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Proteome profiling illustrated by a large-scale fed-batch fermentation of *Penicillium chrysogenum*

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ABSTRACT

Filamentous fungi are employed for the large-scale production of value-added products, including organic acids, enzymes, and antibiotics and bioprocess characterization is essential for production optimization but relies on empiricism-based strategies. Protein expression profiles in an industrial scale, 180 h fed-batch fermentation of *Penicillium chrysogenum* are presented. The biomass of *P. chrysogenum*, as well as the specific penicillin V production rate and fungal morphology were monitored during fermentation to be compared with obtained protein profiles. Our results demonstrate a correlation between proteomics data and biomass concentration, morphological changes, and penicillin production.

1. Introduction

Filamentous fungi are indispensable in pharmaceutical and biotechnological industries due to their ability to produce important metabolites. In the middle of the nineteenth century, it was first discovered that fungi can be used as active agents in the chemical industry (i.e., gallic acid industry) [1]. Since then, the number of fungal products has rapidly increased. To date, many of these products, such as organic acids [2], enzymes [3], and antibiotics [4] are of great importance because of their use in the food and pharmaceutical industries, and other industrial fields. The fermentation of *Penicillium chrysogenum* and its associated penicillin production is an important research topic, particularly in the biotechnology and biopharmaceutical industries. The therapeutic and commercial importance of penicillin and its derivatives demand a highly controlled fermentation process, as small changes in process parameters can lead to severe changes in fungal morphology, growth behavior, and particularly the production efficiency of antibiotics [5]. After a biomass is produced by batch fermentation, industrial fermenters are inoculated by a fed-batch process in which fungal cells are constantly subject to stress, thereby producing high amounts of a particular antibiotic or precursor. In batch and fed-batch processes, fungal growth behavior is constantly changing, leading to subsequent varying morphology. It is generally accepted that phenotypic changes reflect variations in the

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organism's proteome. This knowledge and the constant need for a better understanding of the underlying molecular mechanisms during large-scale cultivation justify the study of global proteome changes during a biotechnologically relevant fermentation process.

The proteome is defined as the total set of proteins expressed at one time point by the genome [6]. According to this definition, the *Penicillium* proteome must continuously change since protein synthesis and degradation are in constant flux to keep cells alive, in contrast to the static genome. During fermentation, the proteome changes even more according to stress levels, and in some cases, extreme external influences such as oxygen supply, carbon feed, pH, temperature, or feed rates. To date, many optimized industrial fermentation processes are simply based on empiricism-driven process improvements; however, the underlying metabolic processes remain poorly characterized.

Proteomics is used to reveal proteome changes in *P. notatum* (Fleming strain) and *P. chrysogenum* NRLL 1951, a strain that has been modified by several improvement processes [7]. However, to the best of our knowledge, the application of proteomics to differentiate expression profiles in industrial fermentation processes has not been reported. Many studies have described the changes that occur from a single species in rather small culture conditions during different growth conditions or due to variations between strains (summarized in the supplementary information). However, monitoring fed-batch fermentation by proteomics is a novel approach. In 2005, Kim et al. [8] described the fed-batch fermentation process of *Escherichia coli* in a bioreactor. The sampling time points were close together, which usually resulted in minimal proteome changes, which allowed the authors to draw comparative proteomic conclusions using difference gel electrophoresis (DIGE) [9]. *Aspergillus oryzae* was one of the first fungal species used for comparing intracellular proteomes at two different time points during fermentation in shake flasks [10]. In 2006, different culture conditions were investigated by comparing the expression profiles during solid-state and submerged cultivation at five different time points [11].

In the current study, two-dimensional gel electrophoresis (2-D GE) was used to assess the up- and down-regulation of proteins and their degradation products during fermentation of *P. chrysogenum*. Minimal changes, as well as significant variations of the proteome were observed, including disappearance and appearance of protein spots. This did not allow implementing DIGE in our process due to a high number of missing values for single spots. However, silver staining provides a wide dynamic range for protein visualization and was therefore the method of choice [12]. Moreover spot intensities were used to estimate increases and decreases in protein concentrations.

2. Materials and methods

2.1. Cultivation conditions and analytical methods for defining samples

All fed-batch samples were directly collected from a 7.5-L stirred bioreactor. Fed-batch process duration was 184 h. Detailed cultivation conditions and classical fermentation analysis were previously described [5], together with the high-throughput recording of microscopic images and computational image analysis for morphological classification and calculation of morphological parameters. Determination of biomass dry weight was performed in duplicate by direct sampling from the bioreactor and centrifuging approximately 10 mL of culture broth in pre-dried falcon tubes. Subsequently, the biomass pellet was washed twice with 10 mL deionized water and dried at 95 °C for 48 h. The weight of the falcon tubes was recorded prior to sampling, after sampling, and after drying the biomass; the biomass dry weight was calculated accordingly. Penicillin V concentration in the supernatant was determined by high-pressure liquid chromatography using a ZORBAX Eclipse Plus C18column – 3.0 mm × 150 mm, particle size 3.5 μm, pore size 95 Å (PN 959963-302, Agilent Technologies, Palo Alto, CA, USA) and an isocratic elution with an aqueous 28% ACN containing 6 mM H_3PO and 5 mM KH_2PO_4 at 1 ml/min flow rate (UV detection at 210 nm).

