Molecular Analysis and Improvement of Protein Production by *Aspergillus oryzae* Grown on Solid Substrates

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Microbial Functionality and Safety

The research program of Microbial Functionality and Safety is directed to understanding, improving and predicting the activity of microorganisms in bioreactors, food products and the gastro-intestinal tract.

Project Solid State Food Fermentations

WCFS researchers at Wageningen University and Research Centre have been studying the process of solid state fermentation at different levels: from the genome and transcriptome level to the level of large-scale fermenters. The research has been focused on two main areas. The first has been the development of non-empirical, model-based methods for designing and operating solid state fermentation processes. The other research focus was on physiological and molecular genetic aspects of the fungal response to specific conditions in the solid state fermentation process, such as temporal and spatial gradients in nutrient, oxygen and water availability. In these studies, *Aspergillus oryzae* has been used as the model organism with wheat and soybean as model substrates, and amylases and proteases as model products.

Contents

CHAPTER 1

General introduction

Introduction to this thesis

Filamentous fungi are able to grow on solid substrates like wheat and rice kernels. Conversion of complex carbohydrate biopolymers (e.g. starch, celluloses) and biomolecules (e.g. proteins) into oligo- and monomeric nutrients is accomplished by secretion of a wide variety of enzymes. The research on industrially relevant enzymes that are produced by filamentous fungi during growth on solid substrates has recently gained increasing attention. This thesis contributes to the basic knowledge of molecular biological mechanisms associated to the production of proteins by the filamentous fungus *Aspergillus oryzae* during growth on solid substrates and describes approaches for improvement of protein production. Hence, this introduction provides a historic overview of fungal solid state fermentation with specific attention for recent studies on protein secretion by and morphology of filamentous fungi. In addition a short outline of this thesis is given.

Mycotechnology: fungi in biotechnology

Filamentous fungi have been used for centuries in diverse biotechnological processes. This so-called mycotechnology (Bennett 1998) is traditionally used in relation to food production. The fermentation technology in Europe involved *Aspergillus* species for the fermentation of cacao beans and *Penicillium* species for the preparation of meat sausages (e.g. salami) and cheeses (e.g. roquefort and camembert). *A. oryzae* and *A. sojae* are being used for centuries for the preparation of *koji* in the Japanese traditional fermentation industry. *Koji* is used as a starter for the production of *sake* (rice wine), *shoyu* (soy sauce), *miso* (soybean paste) and *shochu* (distilled spirits) (Machida 2002, Kitamo 2002). The industrial application of filamentous fungi has expanded rapidly last century since the discovery that *A. niger* was an excellent citric acid producer in submerged fermentation (Wainwright 1992, Grewal and Kalra 1995). Also other fungal products have contributed to the rapid expansion of filamentous fungal applications; excellent examples of this are the antibiotic penicillin and purified enzymes.

Amylase from *A. oryzae* was the first microbial enzyme used in industrial processes (Takamine 1894, Bennett 1998). Takamine grew *A. oryzae* cultures in thin layers on steamed rice and wheat bran and suggested new uses for the partially purified fungal diastase as a substitute for malting enzyme, and as a digestive aid for the treatment of dyspepsia (Takamine 1894, Takamine 1914, Bennett 1998). Also other filamentous fungi have been explioted because of their enormous potential to secrete a variety of proteins (van den Hondel et al. 1992, Punt et al. 1994, Pandey et al. 1999). Currently, a wide range of industries use (partly) purified filamentous fungal proteins and these include proteases, cellulases, dextranases, galactosidases,

glucoamylases, glucose oxidases, xylanases, lipases, invertases, pectinases, tannases, catalases, asparaginases, hesperidinases, naringinases, phytases (Bennett 1998, Pandey et al. 1999).

