

# Proteomics Methodology Applied to the Analysis of Filamentous Fungi - New Trends for an Impressive Diverse Group of Organisms

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## 1. Introduction

Life comprises three domains: the Bacteria, the Archaea and the Eukaryota. Within the last one, the Fungi kingdom forms a monophyletic group of the eukaryotic crown group, which includes the largest group of organisms, where more than 500,000 species have been described and more than one million might exist. The Fungi kingdom consists of a heterogeneous group that includes yeasts, moulds and mushrooms characterized by their lack of photosynthetic pigment and their chitinous cell wall (Hawksworth, 1991). Fungi are one of the most important groups of organisms on the planet, for they play remarkable roles in several ways.

From the environmental point of view fungi, together with bacteria, are responsible for most of the organic matter decomposition process, thus recycling dead material and recalcitrant compounds such as lignin; thereby the organic material utilization by the microbial community is enhanced. This fungal recycling activity has a negative effect when it is exerted on food. Fungi can be responsible for large losses of stored food, particularly food which contains any moisture. This may be a major problem where large quantities of food are being produced seasonally and then require storage until they are needed.

Biocontrol, an alternative to chemical pesticides, is another important role played by fungi. Some species of this kingdom parasitize insects, which can be extremely useful for controlling insect pests of crops, such as the Colorado potato beetles, spittlebugs, leaf hoppers and citrus rust mites. In addition, mycorrhizal associations between plants and fungi are necessary for optimal growth of most plants, such as crops. However, some fungi can also cause negative effects on crops, since many plants are susceptible to fungal attack.

A wide range of fungi also live on and in humans, other animals or plants, but most coexist harmlessly. Nevertheless, some fungal species are involved in disease interactions, either by means of their direct action as disease agents or through the production of secondary metabolites (eg: host-specific toxins). In fact, filamentous fungi produce a diverse array of secondary metabolites, which have a tremendous impact on society because they are exploited for their ability to produce antibiotic (penicillins, cephalosporins, etc) or pharmaceuticals (cyclosporin and other immunosuppressants). Indeed, fungi are the second group, after Actinobacteria, of secondary metabolites producers with industrial application.

Among secondary metabolites, antibiotics (and more precisely  $\beta$ -lactam antibiotics) are especially relevant. The discovery of  $\beta$ -lactam antibiotics is one of the most significant milestones of the human history and entailed a revolution in modern chemotherapy. Members of this family of antibiotics are commonly prescribed world widely due to their high activity and low toxicity and have helped medicine to drastically reduce the mortality rate.

Finally, we should not forget that some fungi are important for food industry. Many mushrooms (basidiomycetes) are edible and different species are cultivated for sale worldwide. Fungi are also widely used in the production of many foods and drinks, such as cheese, beer, wine, bread, juices, cakes, some soya bean products, due to their ability to produce useful enzyme cocktails for white biotechnology.

Several new fungal species are discovered every year, which indicates that a huge number of unrecognized and unidentified fungal species (more than 90%), could be found in association with plants, insects, animals, as lichen-forming fungi or in undisturbed areas (Hawksworth 2001). The three main fungal phyla are: Ascomycota (comprises the majority of all described fungi), Zygomycota and Basidiomycota, which theoretically diverged from the Chytridiomycota approximately 550 million years ago. The split between Ascomycota and Basidiomycota happened 400 million years ago, which was an evolutionary step previous to the land invasion of the plants. Intriguingly, the antiquity of the group strongly contrasts with the current low number of complete fungal genomes deposited in the databases, which is usually the main key that opens the door to the Proteomics application. Since 1996, when the first completed fungal genome of the ascomycetous yeast *Saccharomyces cerevisiae* was released and until 2011, sequencing of around 20 complete fungal genomes had been completed. These sequencing data support the incredible increment of Proteomics applied manuscripts related to filamentous fungi published just in the last decade. It was not until 2004 when the analysis of the secreted proteins of *Aspergillus flavus* was described, or 2005 when the secretome analysis of the edible fungus *Pleurotus sapidus* was published. These data support the interest of a methodological review of filamentous fungal Proteomics. This approach can be done using the three Proteomics levels analysed until our days: i) intracellular, ii) extracellular and iii) membrane and organelle proteomes.

## 2. Proteomics, a useful tool for the analysis of fungi

Proteomics, as the analysis of the protein components of organisms, is a well-known tool providing a global perspective (whole-organism approach) of cellular physiology that allows understanding the cellular protein expression alterations in response to various biotic and abiotic stresses. Due to its capacity to yield definitive information on protein identity, localization, posttranslational modification and the accuracy of *in silico* gene model prediction in fungi, Proteomics has become an integral component of all large-scale “omic” and systems approaches to understanding the rich complexity of fungal biochemistry (Doyle, 2011). The increase of plant-pathogen interaction analysis, focused on the plant-fungus association is a topic of rising interest in the last five years; even when the limitation in sequence availability in public databases is also challenging (Mehta et al., 2008). These studies are concerning to the understanding of the pathogen interaction with their respective hosts to combat the crop diseases, inductor effect of the susceptible host root plant extracts over pathogenic fungi or novel sources of host resistance and the design of

increased disease resistance in crop plants. In addition to these modern Proteomics analyses, traditionally the research about fungal diseases has been applied to humans (eg: invasive pulmonary aspergillosis), plants (eg: grapevine decline) or other animals (eg: entomopathogenic fungus). Two main approaches are used for Proteomics: i) individual protein identification by MALDI-TOF/TOF or ii) tandem LC-MS. These techniques not only allow the elaboration of proteome reference maps, but also the comparative analysis between different experimental conditions, strains or mutants.

Due to the role that ascomycetes play in causing animal diseases and to their ability to be used as cell factories for biotechnology, proteomic studies focused on ascomycetes exceed those carried out using basidiomycetes. All fungal data sets include either "predicted proteins" or "hypothetical proteins" (Martin et al., 2008; Ferreira de Oliveira et al., 2010) and this information should be replaced by "proteins of unknown function" since it is rather clear that if these proteins have been identified, they do exist and are no longer "hypothetical". Providing a function to those "proteins of unknown function" represents one of the major challenges in fungal Proteomics.

### 3. Analysis of the intracellular proteome

The intracellular proteome of a eukaryotic organism mainly consists of those proteins that are present in the cytosol and inside organelles. Due to the heterogeneity of the fungi kingdom, the most basic procedures, such as good quality protein extraction for bidimensional analysis, should be updated for each species. Thus, a general overview of sample preparation, as well as proteomics applications to fungal analysis are developed along the next sections.

#### 3.1 Sample preparation

Since most fungi possess a robust cell wall, the protein extraction protocol is a crucial step for fungal intracellular proteins analysis. A bibliographic search shows different procedures for protein extraction, precipitation or protein solubilisation that should be adapted for each fungus. These extraction procedures for intracellular proteomes, in the case of free living microorganisms, have some common steps, such as the mechanical breaking, the use of protease inhibitors, reducing agents and protein precipitation.

The process to obtain cytoplasmic proteins should include an initial step to discard the culture media, unbroken mycelia, cell wall and membrane contaminants in order to obtain the intracellular proteome as clean as possible. Thus, when cultures reach the desired conditions, the most common mycelia collection process is filtration, since centrifugation of mycelial microorganisms does not generate compact pellets that make the subsequent washing steps difficult. This procedure can be done by filtering through a Whatman 3MM paper (Whatman, Maidstone, England) or a nylon cloth filters (Nytal Maissa, Barcelona, Spain) (Lim et al., 2001; Fernández Acero et al., 2006; Coumans et al., 2010; Jami et al., 2010a; Yildirim et al., 2011). Hence, mycelia is collected and washed and the media can be stored for further analysis of secreted proteins. Washing steps, which allow the media elimination that can interfere in the protein purification, are usually done at 4°C in order to diminish the protein lysis by intracellular proteases. The most commonly employed washing solutions are: i) distilled water (Kniemeyer et al., 2006; Shimizu et al., 2009; Coumans et al., 2010); ii) phosphate buffered saline (PBS) (Lu et al., 2010) or iii) combination of 0.9 % sodium chloride and water (Jami et al., 2010a). In order to prevent protein degradation, samples collected at

different time-points or conditions are washed, paper-dried and immediately stored at  $-20^{\circ}\text{C}$  to  $-80^{\circ}\text{C}$  for several months.

Two common steps included in all the protocols that are crucial for intracellular protein extraction are keeping the temperature as low as possible and adding protease inhibitors. Thus, the use of liquid nitrogen to decrease warming of the disruption systems is a widely used method. The main cell-breaking system is the traditional pre-chilled mortar grinding due to its efficiency against the fungal cell wall (Cobos et al., 2010; Lu et al., 2010; Vödisch et al., 2011), although waring blender machines (Lim et al., 2001), or glass bead beating systems, either combined with a 10 mM Tris-HCl buffer (Oh et al., 2010) or with a phenol buffer (Coumans et al., 2010; Vödisch et al., 2011), have been successfully applied.

After the breaking step, the protein solubilisation buffer always includes a protease inhibitor [e.g.: protease inhibition cocktail for fungi and yeast (Sigma), COMPLETE (Roche)] (Fernández-Acero et al., 2006, Jami et al., 2010a), in addition to a reducing agent [e.g.: 2 mercaptoethanol (2ME), dithiothreitol (DTT)] that reduces the disulfide linkages between two cysteines (Fernández Acero et al., 2006, Kniemeyer et al., 2006). The reducing agent is frequently also added to the precipitation solution (0.093% 2ME, 0.14% DTT). This precipitation step (previous to resuspension in sample solution) is used to selectively purify proteins from contaminants such as salts, nucleic acids, detergents or lipids, which interfere with the final bidimensional analysis. Although the combination of trichloroacetic acid (TCA) and acetone is usually more effective than either TCA or acetone alone (Bhadauria et al., 2007), other mixtures, such as 100 mM ammonium acetate in methanol, have been described for fungi (Vödisch et al., 2011). Optionally, the sample can be treated, previously to precipitation, with a nuclease mix [0.5 mg mL<sup>-1</sup> DNase, 0.25 mg mL<sup>-1</sup> RNase and 50 mmol L<sup>-1</sup> MgCl<sub>2</sub>; or commercially available e.g.: Benzonase (Merck)] (Lu et al., 2010; Barreiro et al., 2005). When the precipitation step is omitted, direct solubilisation in homogenization buffer is done, which includes fungal DNase/RNase as described by Oh and co-workers (2010).