2.2. Sample preparation

Samples collected directly from the fermenter were stored at −20 °C. An aliquot of 1 mg biomass was washed three times using ultrapure water (18.2 MΩ cm resistivity at 25 °C) with short centrifugation steps (13,200 rpm, 4 min, room temperature) between washes to remove interfering substances from the fermentation medium. The final pellet was diluted in lysis buffer (50 mM Tris–HCl, 10% glycerol, 0.5% Tween 20, 150 mM NaCl, 2 mM DTT, pH 8.0, protease inhibitor cocktail (Halt protease inhibitor cocktail kit; ThermoFisher Scientific, Waltham, MA, USA) containing 10% toluol. The samples were disrupted by an ultrasonic sonotrode using a Duty Cycle of 40% and an Output Control value of 2.5; this was repeated two times, 20 seconds each, with a 1 min break. The cell suspension was centrifuged (14,800 rpm, 10 min), and the supernatant was used for subsequent trichloroacetic acid (TCA)/acetone precipitation. Proteins were depleted with ice-cold acetone/20% TCA at a ratio of 1:8 (v:v). The obtained protein pellet was washed with 80% and 100% acetone, and dissolved in isoelectric focusing (IEF) buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, and protease inhibitors (all chemicals were from Sigma–Aldrich, St. Louis, MO, USA). Finally, the protein concentration was determined using the Bradford assay and bovine serum albumin (BSA) for calibration. The samples were stored at −20 °C in aliquots of 120 μg protein.

2.3. 2-D gel electrophoresis

18 cm immobilized pH gradient (IPG) strips with a nonlinear gradient from pH 3 to 10 (GE Healthcare Life Sciences, Pittsburgh, PA, USA) were rehydrated in 340 μl IEF buffer containing 120 μg protein, 20 mM DTT, 2% ampholytes, 0.002% bromophenol blue, 7 M urea, 2 M thiourea and 2% CHAPS. Isoelectric focusing was carried out with at least 50 kVh on a Multiphor II system (GE Healthcare Life Sciences, Pittsburgh, USA). For the second dimension, the strips were equilibrated in 50 mM Tris–HCl, pH 8.8, 6 M Urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue containing 1% DTT for the first and 2.5% iodoacetamide for the second incubation (each for 15 min).

The second dimension was performed on 14 cm isocratic SDS-PAGE gels (15% T) using a SE600 standard dual cooled vertical electrophoresis chamber (Hoefer, Holliston, MA, USA). Protein spots were detected by acidic silver nitrate staining according to Shevchenko et al. [12].

2.4. *Enzymatic treatment*

Spots obtained from the 2-D gels were cut out with a clean scalpel, destained with 100 mM $Na_2S_2O_3$/30 mM $K_4[Fe(CN)_6]$ (1:1, v:v) and washed with ultrapure water and a ultrapure water/acetonitrile (1:1, v:v) (each step for 15 min). Gel particles were further treated with acetonitrile for 5 min, followed 100 mM NH_4HCO_3 (pH 8.5) for 5 min, and finally acetonitrile/100 mM NH_4HCO_3 (1:1, v:v) for 15 min. After drying the gel particles were reduced with 10 mM DTT in 100 mM NH_4HCO_3 (for 45 min at 54 °C) and carbamidomethylated with 55 mM iodoacetamide in 100 mM NH_4HCO_3 (for 30 min, room temperature). After washing with 100 mM NH_4HCO_3 for 5 min and a 100 mM NH_4HCO_3/acetonitrile (1:1, v:v) for 15 min, the gel particles were dried and digested overnight with 12 ng trypsin/μl (porcine trypsin; Promega, Mannheim, Germany) in 50 mM NH_4HCO_3 (pH 8.5). Peptides were extracted with 50 mM NH_4HCO_3/acetonitrile (1:1, v:v) and 1% HCOOH/acetonitrile (1:1, v:v). The pooled extracts were dried in the vacuum centrifuge before they were re-dissolved in 0.1% aqueous TFA. The obtained peptides were purified with ZipTips C18 (Millipore, Billerica, MA, USA).

2.5. *Mass spectrometry*

The samples were spotted together with α-cyano-4-hydroxy cinnamic acid (3 mg/ml in acetonitrile/0.1% TFA, v:v, 1:1) on an Anchorchip target (Bruker Daltonik, Bremen, Germany) and were measured with a MALDI-TOF/RTOF system (UltrafleXtreme, Bruker Daltonik) in the reflectron mode. Mass spectra were recorded in the positive ion mode, and mass spectra were not smoothed for further analysis. Calibration was performed with a mixture of different peptides in a mass range from m/z 573.31 to 3657.92 ($[MH]^+$). Each peptide mass fingerprint (PMF) was confirmed with MS/MS from at least three independent peptides using laser induced peptide dissociation (Ultraflexreme™: LIFT-Mode without gas).