In the last decades, the development of recombinant DNA technology has enabled the construction of filamentous fungal strains for the production of heterologous proteins (Jeenes et al. 1991, Conesa et al. 2001, Ishida et al. 2001, Machida 2002, Joosten et al. 2003). In parallel to this development, the research on protein secretion by *Aspergillus* species has focussed on (post-)transcriptional regulation of protein production, the transit of proteins through the secretion pathway and the structure of the proteins produced (reviewed in Archer and Pederby 1997 and Conesa et al. 2001). In the past three years the sequencing of the genomes of *A. niger* and *A. oryzae* has been completed (reviewed by Archer and Dyer 2004, Machida 2002). The available genome sequence enables identification of genes and functionally important regions of the genome. It has directed the research focus towards development of genome-wide analysis methods for exploiting that information (Archer and Dyer 2004, Hofmann et al. 2003, Maeda et al. 2004), also in relation to fungal product formation and protein secretion (Archer and Dyer 2004, Askenazi et al. 2003).

Secretion of proteins and growth phenotype of filamentous fungi

In its natural environment *A. niger* is involved in degradation of plant cell material by producing highly specialised enzymes like pectinases, (hemi)cellulases, and xylanases (de Vries and Visser 2001, de Vries et al. 2002, de Vries 2003). Enzymes that are produced by filamentous fungi are transported to the exterior of the cell via the secretion pathway. Compared to the knowledge of other eukaryotic

organisms, little is known about the filamentous fungal secretion pathway through which secretory proteins are transported to the exterior of the cell (reviewed by Conesa et al. 2001, Archer and Peberdy 1997, Punt et al. 1994). However, it is generally accepted that the secretion pathway is similar to those of yeasts and higher eukaryotes (Mior and Mao 1990, Conesa et al. 2001). Therefore, in the secretion pathway, posttranslational modifications such as formation of disulfide bond, glycosylation, and folding of the amino acid chain, are expected to involve similar protein activities in all eukaryotic organisms.

Filamentous fungi have a better capacity to secrete proteins compared to *Saccharomyces cerevisiae*. A yield of 30 g/l of extracellular protein can be obtained with certain *Aspergillus* and *Trichoderma* strains (Durand et al. 1988, Finkelstein et al. 1989). In contrast, yields in the grams-per-liter range are only obtained with a few yeast strains (*Pichia, Hansenula*) used for extracellular protein production (Werten et al. 1999, Wyss et al. 1999). Filamentous fungi grow by extension of hyphal tips (Trinci 1974, Prosser 1994, Dynesen and Nielsen 2003) involving the actin cytoskeleton, signal transduction pathways, cell wall formation, membrane biosynthesis and the secretion pathway (Trinci 1978, Seiler and Plamann 2003, Shi et al. 2004). Particularly, this hyphal growth phenotype of filamentous fungi, which is not found in either *S. cerevisiae* or in higher eukaryotic organisms, may contribute to the higher protein secretion capacity of filamentous fungi compared to *S. cerevisiae*.

Recently, the morphological growth phenotype of *A. oryzae* has been suggested to affect protein secretion capacities during growth on a solid substrate (te Biesebeke et al. 2004c and 2004d). Different studies have observed a difference in product formation during filamentous fungal growth on solid substrates and in liquid substrates. In most of these studies differential enzyme activities were measured, not

only for the amylase, glucoamylase and protease activities (Pandey et al. 1999, Machida 2002, te Biesebeke et al. 2004a), but also for enzyme activities of feruloyl esterase (Asther et al. 2002), ferulate esterase (Asther et al. 2002), beta-fructofuranosidase (Ashokkumar and Gunasekaran 2002), pectinesterase (Maldonando and Stasser de Saad 1998), polygalacturonase (Maldonando and Stasser de Saad 1998), beta-fructofuranosidase (Ashokkumar et al. 2001), pectinase (Solis-Pereyra et al. 1993, Solis-Pereyra et al. 1996), tannase (Aguilar et al. 2001) and exopectinase (Diaz-Godinez et al. 2001). In most studies, production of enzymes was more efficient during solid state fermentation. Different mechanisms have been proposed for the observed difference in protein productivity during submerged growth and growth on a solid substrate. It is suggested that during filamentous fungal growth on a solid substrate, catabolite repression is less pronounced than during submerged fermentation (Solis-Pereyra et al. 1993 and 1996, Ashokkumar and Gunasekaran 2002, Bakri et al. 2003, Kamra and Satyanarayana 2004, Holker et al. 2004). Other studies suggest a difference in biomass development (Diaz-Godinez et al. 2001) and also a lower protease activity has been shown to correlate to high enzyme productivity (Aguilar et al. 2001, Diaz-Godinez et al. 2001, Aguilar et al. 2002). Although most of these studies concerned *A. niger, A. oryzae* is a good model-organism to study the relation between cellular morphology and protein production due to its pronounced filamentous fungal growth phenotype (te Biesebeke et al. 2002, Kitamo 2002, Machida 2002).