Proteins may be difficult to resolubilize and may not completely resolubilize after TCA precipitation [“2-D Electrophoresis. Principles and Methods” GE Healthcare]. Thus, residual TCA must be intensively removed by cold acetone washing steps, e.g.: i) 2x acetone plus 0.07% DTT and 1x 80% acetone (Jami et al., 2010a); ii) 2x acetone containing 1% 2ME (Kniemeyer et al., 2006; Shimizu et al., 2009); iii) 3x acetone/0.3% DTT (wt/vol) (Vödisch et al., 2011). A helpful tip after TCA precipitation and washing is to avoid long drying periods of time in order to improve protein resuspension (1-2 minutes is enough).

The main components of the final sample buffer or homogenization buffer described in fungal bibliography have urea (7-9 M), thiourea (0-2 M), CHAPS (1-4% w/v) and ampholyte (0,5-2%), which are combined as indicated in Table 1.

The protocol described by Kniemeyer and co-workers (2006) for protein extraction of *Aspergillus fumigatus* summarized part of the steps described above, since after the mycelia homogenization in a pre-cooled (in liquid nitrogen) mortar they did the extraction, cleaning and precipitation steps together, using the Clean Up kit (GE Healthcare). This procedure produced quite good results when it was applied to *P. chrysogenum* showing a good protein yield (Jami et al., 2010a). However, some minor problems, such as the poor representation of large proteins, stripping in the basic region and the improper cleaning of the middle sized protein region (50-100 kDa), were observed. Nonetheless, this procedure is highly recommendable for fungal protein extraction.

Based on the methods described by Fernandez-Acero and co-workers (2006) for *Botrytis cinerea*, the mortar grinding approach combined with a phosphate buffer extraction was

used for *P. chrysogenum* (Jami et al., 2010a). This method improved the final bidimensional gel results, avoiding the problems observed in the direct Clean Up kit method. In addition, this procedure yielded large protein amounts, which allowed Blue silver Coomassie colloidal staining (Candiano et al., 2004), thus simplifying the 2D gels handling. In fact, Coomassie staining of proteins is still the visualization method of choice for the 2D analysis of fungal proteomes.

HOMOGENIZATION BUFFER	REFERENCE
8 M urea, 2% (w/v) CHAPS, 20 mM DTT, 0.5% (v/v) biolytes	Fernández-Acero et al., 2006
7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.8% (v/v) ampholytes, 20 mM Tris, 20 mM DTT	Kniemeyer et al., 2006
7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT, 1.0% (v/v) IPG buffer	Shimizu et al., 2009
7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, 20 mM Tris, 1% (v/v) Pharmalyte pH 3-10	Lu et al., 2010
8 M urea, 4% (w/v) CHAPS, 40 mM Tris-pH 7.4, 100 mM DTT, 0.2% (w/v) ampholite	Oh et al., 2010
7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 1% (w/v) Zwittergent 3-10, 20 mM Tris	Vödtsch et al., 2011
8 M urea, 2 M thiourea, 1% (w/v) CHAPS, 20 mM DTT, 0.5% (v/v) ampholyte 3-10	Yildirim et al., 2011

Table 1. Different solubilisation buffers used for the Proteomics analysis of fungi

### 3.2 Topics analysed using the proteomics approach on intracellular proteins

Two main technologies are used for the identification of proteins: i) individual protein identification by MALDI-TOF/TOF following SDS-PAGE or 2D-PAGE fractionation and ii) "shotgun" Proteomics, where total protein digests of fungal origin are analysed by tandem LC-MS to generate constituent protein data sets.

#### 3.2.1 Intracellular reference maps

Proteome reference maps provide an impression of the kind and quantity of proteins detectable by either of these two approaches. Furthermore, proteomic data has the potential to improve gene annotation and to discover alternative splice variants of transcripts and isoforms of proteins (Wright et al., 2009; Chang et al., 2010).

The availability of multiple fungal genome sequences during the last five years (over 20 fungal genomes have been completed) has been the key of the large progress of fungal Proteomics research. As a consequence, the number of proteins successfully identified from one specific fungus has drastically increased from some few to several hundreds. Now, the major challenge in modern fungal biology is to understand the expression, function and regulation of the entire set of proteins encoded by fungal genomes.

The combination of 2D-PAGE and mass spectrometry for the identification of proteins from filamentous fungi was first used by Grinyer and co-workers (2004), although reports on 2D-PAGE visualization of fungal proteins had been previously carried out (Hernández-Macedo et al., 2002). Combination of 2D-PAGE with both MALDI-TOF (Kratos Analytical AXIMA CFR plus) and LC-MS/MS [LCQ Deca IT MS (Thermo Finnigan)] techniques, allowed the initial identification of 25 proteins (out of 96 attempted) from the hundreds of proteins

resolved in the initial proteome map of *Trichoderma harzianum*, which was performed using whole-cell protein extracts.

*Aspergillus* sp. is a model organism for filamentous fungi and genome sequences from several species of this genus have been released in the last years (Galagan et al., 2005; Machida et al., 2005; Nierman et al., 2005; Payne et al., 2006; Pel et al., 2007). However, the number of global protein expression studies on *Aspergillus* is still relatively low. The majority of the *Aspergillus* proteome research is still represented by quantitative 2D studies (Carberry and Doyle, 2007; Kim et al., 2008; Kniemeyer, 2011) and less than 10% of the predicted whole proteome of *Aspergillus* sp. has been identified and quantified until now. The mycellial intracellular proteome of the pathogenic fungus *A. fumigatus* was characterized in two studies; the first one identified 54 proteins (Carberry et al., 2006) by 2D-PAGE and Ettan MALDI-TOF (Amersham Biosciences) and more recently, the second one identified 381 spots representing 334 proteins (Vödisch et al., 2009) using the same approach and an Ultraflex MALDI-TOF/TOF (Bruker Daltonics). The majority of those proteins were involved in cellular metabolism, protein synthesis, transport processes and cell cycle. The intracellular proteome of dormant conidia (asexually produced spores) of *A. fumigatus* was carried out by Teutschbein et al. (2010), who used 2D-PAGE and MALDI-TOF techniques to find that these structures contained in particular high amounts of proteins, which are required for stress tolerance and rapid reactivation of metabolic processes. The cytosolic proteome reference map of the well-known producer of primary metabolites and extracellular proteins *Aspergillus niger* was also characterized. 2D electrophoresis combined with a mass spectrometry analysis [Ultraflex MALDI-TOF/TOF (Bruker Daltonics) and ESI-QqTOF MS/MS (Micromass)] allowed the identification of around 100 proteins (Lu et al., 2010). In all studies, proteins primarily involved in translation, energy metabolism, transport processes and the stress response were most abundant.

*Penicillium chrysogenum*, the microorganism industrially used for the production of penicillin, is another example of filamentous fungus whose genome sequence has been recently released (van den Berg et al., 2008). In this specific case, a first study was reported about the intracellular proteome reference map (Jami et al., 2010a). This map was carried out using 2D-PAGE ([http://isa.uniovi.es/P\\_chrysogenum\\_proteome/](http://isa.uniovi.es/P_chrysogenum_proteome/)) and further individual protein identification by means of a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems), which allowed the identification of almost 1000 proteins and isoforms, the majority of them being involved in energy metabolism and the stress responses. Although this is an adequate number of proteins for 2D-PAGE analysis, it represents less than 10% of the predicted whole proteome of *P. chrysogenum*. Therefore, proteome research on filamentous fungi, such as *Aspergillus* sp. or *P. chrysogenum*, clearly lag behind the deep proteome analyses of *S. cerevisiae* and other microbial model organisms with identification of around two-third of the predicted open reading frames (de Godoy et al., 2008; Picotti et al., 2009). However, it is likely that in the more complex filamentous fungi only a fraction of the total genome gives rise to proteins under the laboratory culture conditions. Other sets of proteins may be expressed under different nutritional and environmental conditions in nature.

The ascomycete *B. cinerea* is a phytopathogenic fungus important for being the causative agent of disease in a number of important crops. A first approach for the characterization of the *B. cinerea* proteome, detected over 400 spots in 2D-PAGE, although only 22 protein spots were identified by means of an Ultraflex MALDI-TOF/TOF (Bruker Daltonics) mass spectrometer or an ESI IT MS/MS [Esquire HCT IT (Bruker-Daltoniks)]. Some of them

corresponded to isoforms of malate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase (Fernández-Acero et al., 2006).

In summary, the 2D-PAGE reference maps for filamentous fungi revealed that especially high-abundant proteins involved in metabolic processes and the general stress response are easily accessible to gel-based proteomic approaches. However, this technique is not appropriate for hydrophobic, membrane and low-abundant proteins (see Section 4 of this chapter).

### 3.2.2 Comparative proteomics

Besides the elaboration of proteome reference maps, the dynamic nature of fungal proteomes in response to different conditions (comparative Proteomics) has been also investigated. For example, the discovery of novel sources of host resistance, the evolution of strains due to the industrial improvement or the effects of carbon sources, antifungal drugs and gene deletion have been analysed in detail (Fernández-Acero et al., 2010; Jami et al., 2010a; Cagas et al., 2011b).

One of the earliest intracellular filamentous fungal proteomic studies was performed on the wood-degrading fungi *Phanerochaete chrysosporium* and *Lentinula edodes* (Hernández-Macedo et al., 2002). These authors used 2D-PAGE to compare cytoplasmic protein expression patterns in the presence or absence of iron and although they visualized 21 proteins related to iron uptake, the identification of such proteins was deficient. A more recent report on *P. chrysosporium* analysed total soluble proteome alterations of this fungus in response to lead exposure using 2D in combination with a 4700 MALDI-TOF Proteome-Analyzer (Applied Biosystems) (Yildirim et al., 2011). This study highlighted the particular role of the elements of DNA repair, post-transcriptional regulation and heterotrimeric G protein signalling in response to different doses of lead.