2.6. *Protein identification*

Proteins were identified using Mascot as a search engine in combination with NCBI (NCBInr 20121124), and were all associated with *P. chrysogenum*. With the help of Kyoto Encyclopedia of Genes and Genomes (KEGG database [13]) and an organism-specific database of metabolic pathways, compounds and reactions; namely, FungiCyc (Broad Institute [14]), the proteins were assigned to their specific metabolic pathways. If proteins were not described in KEGG or FungiCyc, PANTHER hidden Markov model sequence scoring [15] was used to find biological processes known for sequence homologues in other species.

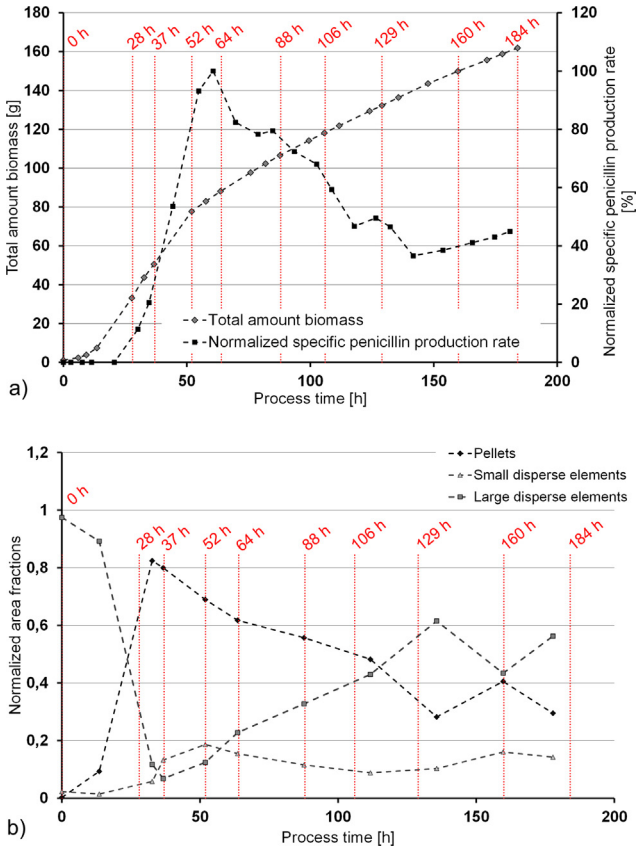


Fig. 1 – (a) Biomass and specific penicillin production rate and (b) morphological behavior of *Penicillium chrysogenum* during a 184 h fed-batch fermentation.

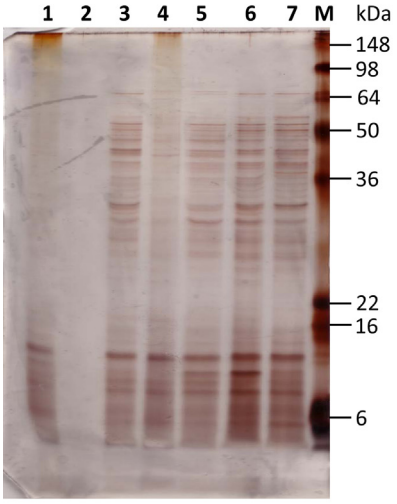
3. Results and discussion

3.1. *Fermentation of Penicillium chrysogenum*

The monitoring of process parameters like penicillin V production rate, biomass, and fungal morphology with respect to pellet formation [16] were used for defining fed-batch fermentation broth samples of *P. chrysogenum*. Morphological changes showed dispersed elements of all sizes, as well as formation of fungal pellets. The comparison of Fig. 1a and b shows that the specific penicillin V production rate increased immediately after pellet formation (28–37 h) starts. This correlation was also observed by Liu et al. [17]. Hence, morphology correlates with penicillin production, and it can be assumed with the utmost probability that morphological changes are the reflection of protein changes. Therefore the proteome of *P. chrysogenum* during fed-batch fermentation was investigated to get a first view on underlying cellular mechanisms and interactions.

3.2. *Investigations of buffer systems for protein extraction*

In a first attempt to unravel the (mechanisms behind the) penicillin production process, which entirely occurs in the cytoplasm, this study focuses on intracellular proteins, extracted from *P. chrysogenum* mycelium harvested at fixed



1 Lysis buffer, Laemmli buffer
2 Lysis buffer, IEF buffer, 80 mM citric acid
3 Lysis buffer, IEF buffer
4 Lysis buffer, 10 % Toluol, Laemmli buffer
5 Lysis buffer, 10 % Toluol, IEF buffer, 80 mM citric acid
6 Lysis buffer, 10 % Toluol, IEF buffer
7 10 % Toluol, IEF buffer

Lysis buffer: 50mM Tris-HCl, 10% glycerol v/v, 0.5% Tween20 v/v, 150mM NaCl, 2mM DTT; pH 8.0, protease inhibitor cocktail

Laemmli buffer: 62.5mM Tris-HCl, 2% SDS, 10% glycerol v/v, pH6.8protease inhibitor cocktail