Fungal cellular morphology

The cellular morphology of filamentous fungi is the result of growth by the extension of existing hyphae and the formation of branches along the existing hyphae

(Trinci 1974, Prosser 1994, Dynesen and Nielsen 2003). The process of filamentous fungal growth is controlled by molecular mechanisms involving many gene products (Seiler and Plamann 2003, Pruyne and Bretscher 2000a, 2004b). In *Neurospora crassa* it has been shown that secretory vesicles utilize the microtubule cytoskeleton as a structural basis for transport (Steinberg and Schliwa 1993) and it is suggested that also the mechanism that drives vesicular transport is conserved in most eukaryotic organisms (Seiler et al. 1997, 1999). The secretory vesicles have, besides their role in transport of secretory proteins, also a role in transport of proteins that maintain cellular morphology (cell wall and membrane assembly, cell signalling). An extraordinary feature of filamentous fungi is a specialised organelle located at the hyphal apex called the *spitzenkorper* (Brunswik 1924) that has been proposed to serve as a vesicle supply center (Girbardt 1969, Bartnicki-Garcia et al 1989). Besides its role in protein secretion, the microtubule-dependent movement and positioning of vesicles of the *spitzenkorper* are essential for generation of shape of the hyphae (Trinci 1974, Riquelme et al. 1998, Bartnicki-Garcia et al 1995, Reynaga-Pena et al. 1997). A schematic drawing that illustrates the relation between protein secretion and cellular morphogenesis of filamentous fungi is presented in Fig. 1.

A requirement for a proper development of every organism is the determination and maintenance of cellular polarity (Seiler and Plamann 2003). In budding yeast, the cytoskeleton (actin, fimbrin and tropomyosin) determines the direction of cell growth that is directed (polarised) to regulatory and cytoskeletal proteins (Pruyne and Bretscher 2000a, 2004b). Although filamentous fungi differ from budding yeast in polarity establishment and maintenance, some of the known proteins from yeast involved in bud emergence are required for filamentous fungal germ tube emergence (Momany 2002). During polarity establishment and early

maintenance of polarity, several key proteins play similar function in yeast and filamentous fungi like Rho GTPases (Cdc42, Rho1, Rho3), septins and Bni1 formins (Momany 2002, Seiler and Plamann 2003, Guest et al. 2004). Germ tube elongation and formation of sub-apical branching hyphae involve the forward movement of the *spitzenkorper* in filamentous fungi (Reynaga-Pena 1997). However, the identity and function of most proteins that are involved in this process are still largely unknown (Momany 2002). It can be concluded that, although filamentous fungi are used since decades for the production of proteins, basic knowledge about cellular morphogenesis of filamentous fungi, that appears to be associated to the secretion pathway, is limited.