The proteome response of *Aspergillus nidulans* to the presence of the macrolide antibiotic concanamycin A was also analysed by 2D electrophoresis coupled to an ESI Qq-TOF mass spectrometer (Micromass) (Melin et al. 2002). In addition to glyceraldehydes-3-phosphate dehydrogenase, three proteins were found down-regulated under these conditions; a homolog to a cadmium-induced protein, a homolog to LovC (involved in the biosynthesis of the secondary metabolite lovastatin) and a homolog to a protein of unknown function. Osmoadaptation was also studied in *A. nidulans* by 2D-PAGE and MALDI-TOF [Autoflex series MALDI-TOF (Bruker Daltonics)] (Kim et al. 2007), revealing that glyceraldehyde-3-phosphate dehydrogenase, aldehyde dehydrogenase and a hypothetical protein with a domain of unknown function DUF1349 were overrepresented, whereas enolase and TCA cycle enzymes were less abundant in osmoadapted cells. These results suggest a shift from energy metabolism to the biosynthesis of glycerol, most likely important for control of cell turgor under osmoadaptation. The major proteome differences found between *A. nidulans* grown under hypoxia or under normal aeration were also analysed by 2D electrophoresis combined with AXIMA MALDI-TOF (Shimadzu) protein identification (Shimizu et al. 2009). Differences in metabolic enzymes and enzymes for energy production together with an overrepresentation of proteins involved in nucleotide salvage under hypoxic conditions, suggested that activation of nucleotide salvage is a fungal mechanism of adaptation to oxygen deprivation. In another study, long-term exposure to menadione was investigated by LC/MSMS Proteomics [3D IT LCQ Fleet (Thermo Fisher)] and transcriptomics in *A. nidulans* vegetative cells. Enzymes in the vitamin B2 and B6 biosynthetic pathways were repressed concomitantly with the repression of some protein folding chaperones and nuclear transport

elements. Under long-term oxidative stress, the peroxide-detoxifying peroxiredoxins and cytochrome C peroxidase were replaced by thioredoxin reductase, a nitroreductase and a flavohemoprotein, and protein degradation became predominant to eliminate damaged proteins (Pusztahelyi et al., 2011). Those proteins involved in early phase of conidia germination were also analysed through Proteomics using 2D in conjunction with a Voyager-DE STR MALDI-TOF (PerSeptive Biosystems) mass spectrometer (Oh et al., 2010). During the early phase of the germination stage, levels of proteins involved in metabolism, protein synthesis and transcription highly increased, confirming the importance of metabolic activation and new protein synthesis for the germination process.

The effect of the deletion of a specific gene has been also investigated in *A. nidulans* by comparative Proteomics of the mutant versus the wild-type strain. Proteomic analysis of a strain deleted in the glutathione reductase gene (*glrA*) by 2D-PAGE revealed that 13 proteins were overrepresented, whereas 7 proteins reduced their expression in the *A. nidulans* mutant compared to the wild-type (Sato et al., 2009). The deleted mutant shifted to a temperature sensitive phenotype with decreased intracellular glutathione and reduced resistance to oxidative stress. Analysis of the upregulated proteins by means of an AXIMA MALDI-TOF (Shimadzu) mass spectrometer identified a thioredoxin reductase, cytochrome c peroxidase and catalase B, in addition to a number of peroxiredoxins. It was concluded that increased levels of those enzymes in the *glrA*-deletion strain revealed interplay between the glutathione system and both the thioredoxin system and hydrogen peroxide defence mechanisms. Moreover, upregulation of an elongation factor 1 $\beta$  (*ElfA*; 2.5-fold) and a glutathione s-transferase (*GstB*; 2.6-fold) was also observed in *A. nidulans*  $\Delta$ *glrA*. Orthologues of these two proteins were also reported to be upregulated in response to oxidative stress in *A. fumigatus* (Burns et al., 2005; Carberry et al., 2006). In addition, increased levels of catalase B and cytochrome c peroxidase may be responsible for the depleted H<sub>2</sub>O<sub>2</sub> levels observed in the deleted mutant. Comparative Proteomics was also applied to another strain of *A. nidulans* deleted in the *hapC* gene, which encodes a component of the transcriptional regulator AnCF controlling redox balancing and coordinating the oxidative stress response. Upregulation of several redox-active proteins, such as thioredoxin, peroxiredoxin A and glutathione was observed by 2D-PAGE combined to mass spectrometry using an Ultraflex I MALDI-TOF/TOF (Bruker Daltonics) mass spectrometer in the mutant compared to the wild-type strain (Thön et al., 2010).

The dynamic nature of the *A. fumigatus* proteome in response to different conditions has been also subject of study. Comparison of the 2D-PAGE maps from *A. fumigatus* grown on either glucose or ethanol as sole carbon sources was carried out and the main enzymes involved in alcohol metabolism were identified by means of an Ultraflex I MALDI-TOF/TOF (Bruker Daltonics) mass spectrometer (Kniemeyer et al., 2006). Ethanol led to upregulation, among others, of an alcohol dehydrogenase and a particular aldehyde dehydrogenase. The latter was suggested to play a role as acetaldehyde dehydrogenase for the production of acetyl-CoA.

Comparison of developmental stages was also carried out in *A. fumigatus*. Analysis of the proteome of germinating conidia with hyphae revealed that a CipC-like protein was one of the major hyphal-specific proteins. Although the biological function of this cytosolic protein has not been elucidated yet, a putative role during invasive growth in the host has been suggested (Bauer et al., 2010). Four stages of early development were evaluated using the gel free system of isobaric tagging for relative and absolute quantitation (iTRAQ) to determine the full proteomic profile of the pathogen *A. fumigatus*. A total of 461 proteins

were identified with a 4800 Plus MALDI-TOF/TOF Analyzer (Applied Biosystems), at 0, 4, 8, and 16 hours and several fold changes for each were established. Ten proteins including the hydrophobin rodlet protein RodA and a protein involved in melanin synthesis Abr2 were found to decrease relative to conidia (Cagas et al., 2011a).

The proteins differentially synthesized in *A. fumigatus* cultivated with the antifungal drug amphotericin B were also investigated in order to identify resistance mechanisms to this antifungal drug (Gautam et al., 2008). Differential expression levels for 85 proteins (76 upregulated and 9 downregulated) were detected by 2D electrophoresis and a 4800 Plus MALDI-TOF/TOF Analyzer (Applied Biosystems). Forty-eight of them were identified with high confidence and included ergosterol pathway proteins (a key amphotericin B target), cell stress proteins, cell wall proteins and transport proteins. Expressions of three genes, a Rho-GDP dissociation inhibitor, a secretory-pathway GDI and Mn SOD, were detectable at both microarray and proteomic levels. Matching between Proteomics and Transcriptomics pointed out the genes encoding those potential target proteins for the development of new antifungal drugs. In a more recent work, the same group followed a similar proteomic-transcriptomic approach to assess the *A. fumigatus* response to artemisinin, an antimalarial drug reported to have antifungal activity against some fungi (Gautam et al., 2011). Proteomic profiles of *A. fumigatus* treated with artemisinin showed modulation of 175 proteins (66 upregulated and 109 downregulated) and peptide mass fingerprinting led to the identification of 85 proteins (29 upregulated and 56 downregulated), 65 of which were unique proteins. The differentially expressed proteins belonged to carbohydrate metabolism, cell stress, amino acid metabolism, translation, ubiquitin-dependent protein degradation, transcription, cytoskeletal proteins, cell wall and associated proteins and others including hypothetical proteins.

Proteome analysis of the enzymatic reactive oxygen intermediates detoxifying system was studied in *A. fumigatus* challenged by H<sub>2</sub>O<sub>2</sub> together with the comparative proteome analysis of a mutant deleted in the transcription factor Afyap1, which is involved in the oxidative stress response (Lessing et al., 2007). Differential gel electrophoresis (DIGE) analysis, followed by mass spectrometry using an Ultraflex I MALDI-TOF/TOF (Bruker Daltonics) mass spectrometer, identified 27 protein spots that displayed an increase and 17 protein spots that displayed a decrease in intensity following *A. fumigatus* exposure to H<sub>2</sub>O<sub>2</sub>. Examples of upregulated proteins were the Allergen AspF3 (putative thioredoxin peroxidase), a mitochondrial peroxiredoxin Prx1 (which likely has thioredoxin peroxidase activity), Cu, Zn superoxide dismutase or spermidine synthase. Proteome analysis of the  $\Delta$ Afyap1 mutant strain challenged with 2 mM H<sub>2</sub>O<sub>2</sub> indicated that 29 proteins are controlled directly or indirectly by AfYap1, including catalase 2.

The modification of *P. chrysogenum* from a Proteomics point of view during the industrial strain improvement process was analysed in detail using high-resolution 2D-PAGE coupled to MALDI-TOF/TOF (Jami et al. 2010a). Three strains were compared in this study; the wild type *P. chrysogenum*, a strain with a small improvement for penicillin biosynthesis that is used as a reference in the laboratory and a penicillin high-producing strain. The used experimental conditions allowed for estimating the main proteome changes between different stages of the industrial strain improvement program. Cysteine (a penicillin precursor) biosynthetic enzymes, enzymes of the pentose phosphate pathway and stress response proteins were overrepresented in the high producer strains, whereas proteins for virulence and biosynthesis of other secondary metabolites different from penicillin (pigments and isoflavonoids) were down-regulated. This interesting study concluded that

the increased penicillin titers reached by the high-producing strains is a consequence of rebalancing among different metabolic pathways.