IEF buffer: 7M urea, 2M thiourea, 2% CHAPS, protease inhibitor cocktail

Fig. 2 – SDS–PAGE analysis of *P. chrysogenum* cell lysates. Lysis buffer with and without toluol was applied during ultrasonic treatment. Different buffer combinations were tested for dissolving protein pellets after precipitation (acetone/trichloroacetic acid). Lane 6 shows the highly effective combination of toluol containing lysis buffer and the finally applied IEF buffer.

time points during a 184 h fermentation process. The fungal mycelium of *P. chrysogenum* is not very rigid, allowing a straightforward assessment of the intracellular proteome. However buffers are critical in any proteomics approach. So after short washing of the collected mycelium, cells were disrupted in various buffers using an ultrasonic sonotrode. After protein precipitation with TCA and ice-cold acetone the effectiveness of different buffers for protein reconstitution, all compatible with subsequent 2-D gel electrophoresis, were tested. Seven protein extraction methods were used to determine the most efficient method for further protein analysis. Lane 6 in Fig. 2 shows the most efficient procedure in comparison to other protein extraction protocols often used for fungal protein extraction. The addition of 10% toluol to the lysis buffer enhances nucleotide removal and improved the extraction method in contrast to the application of standard lysis buffers (Lane 3). This was especially observed for proteins in the lower molecular mass range. The use of a chaotropic IEF buffer (Lane 1) instead of Laemmli buffer (Lane 4) increased the number of detected proteins in the higher molecular mass range. Lysis buffers containing a low concentration of mild detergents were not efficient for protein extraction, and the Laemmli system was not effective for dissolving precipitated proteins. Based on these results we decided to perform further experiments with lysis buffer in combination with 10% toluol (50 mM Tris–HCl, 10% glycerol, 0.5% Tween 20, 150 mM NaCl, 2 mM DTT, pH 8.0, protease inhibitor cocktail) and IEF buffer (7 M urea, 2 M thiourea, 2% CHAPS, protease inhibitor cocktail) for the solubilization of proteins after TCA/acetone precipitation.

3.3. *2-D gel electrophoresis and data interpretation*

The 2-D gel electrophoresis approach in combination with acidic silver nitrate staining was used to examine protein expression profiles during the fed-batch fermentation of *P. chrysogenum*. The intensities of several spots (e.g., spot numbers 4, 15, 30) show an increasing trend with fermentation time (Fig. 3), whereas other intensities tend to decrease (e.g., spot numbers 1, 5, 10) over time. Furthermore, some proteins were constantly expressed (e.g., spot numbers 23, 30) during the entire fermentation process. The spots from Fig. 3 were identified as described, and are listed in Table 1. Table 1 also gives relative-qualitative information on spot intensities highlighting the variation of expression levels for the whole process.

At 37–52 h of fermentation (when the rate of penicillin production rate is increasing), the intense spots on the gel, which represent proteins like glutamate dehydrogenase (spot number 1) and dihydrolipoyl dehydrogenase (spot number 14), appear down-regulated. The first protein is associated with nitrate assimilation and glutamate biosynthesis, and thus represents a branch-point enzyme between carbon and nitrogen metabolism [18]. Glutamate dehydrogenase is essential for penicillin production, as was demonstrated by a study that investigated production with a *Penicillium* strain with deletion depletion of the glutamate dehydrogenase gene [19]. Mitochondrial dihydrolipoyl dehydrogenase is used during the TCA cycle and glycolysis, in addition to glycine, serine, and threonine catabolism. The presence of both proteins during biomass production (Fig. 1a) was corroborated by their assigned functionality.

The abundance of a GroEL-like type I chaperonin (spot number 10) and peptidylprotyl isomerase (spot number 11), in the first phase of fermentation, both proteins associated with protein folding, seems consistent with increased biomass production, but maybe also by pellet formation. The latter is also assigned to intracellular protein transport by PANTHER classification. The unclassified protein NMT1-like family of proteins (spot number 16) shows homology to a putative pyrimidine precursor biosynthesis enzyme thi11-related

Table 1 – Identified intracellular proteins of *Penicillium chrysogenum* during fed-batch fermentation over 184 h. ++, +, ∼ and − indicate spot intensities (symbols are representing the relative protein amount starting with a high level of proteins (++) and ending with the disappearance of it (−)).