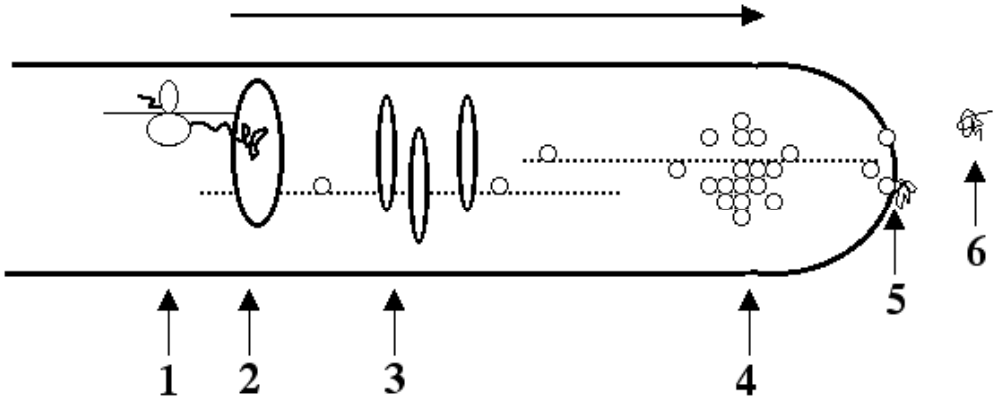


Fig. 1 *Schematic drawing of the filamentous fungal hyphal tip illustrating the relation between protein secretion and cellular morphogenesis. The horizontal arrow indicates the direction of hyphal growth. Secretory proteins are produced by the ribosomal complex (1) and enter the endoplasmic reticulum (ER) where protein folding takes place (2). Secretory proteins might be post-translationaly modified in the ER or in the Golgi-complex (3). Secretory vesicles (circles) originating from the Golgi-complex are transported to be incorporated into the spitzenkorper (4) and then fuse to the cell membrane (5) releasing secretory proteins to the exterior of the growing hyphal tip (6). The fused vesicles become part of the cell membrane. The dotted lines illustrate the actin cytoskeleton.*

Solid state fermentation

The process of cultivating of filamentous fungi on a moist solid substrate in the absence of free-flowing water is called solid state fermentation. The work that is presented in this thesis has been part of a larger research project that aims to determine the fungal response to specific conditions during solid state fermentation process to optimise this industrial process. The project consisted of different subprojects that all have a different focus ranging from fungal molecular genetic and physiological aspects (te Biesebeke et al. 2002) towards developing non-empirical engineering methods for development of solid state fermentation methods (Schutyser 2003). *A. oryzae* was chosen as a model organism in the project for it is known for its excellent potential to grow on a solid substrate. Wheat and rice kernels were used as model substrates. The choice for these substrates has been based on the fact that they are also used in industrial solid state fermentation. Amylases and proteases have been used as examples of hydrolytic enzymes, because they are indispensable for growth of *A.oryzae* on polymeric substrates. Moreover, these enzymes are also of significant importance to the food and feed industry.

Outline of this thesis

The general aim of this thesis was to provide basic knowledge about gene expression, cellular morphology and protein production of filamentous fungi grown on solid substrates. This knowledge was also used to improve protein production of filamentous fungi and to improve the solid state fermentation process. To achieve these goals we used a molecular genetic approach including genomics-based technologies. Chapter 2 describes the general issues that are important during growth of *A. oryzae* in the solid state fermentation process compared to growth in a liquid

medium. Chapter 3 describes the growth of *A. oryzae* on wheat kernels and compares it to growth on rice kernels. Moreover, different proteins that were secreted by *A. oryzae* during growth on the wheat kernel were identified. Several of these proteins were found to be associated to penetration and conversion of the substrate. Chapter 4 describes the control mechanisms that regulate the transcription of 3 genes encoding the hydrolytic enzymes glucoamylase B, alkaline protease A and neutral protease B during growth on a solid surface in contrast to growth in a liquid medium. Besides our interest in control mechanisms involved in the differential regulation of these hydrolytic enzymes, we were interested in other classes of genes that were differentially expressed during growth of *A. oryzae* compared to growth in a liquid medium. Chapter 5 shows that most of the differentially expressed genes encode proteins involved in polarized growth and morphology. To improve the fungal solid state fermentation process, we decided to construct strains with an altered morphology. These hyper-branching strains were found to have a higher production of hydrolytic enzymes (Chapter 6). Besides morphology, an important aspect of growth of *A. oryzae* on a solid substrate is oxygen supply that is often limited at the level of the penetrative hyphae. In Chapter 7 we have focussed on a newly identified class of filamentous fungal flavohemoglobins. The expression of an *A. oryzae* flavohemoglobin appeared to be correlated to polarised hyphal growth. The intracellular production of the hemoglobin domain derived from a fungal flavohemoglobin gene could improve biomass formation, growth rate and enzyme production of *A. oryzae* (Chapter 8) during growth on a solid substrate. Fig. 2 illustrates the strategies followed to study protein production of *A. oryzae*. Chapter 9 provides a summary and discussion of the major results of this thesis.