Combination of proteomic analysis with metabolic measurements is a very interesting approach for the study of fungal metabolic processes. Matsuzaki and co-workers (2008) analysed the intracellular processes of the white-rot basidiomycete *P. chrysosporium* involved in the metabolism of benzoic acid at the proteome and metabolome levels by high-resolution 2D electrophoresis coupled to LC-MS/MS. Aryl-alcohol dehydrogenase, aryl-aldehyde dehydrogenase, and cytochrome P450s were upregulated after the addition of exogenous benzoic acid. Intracellular metabolic shifts from the short-cut TCA/glyoxylate bicycle system to the TCA cycle and an increased flux in the TCA cycle indicated activation of the heme biosynthetic pathway and the production of NAD(P)H. In addition, combined analyses of proteome and metabolome clearly showed the role of trehalose as a storage disaccharide and that the mannitol cycle plays a role in an alternative energy-producing pathway. Conclusions could be drawn for the process of lignin degradation based on enzyme expression values (assessed by Proteomics) and the amount of chemical species related to these enzymes (assessed by Metabolomics).

In another example of combination of Proteomics with metabolomics the analysis of the effect of lactate and starch on fumonisin B2 biosynthesis (a mycotoxin) in *A. niger* was carried out using 2D-PAGE and a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems) (Sørensen et al., 2009). These authors reported that there is a specific relation between the increase in fumonisin B2 and the enzymes affecting the intracellular levels of acetyl-CoA.

#### 4. Proteome analysis of fungal secretomes

Greenbaum and co-workers (2001) defined the secretome as the population of gene products that are secreted from the cell. In 2010, Makridakis and Vlahou specified the definition as the rich complex set of molecules secreted from living cells, which in a less strict definition includes molecules shed from the surface of living cells. Thus, the secretome or extracellular proteome consists of the totality of the proteins secreted from a cell, organism or entity. These proteins can be subdivided into freely released proteins and proteins associated to the outer cell wall. Even the cellular machinery involved in the protein secretion was proposed as part of secretome (Tjalsma et al., 2000).

Many fungi secrete a huge number of proteins to facilitate their saprotrophic lifestyle, which justifies the secretome-related studies to understand this filamentous fungi way of life. Thus, Bennett (2006) wrote that “animals eat their food and then digest it; fungi digest their food and then eat it”, which illustrates the vast number of extracellular enzymes necessary to assimilate the whole potential substrates. Studies of the secreted proteins from filamentous fungi show that they are not only natural toolboxes for white biotechnology (degradation process, industrial application, etc.), but also that they are the most important indoor allergen generators. Besides, some of these proteins are important as pathogenicity biomarkers in humans and plants, while others have demonstrable potential for the control of insect pests.

The considerable number of available publications on the analysis of human pathogenic bacteria and yeasts contrasts with the limited number of secretome studies on filamentous asco- and basidiomycetes. Since Medina and co-workers (2004) described the extracellular proteins involved in degradation of the flavonoid rutin by *A. flavus*, a few publications in the last years have described the fungal secretomes applied to different fields as described in the next sections.

#### 4.1 Fungal allergen identification by 2D analysis

Naturally and synthetically produced compounds are responsible for the increase of human health conditions by allergies. Successful diagnosis and therapy are conditioned by the identification of the allergenic compounds, which mainly come from food and inhalation. Thus, pollens of grass and trees are the most important outdoor airborne allergens. In addition, other important allergenic source is airborne mould (Benndorf et al., 2008). A significant association between asthma and sensitization to *Alternaria* and *Cladosporium* (up to 20%) has been described (Zureik et al., 2002). These fungal species occur typically outdoors with a maximum level of spore concentration in summer and autumn, whereas species such as *Aspergillus* and *Penicillium*, especially *Aspergillus versicolor*, *Penicillium expansum* and *P. chrysogenum*, dominate indoors in winter in humid and cold climates (Cruz et al., 1997; Benndorf et al., 2008). Commercialized test systems to diagnose allergic reactions to this mould species are not properly developed. The allergens studied by means of Proteomics combining the resolution of 2D electrophoresis, the specificity of the immunoblotting and the mass spectrometry-based protein identification allow exploring the so-called immunoproteome or “immunome” (Doyle, 2011). This approach, which is usually performed using immunoglobulin E (IgE) from sera of sensitive patients, is an emerging strategy for the identification of immunoreactive fungal antigens (Benndorf et al., 2008; Ishibashi et al., 2009). In fact, this allergen screening system is only possible by means of the bidimensional electrophoresis, since it is the proper way to combine several sensitive sera over the same antigens. This procedure has been used for allergen identification of atopic dermatitis caused by *Malassezia globosa* [using an oMALDI Qq-TOF (Applied Biosystems) and N-terminal sequence analysis in a Procise 494 protein sequencer (Applied Biosystems) (Ishibashi et al., 2009)], enolase antigen from *Penicillium citrinum* and *A. fumigatus* [Edman degradation (Lai et al., 2002)], spore allergens from the indoor mould *A. versicolor* [by means of a LC/MSD TRAP XCT system (Agilent Technologies) (Benndorf et al., 2008)], or antigen of *A. fumigatus* from allergic broncho-pulmonary aspergillosis patients [using a Q-TOF Ultima Global mass spectrometer (Micromass) (Singh et al., 2010)]. Figure 1 shows the antigen detection system by 2D electrophoresis. As first step, the sera from sensitive patients are tested in order to find common allergens by monodimensional electrophoresis. Those common reactive sera can be used over the same blotted 2D gel (stripping for reprobing) to locate the antigenic protein, which is identified by mass spectrometry or N-terminal sequencing (Edman degradation). Thus, the peptides obtained are used as the template for degenerated primer design. These primers combined with the rapid amplification of cDNA ends method (RACE) allow the gene cloning from cDNA.

The characterization of secreted proteins has also other medical applications since surface-associated and secreted proteins represent primarily exposed components of fungi during host infection. Thus, several secreted proteins contribute to pathogenicity since its role in defence mechanisms or immune evasion is known. These studies lead to the identification of possible biomarkers for the verification of diseases caused by *Aspergillus* species as *A. fumigatus* combining 2D electrophoresis and mass spectrometry identification [Ultraflex I MALDI-TOF/TOF (Bruker Daltonics)] (Wartenberg et al., 2011).

#### 4.2 The environment and fungal secretomes

Over 8,000 species of fungi cause more plant diseases than any other group of plant pests. As a result, some of the world's great famines, human suffering and largest human migrations on the planet have been due to plant-pathogenic fungi. The destruction of wheat

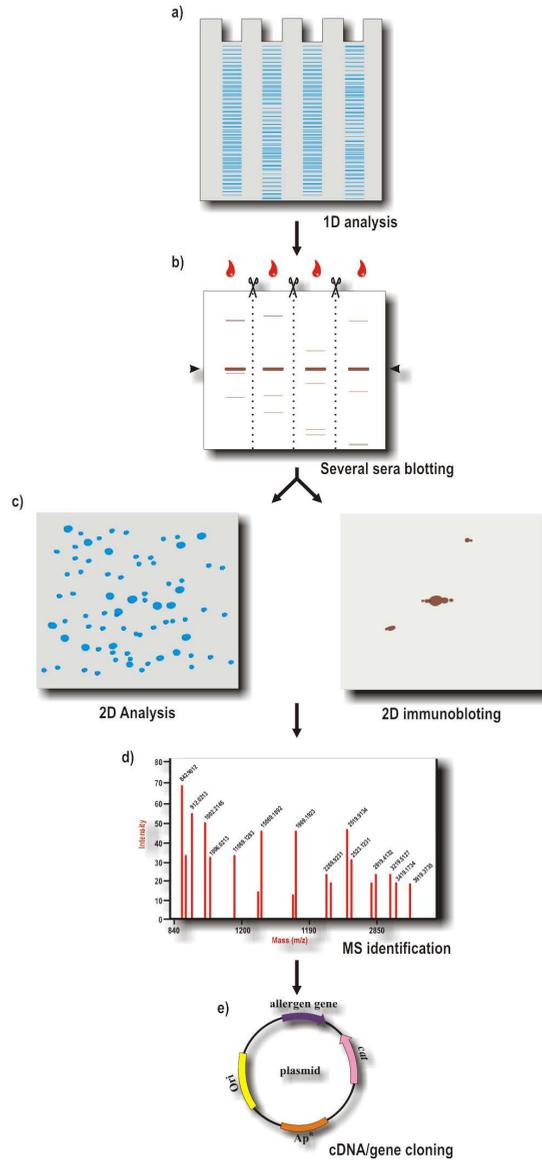


Fig. 1. Proteomics approach to allergen identification in fungi. The identification of fungal antigens needs several sera from different sensitive patients. After protein extraction, a monodimensional gel allows protein separation (a). The immunoblotting using different sera allows allergen localization and western condition optimization (b). 2D electrophoresis is used for individual antigen identification. The Coomassie stained gel (control) is used for protein isolation (c). Mass spectrometry is carried out for peptide identification (d). As final step, degenerated primers are designed and the gene encoding the allergen is cloned by combination of these primers and RACE methodology (e).

crops in the Middle Ages caused by the fungus called bunt or stinking smut (*Tilletia* spp.), the potato blight in Ireland and northern Europe caused by *Phytophthora infestans* or the strike in the grape vineyards of central Europe due to the downy mildew (*Plasmopara viticola*) in the 1870s are clear examples of the strong influence of the fungi over mankind (Ellis et al., 2008). Therefore, the interaction fungi-plant is in one the most interesting clues to be faced up for Proteomics due to its global economic implications.

The recognition plant-pathogen is either host specific or almost generalist. Thus, the genus *Botrytis*, which presents some species that seem to have host specificity, also includes *B. cinerea*. This fungus is able to infect more than 200 different plant species, at any plant stage or tissue, including fruits during storage and distribution. As a result, it inflicts high economic damage at various levels in the food industry (Fernández-Acero et al., 2010). The ability for *B. cinerea* to infect is due to its wide variety of infection strategies, which are facilitated by a set of pathogenicity/virulence factors. The study of the secreted proteins has been faced up by means of different carbon sources from fruit (tomato, strawberry, kiwifruit, glucose, carboxymethyl cellulose, starch, pectin or tomato cell walls) and using solid and liquid cultures (Shah et al., 2008; Espino et al., 2010; Fernández-Acero et al., 2010). The identified proteins by 2D electrophoresis and MALDI TOF-TOF [Autoflex MALDI-TOF or UltraflexIII MALDI TOF/TOF (Bruker-Daltonics) ] or LC-MS/MS [LTQ linear IT (Thermo Fisher)] were involved in the degradation of plant defensive barriers, proteases, pathogenicity factors and virulence factors (e. g.: pectin methyl esterases, xylanases and proteases). This comparative approach is a promising strategy for discovering new pathogenicity factors and dissecting infection mechanisms.