			Fermentation time [h]											
Spot	Protein name	NCBI Database entry	0	28	37	52	64	88	106	129	160	184	Function (KEGG Database)	Function (FungiCyc Database)
1	Glutamate dehydrogenase	gi|255949800	+	+	++	+	+	-	∼	∼	∼	∼	Unclassified	D/U/A, AA biosynthesis
2	Allergen Pen n18	gi|7963902	++	+	++	++	++	+	+	+	+	+	Unclassified	Unclassified
3	S-formylglutathione hydrolase	gi|255931739	+	+	+	+	+	+	+	∼	+	∼	Energy metabolism	D/U/A
4	Dipeptidyl aminopeptidase	gi|255935533	−	−	−	∼	∼	+	∼	−	++	+	Unclassified	ORF
5	Succinate-semialdehyde dehydrogenase	gi|255936363	+	∼	+	+	-	−	∼	-	-	-	AA and CARB metabolism	D/U/A
6	Aspartyl protease	gi|255936729	+	+	++	++	+	++	++	+	++	++	Unclassified	Unclassified
7	Proteasome endopeptidase complex	gi|255938700	∼	∼	+	∼	-	−	∼	-	+	-	Folding, sorting and degradation	Unclassified
8	Threonine dehydrogenase	gi|255940794	+	+	++	++	++	+	++	++	+	+	Unclassified	ORF
9	Histidine kinase	gi|255941286	∼	+	++	++	++	∼	+	+	+	+	Unclassified	Unclassified
10	GroEL like type I chaperonin	gi|255941288	+	+	+	∼	∼	∼	∼	∼	∼	∼	Folding, sorting and degradation	ORF
11	Peptidylprolyl isomerase	gi|255941672	+	∼	+	∼	∼	∼	-	∼	-	∼	Unclassified	Unclassified
12	Zn-dependent oligopeptidase	gi|255943861	∼	∼	∼	∼	-	-	-	-	-	-	Unclassified	ORF
13	Aspartate-semialdehyde dehydrogenase	gi|255944155	∼	+	++	+	+	∼	+	∼	∼	∼	SEC, AA metabolism	AA biosynthesis
14	Dihydrolipoyl dehydrogenase	gi|255944493	+	+	+	∼	∼	∼	∼	∼	∼	∼	AA and CARB metabolism, SEC	D/U/A
15	Beta-N-acetylhexosaminidase	gi|255945521	∼	-	∼	++	++	+	++	+	++	++	Glycan metabolism, CARB metabolism, SEC	Unclassified
16	NMT1-like family protein	gi|255955655	+	+	++	+	∼	-	-	-	∼	∼	Unclassified	ORF
17	Nucleoside diphosphate kinase	gi|255955689	+	∼	+	∼	∼	+	+	∼	+	∼	Nucleotide metabolism, SEC	Nucleosides and nucleotides biosynthesis
18	Copper/zinc superoxide dismutase	gi|255955883	+	+	+	+	+	+	+	+	+	+	Transport and catabolism	Detoxification
19	Vacuolar serine proteinase	gi|255955889	+	+	++	++	++	+	+	∼	+	∼	Unclassified	Unclassified
20	2-isopropylmalate synthase	gi|255957077	-	∼	+	∼	-	-	∼	-	∼	-	CARB and AA metabolism, SEC	AA biosynthesis
21	Hypothetical protein	gi|255947940	+	∼	+	∼	-	-	-	-	-	-	Unclassified	ORF
22	Rossman fold NAD(P) binding protein	gi|255948090	+	+	+	∼	-	-	-	-	-	-	Unclassified	ORF
23	N-acetylglucosamine-6-phosphate deacetylase	gi|255948486	-	-	∼	∼	∼	∼	+	∼	∼	∼	CARB metabolism, SEC	D/U/A
24	Glucosamine-6-phosphate deaminase	gi|255948492	-	-	-	+	+	+	+	+	+	∼	CARB metabolism, SEC	D/U/A
25	Aspartate aminotransferase	gi|255948946	∼	∼	+	∼	-	-	-	-	-	-	SEC, AA metabolism	D/U/A, AA biosynthesis
26	Aspartyl aminopeptidase	gi|255949104	+	+	++	+	∼	-	-	-	∼	∼	Unclassified	ORF
27	X-Protyl aminopeptidase	gi|255949480	+	+	+	∼	-	-	-	-	-	-	Unclassified	Unclassified
28	Homocysteine methyltransferases	gi|255950002	-	-	+	-	-	-	-	-	-	-	SEC, AA metabolism	AA biosynthesis
29	Glucose-6-phosphate isomerase	gi|255950216	-	-	∼	∼	+	∼	+	-	∼	∼	SEC, CARB metabolism	CAR biosynthesis, Generation of precursor metabolites and energy
30	Glycosyl hydrolase family 20 protein	gi|255951202	-	-	-	∼	∼	∼	∼	-	+	+	Unclassified	Unclassified

AA = Amino acid; CARB = Carbohydrate; SEC = Bioynthesis of secondary metabolites; D/U/A = Degradation/Utilization/Assimilation.

