kim Y., M.P. Nandakumar & M.R. Marten. 2008. The state of proteome profiling in the fungal genus Aspergillus. Brief. Funct. Genom. Proteom. 7: 87-94.

Kurland C.J., L. Collins & D. Penny. 2006. Genomics and the irreducible nature of eukaryote cells. Science 312: 1011-1014.

Lowry O.H., N.J. Rosebrough, A.L. Farr & R.J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Machida M., K. Asai, M. Sano, et al. 2005. Genome sequencing and analysis of Aspergillus oryzae. Nature 438: 1157-1161.

Medina M.L., U.A. Kiernan & W.A. Francisco. 2004. Proteomic analysis of rutin-induced secreted proteins from Aspergillus flavus. Fungal Genet. Biol. 41: 327–335.

Medina M.L., P. Haynes, L. Breci & W.A. Francisco. 2005. Analysis of secreted proteins from Aspergillus flavus. Proteomics 5: 3153-3161.

Meyer V. 2008. Genetic engineering of filamentous fungi-progress, obstacles and future trends. Biotechnol. Adv. 26: 177–185.

Mogensen J., H.B. Nielsen, G. Hofmann & J. Nielsen. 2006. Transcription analysis using high-density micro-arrays of Aspergillus nidulans wild-type and creA mutant during growth on glucose or ethanol. Fungal Genet. Biol. 43: 593–603.

Moreno A.B.; A. Martínez del Pozo & B. San Segundo. 2006. Biotechnologically relevant enzymes and proteins. Antifungal mechanism of the Aspergillus giganteus AFP against the rice blast fungus Magnaporthe grisea. Appl. Microbiol. Biotechnol. 72: 883-895.

Nierman, W.C. A. Pain, M.J. Anderson, et al. 2005. Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. Nature 438: 1151-1156.

Oda K, D. Kakizono, O. Yamada, et al. 2006. Proteomic Analysis of Extracellular Proteins from Aspergillus oryzae Grown under Submerged and Solid-State Culture Conditions. Appl. Environ. Microbiol. 72: 3448–3457.

Olmo N., J. Turnay, G. Gonzalez de Buitrago, I. López de Silanes, et al. 2001. Cytotoxic mechanism of the ribotoxin α-sarcin: Induction of cell death via apoptosis. Eur. J. Biochem. FEBS 268: 2113-2123.

Pel H.J., J.H. de Winde, D.B. Archer, et al. 2007. Genome sequencing and analysis of the versatile cell factory Aspergillus niger CBS 513.88. Nat. Biotechnol. 25: 221-231.

Reinders J., U. Lewandrowski, J. Moebius, Y. Wagner & A. Sickmann. 2004. Challenges in mass spectrometry-based proteomics. Proteomics 4: 3686-3703.

Semova N., R. Storms, T. John & P. Gaudet. 2006. Generation, annotation, and analysis of an extensive Aspergillus niger EST collection. BMC Microbiol. 6:7, doi:10.1186/1471-2180-6-7.

Shimizu M., T. Fuji, S. Masuo, K. Fujita & N. Takaya. 2009. Proteomic analysis of Aspergillus nidulans cultured under hypoxic conditions. Proteomics 9: 7-19.

Stoeckert C.J. Jr. 2005. Functional genomics databases on the web. Cell. Microbiol. 7: 1053-1059.

Stotz K.C., A. Bostanci & P.E. Griffiths. 2006. Tracking the Shift to 'Postgenomics'. Community Genet. 9:190–196.

Ström K., J. Schnürer & P. Melin. 2005. Co-cultivation of antifungal Lactobacillus plantarum MiLAB 393 and Aspergillus nidulans, evaluation of effects on fungal growth and protein expression. FEMS Microbiol. Lett. 246: 119–124.

Swedlow J.R., S.D. Lewis & I.G. Goldberg. 2006. Modelling data across labs, genomes, space and time. Nat. Cell Biol. 8: 1190-1195.

van den Hombergh J. P. T. W., P. J. I. van de Vondervoort, L. Fraissinet & J. Visser. 1997. Aspergillus as a host for heterologous protein production: The problem of proteases. Trends Biotechnol. 15: 256–263.

Vashist S., C.G. Frank, C.A. Jakob & D.T.W. Ng. 2002. Two distinctly localized P-type ATPases collaborate to maintain organelle homeostasis required for glycoprotein processing and quality control. Mol. Biol. Cell 21: 3955–3966.

Villena G.K. & M.Gutiérrez-Correa. 2006. Production of cellulase by Aspergillus niger biofilms developed on polyester cloth. Lett. Appl. Microbiol. 43: 262-268.

Villena G.K., T. Fujikawa, S. Tsuyumu & M. Gutiérrez-Correa. 2009. Differential gene expression of some lignocellulolytic enzymes in Aspergillus niger biofilms. Rev. peru. biol. 15: 97- 102.

Ward O.P., W.M. Qin, J. Dhanjoon, J. Ye & A. Singh. 2006. Physiology and Biotechnology of Aspergillus. Adv. Appl. Microbiol. 58: 1-75.

Wessel D. & U.I. Fluegge. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. 138: 141-143.

Wittmann C. 2007. Fluxome analysis using GC-MS. Microb. Cell Fact. 6: 6, doi:10.1186/1475-2859-6-6.

Wright J.C., D. Sugden, S. Francis-McIntyre, I. Riba-Garcia, S.J. Gaskell, et al.. 2009. Exploiting proteomic data for genome annotation and gene model validation in Aspergillus niger. BMC Genomics 10: 61, doi: 10.1186/1471-2164-10-61.

Zhu L.Y., C.H. Nguyen, T. Sato & M. Takeuchi. 2004. Analysis of secreted proteins during conidial germination of Aspergillus oryzae RIB40. Biosci. Biotechnol. Biochem. 68: 2607-2612.