The ultrafast genome sequencing is a boosted fashion trend, which is generating thousands of gene sequences every day. In spite of this fact, several fungi which are producers of severe infections in fruit trees (fruit blight) and grapevines, like *Diplodia seriata*, keep fully unsequenced. Despite this limitation, 2D electrophoresis application combined with *de novo* sequencing and BLAST similarity search by a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems) led to the protein identification of virulence factors described in other fungal strains when they grew in medium supplemented with carboxymethyl cellulose (Cobos et al., 2010). This fact shows the wide range of Proteomics applications.

The use of fungi as effective biocontrol agents is a promising field. Thus, *Trichoderma* spp., which are mycoparasites of several soil-borne fungal plant pathogens, have been analysed using the Proteomics approach. Suárez and co-workers (2005) visualized on 2D gels more than 250 extracellular proteins when chitin served as a carbon source, but comprehensive protein identification by mass spectrometry was not performed. Recently, Monteiro and co-workers (2010) studied the overgrowth, mycelia degradation and protein secreted by *T. harzianum* on different plant pathogens (*Macrophomina phaseolina*, *Fusarium* sp. or *Rhizoctonia solani*) by means of 2D electrophoresis and mass spectrometry [MALDI-TOF Reflex IV (Bruker Daltonics)]. Intriguingly, hyphae of *T. harzianum* did not present coiling around *Fusarium* sp. supporting the idea that mycoparasitism may be different among plant pathogens. These data were concordant with the differently observed secretion patterns, which are related to phytopathogen cell-wall composition.

Tan spots are associated with the development of extensive chlorotic and/or necrotic lesions on the leaves of susceptible host genotypes caused by the fungus *Pyrenophora tritici-repentis*. It is an economically important foliar disease of wheat with a worldwide distribution (Cao et al., 2009). These authors compared the secretomes of avirulent *versus* virulent race isolates of the pathogen by means of 2D electrophoresis and later ESI-q-TOF MS/MS (Micromass)

identification. Therefore, various metabolic enzymes in addition of  $\mu$ -mannosidase, exo-b-1,3-glucanase, heat-shock and BiP proteins were found as up-regulated in the virulent race. Such differences could reflect an adaptation to a saprophytic habit in avirulent isolates of the fungus and it may suppose the application of avirulent races as biological control system to displace the virulent ones.

An environmentally friendly alternative to chemical insecticides for agricultural pest control are those fungi involved in insect-fungus interactions. These can result in biological insecticides deduced from entomopathogenic fungus. Thus, *Metarhizium* spp. are a good model system for studying these interactions and a resource of genes for biotechnology. Previously to the genome sequence publication of *Metarhizium anisopliae* and *Metarhizium acridum* (Gao et al., 2011), the intercellular proteome from conidia and mycelia of *M. acridum* was analysed by 2D electrophoresis and mass spectrometry [MALDI-TOF/TOF-MS (Axima Performace) or MALDI MS/MS (Waters)] (Barros et al., 2010) observing clear differences in the expression of identical proteins and isoform occurrence between conidia and mycelia. The secretome analysis of *M. anisopliae* combining 2D electrophoresis and mass spectrometry [UltraFlex II MALDI-TOF/TOF (Bruker Daltonics)] showed the presence of proteases, reductases and acetyltransferase enzymes in presence of the exoskeleton of *Callosobruchus maculatus* (main pest of cowpea) (Murad et al., 2006). In addition, the genome sequencing of fungal pathogens of trees that live as beetle symbionts such as *Grossmannia clavigera* (DiGuistini et al., 2011) will facilitate the analysis of the secretome evolution and its applicability to pest control. This analysis of secreted proteins of entomopathogenic fungi is a poorly explored field, which supposes one of the next and promising Proteomics challenges.

### 4.3 Proteomics of industrially interesting fungi

Filamentous fungi have an extraordinary ability to secrete proteins, secondary metabolites and organic acids to the culture medium. Thus, in comparison to other eukaryotic expression systems, such as yeast, algae or insect cells, filamentous fungi possess the exceptional advantage of an unbeatable secretion capacity. This is the reason why filamentous fungi have been used for food maturation and beverage processes for more than 1,500 years. Therefore, this traditional use has been transformed in commercial applications for food and beverage industries (Peberdy, 1994; Punt et al., 2002; Cardoza et al., 2003). Specially interesting is the list of commercial enzymes produced by Aspergilli that includes amylases, chymosin, glucose oxidases, catalases, cellulases, pectinases, lipases, proteases, phytases and xylanases in food, detergent, textile, pulp and paper industries (see review: Fleißner & Dersch, 2010). As an example, the production of fermented foods like miso, sake or soy sauce is traditionally performed by the ascomycete *Aspergillus oryzae*, which presents high protein secretion levels. This fungus is also an interesting host for enzyme production (homologous and heterologous) (Bouws et al., 2008). The extracellular proteome analysis of *A. oryzae* at different times in submerged and solid-state cultures containing wheat bran showed an impressive number of enzymes related to cell wall degradation (glucoamylase A, xylanase G2,  $\alpha$ -glucosidase A, cellulase B,  $\alpha$ -amylase,  $\beta$ -glucosidase and xylanase F3) (Oda et al., 2006). Thus, *A. oryzae* is also used in modern biotechnology due to this array of secreted proteins (Machida et al., 2005).

Even when the analysis of the extracellular proteome does not suggest a clear relation with the antibiotics production in *P. chrysogenum*, it can offer new clues either for the general understanding of the fungal metabolism or for possible industrial applications of the

secreted proteins. Thus, some secreted proteins identified in the culture medium of *P. chrysogenum* (isoamyl alcohol oxidase, sulfydryl oxidase, dihydroxyacid dehydratase, polygalacturonases, pectate lyases, ferulic acid esterases) by 2D electrophoresis and mass spectrometry [4800 MALDI-TOF/TOF Analyzer (Applied Biosystems)] are especially relevant because of their interest for food industry as sake and other alcoholic beverages production, burnt flavor removal from sterilized milk, preparation of juices or health product preparation (Jami et al., 2010b).

The public recognition that environmental pollution is a worldwide threat to public health has given rise to a new massive industry for environmental restoration. Hence, paper and pulp industries, as well as food and feed, beverage, textile and several other industrial production processes are linked by the importance of the degradation of plant cell wall polysaccharides. The most efficient degraders of lignocelluloses are saprophytic asco- and basidiomycetes, since they are robust organisms that have a high tolerance to toxic environments. These wood degraders are typically divided in: i) white rot fungi (degradation of hemicelluloses and lignin by basidiomycetes and ascomycetes) and ii) brown rot fungi (attack of cellulose and hemicelluloses, exclusively, accomplished by basidiomycetous) (Bouws et al., 2008). *Postia placenta* is a brown-rot fungi common inhabitant of forest and also largely responsible for the destructive decay of wooden structures. Its genome, transcriptome and secretome have been recently studied revealing unique extracellular enzyme systems, which include an unusual repertoire of extracellular glycoside hydrolases (Martínez et al., 2009). Interestingly, the comparisons with the closely related white-rot fungus *P. chrysosporium* sustain an evolutionary shift from white-rot to brown-rot showing the loss of the capacity for efficient depolymerization of lignin.

Biopulping is an industrial biotechnology application of natural fungi to convert wood chips to paper pulp in an ecosafety process diminishing the chemical and mechanical problems of paper production. Thus fungal pre-treatment of wood led to a significant increase in pulp yield and a better bleachability of the pulp, which can be explained by the production of specific lignocellulose-degrading enzymes through the Proteomics analysis combining 2D electrophoresis and MALDI-QTOF MS (Applied Biosystems)] (Ravalason et al., 2008).

#### **4.4 Guaranteeing the secretome quality: how to distinguish secretome from 'degradome'?**

The previous sections have shown the relevance of the extracellular proteome analysis, but the crucial step for the secreted protein analysis is to distinguish these proteins really secreted from those "contaminant" proteins resulting from cell lyses events. This capital process can be sequentially tackled from three different points of view: i) sample collection; ii) post-identification protein analysis; iii) analysis of "moonlighting proteins" and unconventional secreted proteins.

Some tips in the collection of secreted protein are shown in the figure 2 following the method described for *P. chrysogenum* (Jami et al., 2010b), such as the careful mycelia elimination process (by the use of nylon filters, rigorous centrifugation and filtration through 0.45 µm filters) or the use of low temperatures to avoid mycelia degradation. Sample collection is also crucial to discard those non-natural secreted protein. The scientific literature describes how the presence of extracellular proteins in the culture medium is directly correlated to the growth phase (Peberdy, 1994). Jami and co-workers (2010b) showed the linear correlation existing between the presence of proteins in the culture medium and the culture growth (biomass formation). Interestingly, the protein levels that

were present in the broth increased even after the culture reached the stationary phase. These data strongly suggest that some of those proteins arrived to the culture medium as a result of cell lysis. Consequently, taking samples at an early time point is recommended in concordance with the optimal moment of extracellular protein secretion. For example, *P. chrysogenum* does not show significant amounts of secreted proteins before 24 hours.