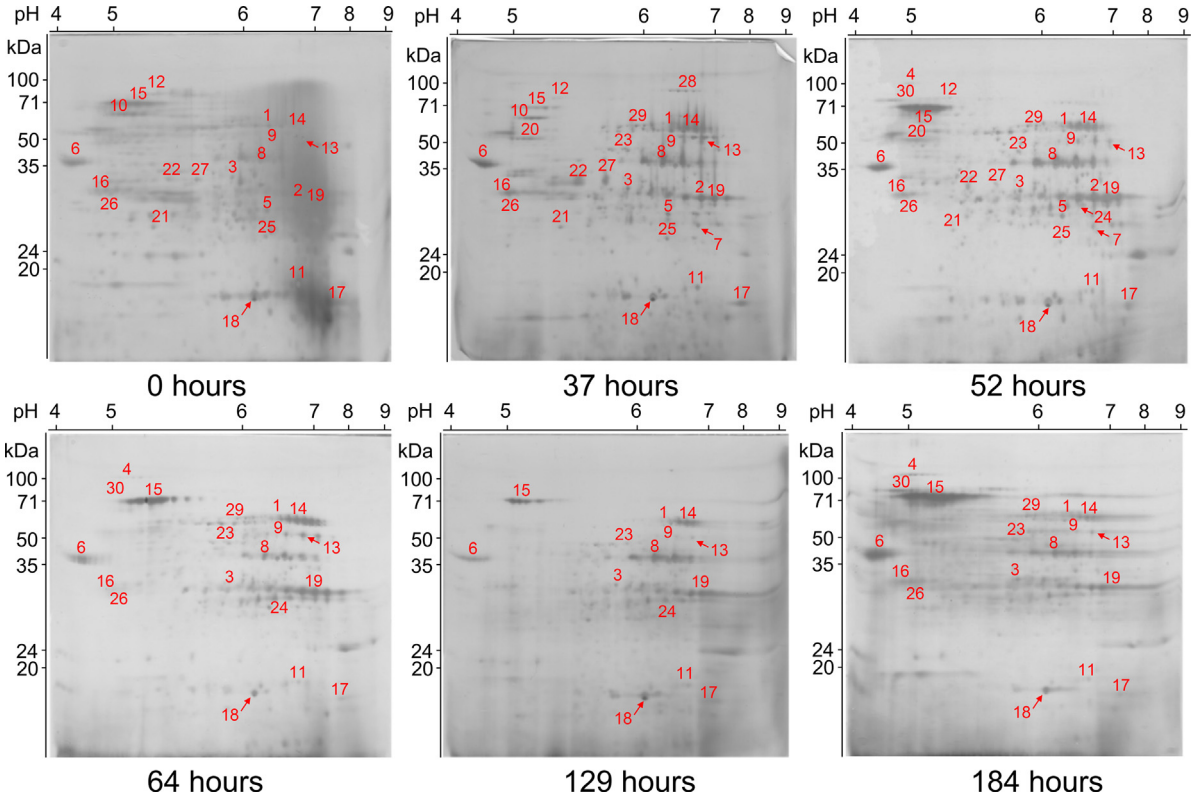


Fig. 3 – 2D gel electrophoresis visualizing proteome changes during a 184 h fed-batch fermentation. Protein spot assigned with numbers refer to identified proteins listed in Table 1.

protein in *Saccharomyces cerevisiae* (PTHR31528:SF1). Generally it can be stated that the NMT1 family is thought to be required for the biosynthesis of the pyrimidine moiety of thiamine, and the peptidylprotyl isomerase interconverts cis and trans isomers of peptide bonds with the amino acid proline [20]. Protein spot 26 is identified as aspartyl aminopeptidase, which is an abundant enzyme class in mammalian cells. Spot number 22 represents a Rossman fold NAD(P) binding protein, which is a thiamine biosynthetic enzyme present in the respiratory electron transport chain. The high abundance of this protein indicates high conversion levels of nutrients into intracellular energy; the biological system is in a highly active state – i.e. is working hard. Spot number 27 represents a proline aminopeptidase, a homologue to protease M24 family members of many species which have nucleic acid binding transcription factor activity. For these proteins (spot number 22 and 27) primary metabolic processes of translation, cellular protein modification processes and proteolysis are described in the Gene Ontology (GO) molecular function annotation; both proteins are only present during the first 37 h of fermentation. The aspartyl aminopeptidase in spot number 26 belongs to the zinc peptidase superfamily and shows a decreasing trend especially after the first 37 h. This protein is imported from the cytoplasm to the vacuole, e.g. in *S. cerevisiae* during macroautophagy, an intracellular catabolic process for proteins and peptides [21]. Aspartate aminotransferase (spot number 25), which is a transaminase using pyridoxal phosphate as a cofactor and an important enzyme for amino acid metabolism, was not prominently expressed during the first 52 h of fermentation, but was nevertheless no longer present during the rest of the fed-batch process. In general, isomerases catalyze structural rearrangement of isomers and aminopeptidases, and transferases are used for essential cellular functions.

After 52 h of fermentation, the expression profile of many proteins changes. Succinate-semialdehyde dehydrogenase (spot number 3), an enzyme that participates in glutamate, butyrate and carbohydrate metabolism, is observed up to 52 h of fermentation, after which it is no longer clearly detected on the gels. β-N-acetylhexosaminidase (spot number 15), glucosamine-6-phosphate deaminase (spot number 24), and glucose-6-phosphate isomerase (spot number 29) are necessary for the biosynthesis of secondary metabolites. While the first two proteins show an increasing trend after maximum penicillin production, the latter protein was only moderately expressed after 37 h. β-N-hexosaminidase is known to influence the branching of hyphae and autolysis of mycelia [22], and therefore plays an important role for the observed morphological changes of the fungus (decreasing occurrence of pellets, Fig. 1).

Proteins that are consistently expressed during fermentation, include threonine dehydrogenase (spot number 8), an unclassified protein of *P. chrysogenum* that is generally responsible for threonine catabolism and is a member of the zinc-requiring, medium chain NAD(H)-dependent alcohol dehydrogenase family (MDR). The copper/zinc superoxide dismutase (spot number 18) catalyzes the conversion of superoxide radicals to molecular oxygen required for transport as

well as catabolism and spot 3 represents S-formylglutathione hydrolase, which is involved in glutathione-dependent formaldehyde oxidation, and spot 6 represents an aspartyl protease, an enzyme belonging to the enzyme family of endonucleases; the presence of these proteins are good indicators for catabolic activities throughout the fermentation process. Interestingly, a histidine kinase (spot number 9) was identified as a consistently expressed protein. Histidine kinases are usually multifunctional, typically transmembrane proteins of the transferase class that plays a role in signal transduction across the cellular membrane. Furthermore, spot numbers 2 and 19 are identified as proteins that belong to the major group of vacuolar serine proteases (major allergens) in *P. chrysogenum*. Some proteins that are yet unclassified for *P. chrysogenum* are identified; namely, glycosyl hydrolase family 20 protein (spot number 30), which is a glycoside hydrolase, and a dipeptidyl aminopeptidase (spot number 4), both of which are more or less consistently regulated during the entire process, with a decreasing trend in the last phase of fermentation, which correlates with a decrease in the rate of penicillin production (160 h).

For other identified proteins, the observed changes are only moderate and biological correlations are sometimes hard to explained. Yet, spot number 21 is a hypothetical protein, decreases in expression after 52 h, the end of the high penicillin production phase, and is homologue to chromosome transmission fidelity (Ctf) protein 8 in *P. roqueforti*. This protein is a component of the Ctf18 RFC-like complex playing a vital role in chromosome cohesion, the onset of cell division. If the observation of change in penicillin production and morphology (decrease in pellet and large disperse element number) is random or meaningful is a finding worth to be followed up. The decreasing trend of expression after high penicillin production of Zn-dependent oligopeptidase (spot number 12), a protein homologue to saccharolysine in *P. roqueforti* responsible for protein catabolism is at the moment inconclusive. Interestingly expression of aspartate-semialdehyde dehydrogenase (spot number 13), a protein involved in biosynthesis of amino acids, increases during the first 37 h, the phase of pellet formation, but seems to decrease, however not constant, for the rest of the process. Nucleoside diphosphate kinase (spot number 17), 2-isopropylmalate synthase (spot number 20), N-acetylglucosamine-6-phosphate deacetylase (spot number 23) and homocysteine methyltransferases (spot number 28), all involved in the biosynthesis of secondary metabolites, do not give conclusive results for increased or decreased expression levels.

The three enzymes directly involved in penicillin biosynthetic pathway; namely, α-aminoadipyl-cysteinyl-valine synthetase, isopenicillin N synthase, isopenicillin N acyltransferase [23], were not observed during this study, but proteins from other pathways correlated with penicillin production. Glutamate dehydrogenase is a protein involved in the nitrogen metabolism of *P. chrysogenum*, and was observed at rather high expression levels during penicillin production. As already described, this protein is in fact essential for the production of penicillin, which underscores the importance of a deeper understanding of fungi metabolism during industrial fermentation. Furthermore, some proteins are related to biomass production; thus, their protein concentration correlated with progression of the fermentation process. Identification of specific proteins during certain stages of fermentation was one point of interest in the current study, as it is thought that specific morphological changes of *P. chrysogenum* are reflected in proteome changes, including important information on its underlying cellular mechanisms and interactions as was shown for the hypothetical protein, homologue to chromosome transmission fidelity (Ctf) protein 8.

4. Conclusions

Proteome profiling during the fed-batch fermentation of *P. chrysogenum* was performed with a 2-D gel electrophoresis-based approach. The correlation between penicillin production, morphology, and biomass concentration during fermentation with protein expression profiles were suggested with this method over a long time period (about 180 h). This kind of time-dependent proteomic study has already been reported in the literature, but not with samples collected over a long period and not with the link to secondary metabolite production and morphology of the filamentous fungus. Proteomic analysis in fungi provides insight into systematic metabolic flux changes, and can be a great tool for controlling a by-product of fermentation. To date, due to complex interactions between process technology, fungal morphology, and overall process performance, the traditional bioprocess design relies on empiricism-based strategies [24]. Therefore understanding relevant industrial processes by modeling interdependencies between process technology, physiology, and morphology in fed-batch cultures is of great importance. A time-resolved proteomics approach can be a novel tool to gain insight into these aforementioned dependencies. During this study, some proteins of an entire fed-batch fermentation process of *P. chrysogenum* were identified with PMF and MS/MS. Furthermore, expression profiles of specific proteins investigated by 2D GE seem to correspond with physiological data in literature. This study was the first to use a gel electrophoresis-based approach to analyze many different samples from one near-industrial fermentation process, with strong differences observed in their biomass concentration, morphology, or secondary metabolite production rate. The number of the identified proteins is rather small in contrast to the proteome of *P. chrysogenum* (129 reviewed and 13.177 unreviewed protein entries in UniProtKB, 27.293 protein entries in NCBI). Yet this approach investigates expression level changes of high abundance proteins which give a first insight into the most obvious and therefore metabolically important proteome changes during a fermentation process very close to industrial scaling and process control. The strongly increasing interest in fungal proteomics confirms that this is an on-going research field which may lead to its application in different industrial research areas, such as bioprocess technology.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euprot.2014.06.002.