Regarding the post-identification protein analysis, Jami and co-workers (2010b) did the comparison between secreted proteins at 40 h *versus* those secreted at 68 h in *P. chrysogenum*. They demonstrated that the expression differences observed during the time course were mainly due to the presence at 40 h of isoforms from those lately expressed proteins or proteins expressed only at late stages. As a conclusion, the amount of “possible” contaminant intracellular proteins in the secretome was reported to be very low (6.09%) in *P. chrysogenum* at 68 h (Jami et al., 2010b). Thus, the knowledge of the protein abundance at the intracellular proteome helps to clarify possible cell lysis events. As an example, malate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase in *B. cinerea* or flavohemoglobin and manganese superoxide dismutase in *P. chrysogenum* should be highly detected in the case of lysis, since these are the most abundant intracellular proteins (Shah et al., 2009; Jami et al., 2010a). However, sometimes it is difficult to ascertain whether a protein is truly secreted or it is present in the culture medium as a consequence of cell lysis. Recently, new bioinformatics tools have been developed to characterize the secreted proteins either in a classical or non-classical way by means of prediction of secretion signal motifs. Therefore, SignalP (for classical secretion signal motifs) and SecretomeP (for non-classical signal motifs) are useful prediction software applications (Bendtsen et al., 2004 a, b) that can be used for real secretion understanding.

Even when the above-mentioned information is taken into account and the researcher has the certainty of the protein sample quality, some unconventional proteins, dubbed “moonlighting proteins” (Jeffery, 1999), can be detected. These proteins present unconventional protein secretion systems (Nickel & Rabouille, 2009) and then, it is difficult to define their authenticity as secreted proteins. One meaning of moonlighting is “to do paid work, usually at night, in addition to one’s regular employment”. Therefore, “moonlighting proteins” is a proper designation for this group of multifunctional proteins, which are widespread among organisms ranging from bacteria to mammals (Gancedo & Flores, 2008). In spite of this ubiquity, more attention has been traditionally given to the “moonlighting proteins” found in higher eukaryotes, but recently the yeasts have caught the attention for studying these proteins in lower eukaryotes. Thus, in many cases, the fusion of two genes that initially encoded proteins with single functions has been the origin of that duality. On the other hand, a significant number of proteins can perform dissimilar functions (see reviews: Gancedo & Flores, 2008; Flores & Gancedo, 2011).

An interesting phenomenon that has been observed in the *P. chrysogenum* secretome is the fact that some extracellular proteins were also previously identified in the microbody matrix of this fungus (Kiel et al., 2009). Therefore, an alternative explanation given to the presence of intracellular proteins in the culture broth was the selective autophagic degradation of peroxisomes (pexophagy), which can give a new sense to the strange “moonlighting proteins” as it was explained in *P. chrysogenum* (Jami et al., 2010b; Martín et al., 2010). It is well known that peroxisome abundance can be rapidly decreased through autophagic pathways, which selectively degrade peroxisomes by fusion to lysosomes or vacuoles (Oku and Sakai, 2010). Thus, integration of peroxisomes into vacuoles may lead to secretion of the proteins located in the peroxisomal matrix by exocytosis, a mechanism that has been

discussed as an alternative route for the release of penicillin from peroxisomes to the culture medium (Martín et al., 2010).

Even when the mycelia lysis is an undesirable event, it can suppose a quite informative source. Thus, the analysis of the secretome of the pathogenic fungus *Fusarium graminearum* *in vitro* and *in planta* demonstrates the presence of thirteen non-secreted proteins only under *in planta* conditions combining SDS-PAGE protein separation and LTQ/FT MS (ThermoElectron) identification (Paper et al., 2007). These proteins, some of which are potent immunogens secreted by animal pathogenic fungi, indicate a significant fungal lysis during plant pathogenesis, which shows the lysis as a useful event for the infection even if it involves partial degradation of the fungal population.

#### 4.5 Bioinformatics tools available for secreted protein predictions

Around 90% of human secreted proteins and almost 90% of the *A. niger* identified extracellular proteins by mass spectrometry contain classical N-terminal signal peptides. These signal peptides, which redirect the ribosomes to the rough endoplasmic reticulum, are typically 15–30 amino acids long and consist of 15–20 hydrophobic amino acid residues cleaved off during translocation across the membrane. Besides, there are also examples of non-classically secreted proteins in fungi, but generally the main group of secreted fungal proteins is processed by the classical secretory pathway (Lum & Min, 2011).

Bendtsen and co-workers (2004a) developed the software SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) for the identification of classical secretion signal motifs presents in proteins. This software is widely extended and commonly integrated in different bioinformatics platforms for secreted proteins identification. On the other hand, Phobius software (Kall et al., 2004) is used to identify signal peptides and to discriminate putative transmembrane domains (<http://phobius.cgb.ki.se/index.html>). The same developers of SignalP 3.0 software created a prediction program of secreted proteins by means of non-classical signal motifs (Bendtsen et al., 2004b). It was called SecretomeP (<http://www.cbs.dtu.dk/services/SecretomeP/>). Even when this program was developed for mammalian applications and later improved for bacteria, it has shown good results in the fungal protein analysis (Shah et al., 2009; Jami et al., 2010b).

The availability of several fungal genomes in databases together with the use of those prediction programs for secretory proteins have allowed the recent development of platforms for the annotation of fungal secretomes, such as the Fungal Secretome Database [FSD (Choi et al., 2010)] or the Fungal Secretome KnowledgeBase [FunSecKB (Lum & Min, 2011)], which allow the proper identification of secreted proteins by the compilation of different software. Thus, the Fungal Secretome Database (<http://fsd.snu.ac.kr/>) is a hierarchical identification pipeline based on nine prediction programs (SignalP 3.0, SigCleave, SigPred, RPS, TMHMM 2.0c, TargetP 1.1b, PSort II, SecretomeP 1.0f and predictNLS), which predict the presence of signal peptides or nuclear localization signals, trans-membrane helices, protein probable location and secretion by non-classical pathways. In contrast, Lum and Min (2011), who suppose that the FSD platform significantly over-estimate the number of secreted proteins in fungi, developed FunSecKB (<http://proteomics.yzu.edu/secretomes/fungi.php>) platform. This system offers a more conservative prediction tool. Nevertheless, the FunSecKB and the FSD databases could complement each other as different data sources, prediction tools and data access utilities.

The connection between the bioinformatics predictions and the really secreted proteins has been analysed in the white-rot basidiomycete *P. chrysosporium* (Wymelenberg et al., 2005;

2006) and in the filamentous ascomycete *A. niger* (Tsang et al., 2009) under different culture conditions. They demonstrated that SignalP results in higher specificity than Phobius, but the combined results from both yielded higher specificity of prediction than separately (Tsang et al., 2009). In addition, the computational secretome is clearly incomplete, but the predicted proteins include many interesting sequences that provide a framework for future investigations (Wymelenberg et al., 2006).

A reference map is a useful tool for the location of secreted proteins in addition to provide a good chance to identify those proteins. Thus, the first secretome reference maps just presented a few dozens of spots as that of *P. sapidus*, a white-rot fungus able to attack lignified biopolymers (Zorn et al., 2005). But the protein purification systems have evolved and several hundreds can be easily observed, as in case of *T. harzianum* (Suárez et al., 2005) or *P. chrysogenum* (Jami et al., 2010b) reference maps. Nowadays, there are several webpages that allow the public deposit of 2D reference maps [e.g.: World-2DPAGE Repository (<http://world-2dpagerepository.org/>)], where the proteins are easily identified clicking on the spot. However, these reference maps are usually from intracellular proteomes. The last step of this useful tool is the development of specific webpages by the research groups. Thus, the yeast *S. cerevisiae* has its own intracellular reference map website [Yeast Protein Map (<http://www.ibgc.u-bordeaux2.fr/YPM/>)]. The extracellular proteome of *P. chrysogenum* that has been fully completed by Jami and co-workers (2010b) and up to our knowledge it has been the first secretome reference map on-line generated by the users. It is available on-line through the website: [http://isa.uniovi.es/P\\_chrysogenum\\_secretome/](http://isa.uniovi.es/P_chrysogenum_secretome/)

#### 4.6 Extraction methods and buffer compositions of filamentous fungi secretomes

Table 2 summarizes some of the most recent methodological updates for the extracellular protein analysis of filamentous fungi.

Fungus	Source/Precipitation	Resuspension condition	Ref.
<i>A. flavus</i>	<b>Liquid:</b> Whatman filtration, lyophilization O/N. Resuspension in 5mL of ddH <sub>2</sub> O, store at -20°C. TCA precipitation (200 g/L) at -20°C, 1 h. centrifugation 5 min, 15000g at 4°C.	8M urea, 2% CHAPS, 50mM DTT, 0.2% (w/v) ampholytes 3/10, trace of bromophenol blue.	Medina et al., 2004
<i>B. cinerea</i>	<b>Solid:</b> Cellophane membranes containing germinated fungal spores were floated onto 37.5 mM sodium acetate buffer (pH 4.4) in the dark for 10 days. 1 ml was frozen / lyophilized to 30 µL	Mix 30 µL of sample plus 10 µL of SDS sample and buffer (Invitrogen). Boil 10 min, cool to R/T. Load 20 µL of mixture onto a NuPAGE 12% Bis-Tris precast gel.	Shah et al., 2008
	<b>Liquid:</b> Centrifugate (15000xg, 4°C, 10 min) the medium. Filtrate supernatant through several layers of filter paper (Whatman No. 1). Froze at -20°C O/N (allow polysaccharide precipitation). Filtrate through filter paper. Add TCA [6% (v/v) final concentration] and precipitate 1 h on ice. Centrifugate (15000xg, 4°C, 10 min). Washed 3 times 96% ethanol. Resuspend in 8M urea. Centrifugate at 15000xg, 5 min to eliminate the insoluble material. Precipitated again with methanol-chloroform. Stored dry at -20°C. Dissolve dry protein in ReadyPrep sequential extraction Reagent 2 to 0.04 mg/mL.	ReadyPrep sequential extraction Reagent 2: 8 M urea, 4% CHAPS, 40 mM Tris, 0.2% Bio-Lyte 3/10 ampholyte.	Espino et al., 2010