REFERENCES

[1] Lockwood L, Moyer A. The production of chemicals by filamentous fungi. Bot Rev 1938;4:140–64.
[2] Goldberg I, Rokem JS, Pines O. Organic acids: old metabolites, new themes. J ChemTechnol Biotechnol 2006;81: 1601–11.
[3] Guimarães LHS, Peixoto-Nogueira SC, Michelin M, Rizzatti ACS, Sandrim VC, Zanoelo FF, et al. Screening of filamentous fungi for production of enzymes of biotechnological interest. Braz J Microbiol 2006;37:474–80.
[4] Nosek J, Radzio R, Kück U. Produktion von β-Lactamantibiotika durch Mikroorganismen. Chem unserer Zeit 1997;31:172–82.
[5] Posch AE, Koch C, Helmel M, Marchetti-Deschmann M, Macfelda K, Lendl B, et al. Combining light microscopy, dielectric spectroscopy, MALDI intact cell mass spectrometry, FTIR spectromicroscopy and multivariate data mining for morphological and physiological bioprocess characterization of filamentous organisms. Fungal Genet Biol 2013;51:1–11.
[6] Wilkins M. Proteomics data mining. Expert Rev Proteomic 2009;6:599–603.
[7] Barreiro C, Martín JF, García-Estrada C. Proteomics shows new faces for the old penicillin producer Penicillium chrysogenum. J Biomed Biotechnol 2012;2012:1–15.
[8] Kim Y-H, Han K, Lee K, Lee J. Proteome response of *Escherichia coli* fed-batch culture to temperature downshift. Appl Microbiol Biotechnol 2005;68:786–93.
[9] Ünlü M, Morgan ME, Minden JS. Difference gel electrophoresis. A single gel method for detecting changes in protein extracts. Electrophoresis 1997;18:2071–7.
[10] Nandakumar MP, Marten MR. Comparison of lysis methods and preparation protocols for one- and two-dimensional electrophoresis of *Aspergillus oryzae* intracellular proteins. Electrophoresis 2002;23:2216–22.
[11] Oda K, Kakizono D, Yamada O, Iefuji H, Akita O, Iwashita K. Proteomic analysis of extracellular proteins from *Aspergillus oryzae* grown under submerged and solid-state culture conditions. Appl Environ Microbiol 2006;72:3448–57.
[12] Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem 1996;68:850–8.
[13] KEGG Database; http://www.genome.jp/kegg/kegg1.html
[14] FungiCyc; http://fungicyc.broadinstitute.org/
[15] Mi H, Muruganujan A, Thomas PD. PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees. Nucleic Acids Res 2012:1–10.
[16] Posch AE, Spadiut O, Herwig C. A novel method for fast and statistically verified morphological characterization of filamentous fungi. Fungal Genet Biol 2012;49:499–510.
[17] Liu H, Zheng Z, Wang P, Gong G, Wang L, Zhao G. Morphological changes induced by class III chitin synthase gene silencing could enhance penicillin production of *Penicillium chrysogenum*. Appl Microbiol Biotechnol 2012:1–10.
[18] Stillman TJ, Baker PJ, Britton KL, Rice DW. Conformational flexibility in glutamate dehydrogenase. Role of water in substrate recognition and catalysis. J Mol Biol 1993;234:1131–9.
[19] Thykaer J, Rueksomtawin K, Noorman H, Nielsen J. NADPH-dependent glutamate dehydrogenase in *Penicillium chrysogenum* is involved in regulation of beta-lactam production. Microbiology 2008;154:1242–50.
[20] Rodriguez-Navarro S, Llorente B, Rodriguez-Manzaneque MT, Ramne A, Uber G, Marchesan D, et al. Functional analysis of yeast gene families involved in metabolism of vitamins B1 and B6. Yeast 2002;19:1261–76.
[21] Yuga M, Gomi K, Klionsky DJ, Shintani T. Aspartyl aminopeptidase is imported from the cytoplasm to the vacuole by selective autophagy in *Saccharomyces cerevisiae*. J Biol Chem 2011;286:13704–13.
[22] Jami M-S, García-Estrada C, Barreiro C, Cuadrado A-A, Salehi-Najafabadi Z, Martín J-F. The *Penicillium chrysogenum* extracellular proteome. Conversion from a food-rotting strain to a versatile cell factory for white biotechnology. Mol Cell Proteomics 2010;9:2729–44.
[23] Martín JF, Ullán RV, García-Estrada C. Regulation and compartmentalization of β-lactam biosynthesis. Microb Biotechnol 2010;3:285–99.
[24] Posch AE, Herwig C, Spadiut O. Science-based bioprocess design for filamentous fungi. Trends Biotechnol 2013;31:37–44.