<i>M. anisopliae</i>	<b>Liquid:</b> 0.3 mm Millipore filtration, ddH <sub>2</sub> O dialysis with a 1.0 kDa cutoff at 4°C. Lyophilized. Store -80°C. Resuspend 2000 mg in 0.005 M Tris/EDTA buffer (1 mM PMSF and 1 mM E-64 protease inhibitors). Precipitate by 2D Clean-Up kit (GE HealthCare).	To 500 mg of protein add 250 ml solution: 2% CHAPS, 8 M urea, 7 mg DTT ml <sup>-1</sup> and 2% IPG buffer.	Murad et al., 2006
<i>L. maculans</i> / <i>L. bicolor</i>	<b>Liquid:</b> i) Ultrafiltrate (Amicon Ultra-15) 15 mL of dialyzed secretome by centrifugation 45 min (4°C, 5000xg) until 500 mL. -TCA precipitation: add 1.5 mL of 10% w/v TCA/0.007% v/v 2-ME in cold acetone to 500 mL of sample. -Phenol extraction: add 500 mL 2M sucrose buffer to 500 mL of the ultrafiltered sample, incubate on ice 15 min. Add 1 mL of Tris-saturated phenol (pH 8). The dried pellet was resolubilized in 500 mL of solution R. ii) TCA/acetone precipitation: mix 10 mL of sample and 40 mL 10% w/v TCA/0.007% v/v 2ME in cold acetone (-20°C). Centrifugate: 30 min, -20°C, 18000xg, discard supernatant. Repeat 3 times adding 10 ml of secretome every time. Wash in 0.002% v/v 2ME/ acetone. Dry pelet 2 h at 200 mbars and resolubilized in 500 mL of solution R. iii) Phenol/ ether extraction: mix 10 mL of sample and 15 ml of phenol. Vortex 20 s; centrifugate 12000xg, 5 min. Discard suppernatant. Add 2 volumes of ether. Vortex 20 s; centrifugate 12000, 5 min. Rerpeat this step. Dry lower aquous phase under vaccum. Resolubilzate in 500 mL of solution R. iii) Lyophilization: freeze-dry 15 mL of sample and resolubilize in 500 mL of solution R.	Solution R: 7M urea, 2M thiourea, 2% w/v CHAPS, 1% w/v DTT, 0.5% v/v proteinase inhibitor mix (GE Healthcare), 0.5% v/v pH 4-7 ampholites and 0.5% v/v pH 3-10 ampholites. 50 mL of solution R per microgram of pellet.	Vincent et al., 2009
<i>P. tritici-repentis</i>	<b>Liquid:</b> Filtrate medium through a 0.22 µm cellulose nitrate filter. Dialyze (cut-off: 1000 Da) against ddH <sub>2</sub> O at 4°C. Lyophilize and resuspend in 1.2 mL rehydration buffer containing 2 mM tributylphosphine (BioRad). Prior to 2D electrophoreis, samples were desalted using a ReadyPrepTM 2-D Cleanup kit (BioRad).	8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte, 0.001% bromophenol blue	Cao et al., 2009
<i>T. harzianum</i>	<b>Liquid:</b> Dialyzed sample against ddH <sub>2</sub> O. Concentrate by ultrafiltration. Precipitate 400 µg of proteins with 2-D clean-up kit (GE Healthcare). Dissolve in rehydration solution.	8 M urea, 0.5% CHAPS, 0.2% IPG buffer, 15 mM DTT and trace amounts of bromophenol blue	Monteiro et al., 2010
<i>A. niger</i>	<b>Liquid:</b> Clarify supernatant by 0.2 µm filtration. Precipitate with TCA (10% w/v) O/N at 4 °C. Wash with ice cold acetone. Resuspend pelet in 0.1% SDS	0.1% SDS buffer	Adav et al., 2010

Table 2. Summary of methodologies used for secretome analyses of filamentous fungi. Microorganism, protein extraction method, buffer composition and reference are indicated. 2ME: 2-Mercaptoethanol (β-mercaptoethanol); TCA: Trichloroacetic acid.

## 5. Proteome analysis of membranes and organelles

Kim and co-workers (2007) propose the term 'subproteomics' to describe proteomic analysis of a defined subset of an organism's protein complement, primarily specific organelles based on the previous review of Cordwell and co-workers (2000). In the case of bacteria: cytosolic, membrane, cell surface-associated and extracellular proteins (secretome) are considered as subproteomes (Hecker et al., 2010). Thus, in eukaryotes the organelle proteomes are a clear example of a subproteome.

The connection of filamentous fungi between the local environment and inner media is provided by the embedded proteins present in the cell wall, plasma and organelle membranes. Thus, the cell wall, which is the largest organelle of a filamentous fungus, represents an essential dynamic structure fulfilling many vital functions, such as physical protection, osmotic stability, selective permeability barrier, immobilized enzyme support, cell-cell interactions and morphogenesis (Pitarch et al., 2008).

To date, most MS-based proteomic analyses of filamentous fungi have had three targets: the whole cell (mycelial extract), the cytosolic proteins and the secretome. Nevertheless, the membranes of organelles, like mitochondria, which includes proteins of the transport systems (e.g.: porins), as well as proteins involved in fusion, fission, morphology and the inheritance of the organelle, remains unknown. Thus, organelle proteome mapping of different cell compartments is an interesting way to reveal various aspects of fungal metabolite production, e.g. penicillin production in the microbodies of *P. chrysogenum* (Ferreira de Oliveira & de Graaff, 2011; Kiel et al. 2009).

### 5.1 Organelle isolation and enrichment for proteome analysis

A crucial step in the organelle Proteomics is organelle enrichment, since downstream analysis is dependent on its good-quality purification. The filamentous fungi idiosyncrasy results in tricky organelle isolation due to their protease production, the polarized growth substantiated by microtubules (increase the organelle separation) and their compact cell wall that difficult cell disruption. Thus, Ferreira de Oliveira and de Graaff (2011) propose two capital steps for organelle enrichment: i) cell disruption; ii) crude organelle separation and enrichment by additional separation techniques. An example of these steps is summarized in figure 2. Firstly, cell disruption should be as homogeneous and reproducible as possible, thus automatic grinders or French pressure cell are recommended. Anyway, bead beating or manual mortar handle can be used. An interesting and popular method to obtain intact organelles is the enzymatic degradation for protoplast formation followed by gentle lysis. In spite of its popularity, the time consumption (2-3 hours) of the process and batch enzymes variability are cons of this method. Secondly, different methods can be used for debris elimination as filtration; low-speed centrifugation; differential detergent fractionation; centrifugal elutriation; ultracentrifugation (linear or density gradients); immunomagnetic separation, which has been used for organelle isolation (mitochondria vacuoles, microbodies, endosomes, vesicles) in other eukaryotes; or miniaturized free-flow electrophoresis (FFE) (Kohlheyer et al. 2008, Ferreira de Oliveira & de Graaff, 2011).

A large number of diseases and developmental abnormalities result from mitochondrial functions. This fact has caught the attention over the mitochondrial biology, since our understanding of mitochondrial molecular biology has been obtained from in *Saccharomyces* and *Neurospora*. The combination of different electrophoretic techniques such as blue native,



2D gels or 2D tricine on *Neurospora* has allowed the identification of 260 mitochondrial proteins, which include 55 previously predicted or hypothetical annotated and 101 proteins not previously identified in mass spectrometry studies [AutoFlex or UltraFlex II instruments (Bruker Daltonics)] (Keeping et al., 2011).

An increase in the number of microbodies enhances antibiotic production by *P. chrysogenum*. This finding has focused the attention on the analysis of microbody matrix proteins (figure 2), since a part of the penicillin biosynthesis pathway is located in the peroxisomal lumen. Kiel and co-workers (2009) by means of LC-MS/MS analysis using a 4800 MALDI-TOF/TOF analyzer (Applied Biosystems) identified 89 microbody proteins, 79 with a putative microbody targeting signal.

### 5.2 Membrane and cell wall proteomes: an almost unexplored field

The hydrophobicity of the membrane or the cell wall embedded proteins cause a difficult isolation and analysis of those proteins, which is consistent with the low number of articles of membrane proteome in filamentous fungi. Initially, Bowman and co-workers (1981) described a protocol for plasma membrane extraction with high ATPase activity of *Neurospora*. Later, Mezence & Boiron (1995) developed a protocol for outer membrane protein extraction for *Madurella mycetomatis*. Both methods were used by Hernández-Macedo and co-worker (2002) for plasma membrane and cell wall protein extraction of *P. chrysosporium* and *L. edodes*, although the proteins were only visualized in one-dimensional SDS-PAGE rather than 2D electrophoresis.

Microbiologists, plant pathologists and commercial companies have paid a considerable attention to the production and application of biological control agents. Thus, *Trichoderma* genus has generated a special attention as active agent for biological control of plant pathogenic fungi. Proteins associated with the cell envelope of *Trichoderma reesei* were analysed by 2D electrophoresis allowing the location of 220 proteins and the identification of 32 spots by nanoelectrospray tandem mass spectrometry [Q-TOF MS (Micromass)] and amino acid sequence (Lim et al., 2001). The most abundant protein was HEX1, the major protein in Woronin body, suggesting that this structure unique to filamentous fungi is linked to the cell envelope.

Glycosylphosphatidylinositol-linked (GPI) proteins anchored to the plasma membrane are known to play a role in fungal morphogenesis (filamentation, mating, flocculation, or adhesion to the external matrix) and an active role in cell wall organization. In order to identify GPI-anchored proteins involved in cell wall biogenesis, a proteomic analysis in *A. fumigatus*, a mould causing most of the invasive fungal lung infections in immunocompromised patients, was carried out. The GPI-anchored proteins were released from a membrane preparation [3 min in a CO<sub>2</sub>-cooled MSK homogenizer with glass beads (1 mm-diameter) and centrifuged at 100000xg for 1 h at 4°C] by an endogenous GPI-phospholipase C, sequentially purified by liquid chromatography, separated by 2D electrophoresis and characterized by MALDI-TOF [Voyager DE-STR MS (PerSeptive Biosystems)] and by internal amino acid sequencing [Finnigan TSQ 7000 (ThermoQuest)]. Thus, nine GPI-anchored proteins were identified in *A. fumigatus* (Bruneau et al., 2001). Following this analysis, de Groot and co-workers (2009) found ten predicted GPI-anchored proteins in cell wall fractions of *A. nidulans*, which consist of six proteins identified as cell wall carbohydrate-active enzymes including three orthologous proteins similar to those described before in *A. fumigatus* by Bruneau and co-workers (2001).

Fungus	Proteome Type	Proteomics approach	Application	Reference
<i>A. fumigatus</i>	Intracellular	2DE / MALDI-TOF	Reference map / Human pathogenicity	Carberry et al., 2006
		2DE / MALDI-TOF/TOF	Human pathogenicity / Alcohol metabolism	Kniemeyer et al., 2006
			Oxidative stress response	Lessing et al., 2007
			Antibiotic resistance	Gautam et al., 2008
		2DE / MALDI-TOF	Reference map / Human pathogenicity	Vödisch et al., 2009
			Dormant conidia / Pathogenicity	Teutschbein et al., 2010
			iTRAQ / 2D LC-MALDI-TOF/TOF	Human pathogenicity / Development
2DE / MALDI-TOF-MS/MS	Antibiotic resistance	Gautam et al., 2011		
<i>A. nidulans</i>	Intracellular	2DE / ESI-QqTOF	Bacterial-fungal interaction	Melin et al., 2002
		2DE / MALDI-TOF	Osmoadaptation	Kim et al., 2007
			Hypoxic responses	Shimizu et al., 2009
			Oxidative stress response	Sato et al., 2009
		2DE / MALDI-TOF/TOF	Conidia germination	Oh et al., 2010
			Oxidative stress response	Thön et al., 2010
2DE / Nano HPCL MS/MS		Pusztahelyi et al., 2011		
<i>A. niger</i>	Intracellular	2DE / MALDI-TOF/TOF	Biotechnological processes	Sørensen et al., 2009
		2DE / MALDI-TOF/TOF / ESI-QqTOF	Reference map / Biotechnological processes	Lu et al., 2010
<i>B. cinerea</i>	Intracellular	2DE / MALDI-TOF/ ESI IT	Reference map / Vegetal pathogenicity	Fernández-Acero et al., 2006
<i>P. chrysogenum</i>	Intracellular	2DE / MALDI-TOF/TOF	Reference map / Penicillin production	Jami et al., 2010a
<i>P. chrysosporium</i>	Intracellular	2DE / LC-MS/MS	Environmental pollution	Matsuzaki et al., 2008
		2DE / MALDI-TOF		Yildirim et al., 2011
<i>T. harzianum</i>	Intracellular	2DE / MALDI-TOF / LC-MS/MS	Reference map / Biocontrol agent	Grinyer et al., 2004
<i>A. fumigatus</i>	Extracellular	2DE / immunoblotting / Q-TOF	Immunome	Singh et al., 2010
		2DE / MALDI-TOF-MS/MS	Human pathogenicity	Wartenberg et al., 2011

Fungus	Proteome Type	Proteomics approach	Application	Reference
<i>A. niger</i>	Extracellular	1D / Nano HPCL MS/MS	Reference map / Biotechnological processes	Lu et al., 2010
<i>A. oryzae</i>	Extracellular	2DE / MALDI-TOF	Food production	Oda et al., 2006
		2DE / MALDI-TOF		Machida et al., 2005
<i>A. versicolor</i>	Extracellular	2DE / immunoblotting / LC-MS/MS	Immunome	Benndorf et al., 2008
<i>B. cinerea</i>	Extracellular	1DE / LC-MS/MS	Vegetal pathogenicity / virulence factors	Shah et al., 2008
		2DE / MALDI-TOF / LC-MS/MS		Espino et al., 2010
		2DE / MALDI-TOF/TOF		Fernández-Acero et al., 2010
<i>D. seriata</i>	Extracellular	2DE / MALDI-TOF/TOF	Vegetal pathogenicity / virulence factors	Cobos et al., 2010
<i>M. acridum</i>	Extracellular	2DE / MALDI-TOF	Biocontrol agent	Barros et al., 2010
<i>M. anisopliae</i>	Extracellular	2DE / MALDI-TOF/TOF	Biocontrol agent	Murad et al., 2006
<i>M. globosa</i>	Extracellular	2DE / immunoblotting / oMALDI Qq-ToF / N-terminal aa sequencing	Immunome	Ishibashi et al., 2009
<i>P. citrinum / A. fumigatus</i>	Extracellular	2DE / immunoblotting / N-terminal aa sequencing	Immunome	Lai et al., 2002
<i>P. chrysogenum</i>	Extracellular	2DE / MALDI-TOF/TOF	Food production	Jami et al., 2010b
<i>P. placenta</i>	Extracellular	1DE / LC-MS/MS	Environmental pollution	Martínez et al., 2009
<i>P. tritici-repentis</i>	Extracellular	2DE / ESI-q-TOF MS/MS	Biocontrol agent	Cao et al., 2009
<i>T. harzianum</i>	Extracellular	2DE	Biocontrol agent	Suárez et al., 2005
		2DE / MALDI-TOF		Monteiro et al., 2010
<i>A. fumigatus</i>	Plasma-membrane anchored proteins	2DE / MALDI-TOF	Biomarker	Bruneau et al., 2001
		1DE / LC-MS/MS		Groot et al., 2009
		1DE / 2D LC-MS/MS		Ouyang et al., 2010
	Conidial surface	2DE / LC-MS/MS	Asif et al., 2005	
	cell wall and plasma membrane	iTRAQ / LC-MALDI-TOF/TOF	Antibiotic resistance	Cagas et al., 2011b
<i>Neurospora spp.</i>	Organelle	2DE / 2DE-tricine / MALDI-TOF	Disease analysis	Keeping et al., 2011
<i>P. chrysogenum</i>	Organelle	1DE / LC-MALDI-TOF/TOF	Penicillin production	Kiel et al., 2009
<i>P. chrysosporium / L. edodes</i>	Plasma membrane / cell wall	1DE	Procedure optimization	Hernández-Macedo et al., 2002
<i>T. reesei</i>	Cell envelope	2DE / ESI-Q-TOF	Biocontrol agent	Lim et al., 2001

Table 3. Summary of the major proteomes identified from filamentous fungi using different proteomic approaches.

In other proteomic analysis, Asif and co-workers (2005) provided the first conidial surface subproteome map of *A. fumigatus* with the goal of finding potential therapeutic targets against this human pathogen. The intact viable *Aspergillus* conidia were extracted with a mild alkaline buffer (0.1 M Tris-HCl-buffer pH 8.5) in the presence of a 1,3- $\beta$ -glucanase containing 1 mM 1,10 phenanthroline. Thus, the combination of 2D electrophoresis and LC-MS/MS [Q-TOF Ultima Global MS (Waters)] allowed the identification of 26 different

proteins of *A. fumigatus*, twelve of which contain a signal for secretion. Among the proteins without a secretory signal the well-known allergen AspF3 was identified.

Recently, Ouyang and co-workers (2010) in close connection with the previous paper of Bruneau and co-workers (2001) attempted to identify membrane proteins associated with cell wall biosynthesis and glycoconjugates of total membrane preparations from *A. fumigatus*. Thus, the combination of 1D gels and 2D LC-MS/MS allowed the identification of 530 proteins that include 9 membrane proteins involved in cell wall biosynthesis and 8 enzymes involved in sphingolipid synthesis. These can potentially be used as antifungal drug targets following the way designed by Asif and co-workers (2005).

The identification of resistance mechanisms to antifungal drugs is an interesting topic, which has been investigated in *A. fumigatus* by determining the fungal proteomic response to drug exposure (Cagas et al., 2011b). These authors analysed the proteomic response of *A. fumigatus* to caspofungin by 2D electrophoresis from 4 subcellular compartment fractions (secreted, cytoplasmic, microsomal and cell wall and plasma membrane) and later gel-free iTRAQ method [using a MALDI-TOF/TOF MS (Applied Biosystems)] and microarrays were performed from the secreted and cell wall and plasma membrane fractions. These analyses presented the high potential to identify biomarkers of the employed techniques that assess the efficacy of caspofungin drug therapy.

On the one hand, the cell wall proteins are often highly glycosylated (hampers MS analysis) and they are difficult to separate from cell wall polysaccharides. On the other hand, membrane proteins are hard to dissolve and difficult to digest by standard methods (hydrophobic parts lack tryptic cleavage sites) due to their hydrophobicity. Besides, they usually present a dramatic low abundance. Thus, despite the technical progresses, the proteomic analysis of cell wall and membrane proteins remains as a tough task in filamentous fungi supposing a trending Proteomics challenge (Kniemeyer, 2011).

## 6. Conclusion

Proteomics is a powerful tool, which has emerged as a result of summarize methodological and technical findings in mass spectrometry, protein visible and fluorescent staining, bidimensional electrophoresis, isobaric labelling, etc. The genome sequence facilities generated in the last years have supported this current trending topic that proteomes and subproteomes analyses represent. Along the present review the gradient of knowledge existing about filamentous fungi Proteomics is clearly presented, which is perfectly correlated with the protein isolation difficulty. Thus, several studies have been carried out over the intracellular proteome that helped to the development of a huge number of procedures for protein extraction and analysis. These analyses collect from technical updates to multiple strain comparison. As far as the protein isolation became more and more complex the numbers of published analyses diminished due to the complexity of the organelle isolation as well as hydrophobic membrane protein analysis. Anyway, the Proteomics approach applied to the filamentous fungi is opening a new world of strain improvement, drug resistance analysis, antigen detection, biomarker discovery and environment applications, which was difficult to predict only ten years ago. Thus, Proteomics application to filamentous fungi is a present reality (summarized in table 3) with a promising future that still has several challenges to be faced up.

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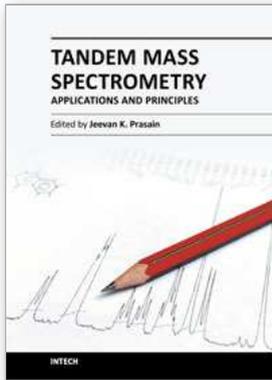
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