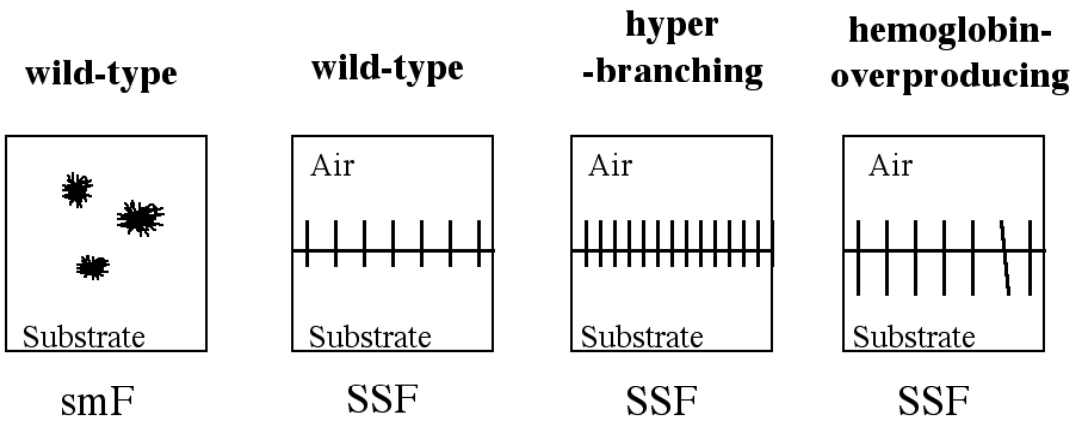


Fig. 2. *Schematic representation of strategies followed to study protein production of Aspergillus oryzae. A. oryzae wild-type grown in submerged fermentation (smF) was compared to solid state fermentation (SSF). Moreover, hyperbranching and hemoglobin-overproducing strains were constructed and compared to the wild-type grown in SSF. The vertical lines represent fungal hyphae.*

CHAPTER 2

Aspergillus oryzae in solid state and submerged fermentations

R te Biesebeke, GJ Ruijter, YSP Rahardjo, MJ Hoogschagen, M Heerikhuisen, A Levin, KGA van Driel, MAI Schutyser, J Dijksterhuis, Y Zhu, FJ Weber, WM de Vos, CAMJJ van den Hondel, A Rinzema, PJ Punt (2002) *Aspergillus oryzae* in solid state and submerged fermentations. Adapted from FEMS Yeast Res 2:245-248.

Abstract

Aspergillus oryzae grown in submerged and solid state fermentations revealed a number of differences in molecular and physiological aspects that are correlated with the different growth conditions. First, the aerial hyphae that occur only in solid state fermentation are mainly responsible for oxygen uptake. Second, solid state fermentation is characterised by gradients in temperature, water activity and nutrient concentration, and inside the hyphae different polyols are accumulating. Third, pellet growth in submerged and mycelial growth in solid state fermentations show different gene expression and protein secretion patterns. With this integrated approach we aim to expand our basic knowledge of mechanisms of growth of filamentous fungi on solid substrates and to exploit the biotechnological applications.

Introduction

Solid state fermentations (SSF) are applied in many traditional food fermentation processes and offer possibilities for improved production of novel as well as existing food products and ingredients (Pandey et al. 2000). In recent developments, SSF is used to produce high yield quantities of pure enzymes. In SSF these enzymes, are much more efficiently produced compared to submerged fermentations (smF) (Pandey et al. 1999, Machida 2002, Holker et al. 2004).

Filamentous fungi play a key role in SSF for their hyphal development allows them to effectively colonise and penetrate the solid substrate (Pandey et al. 2000, Machida 2002). As a consequence of hyphal growth the fungus is confronted with gradients in concentration of substrates and enzymes, the presence of a substrate-air interface and gradients in water and temperature (Hata et al. 1998). Water is frequently a limiting factor for fungal growth in SSF and heat removal is one of the

major problems in large-scale SSF. These problems do not occur with smF of fungi. In smF, water is abundantly present and gradients in temperature, oxygen concentration and nutrients are absent. The focus of SSF research has been primarily on process and fermenter design where the organism involved is regarded as a black box (Weber et al. 1999). The molecular mechanisms underlying the behaviour of fungi in SSF are not described in detail. *A. oryzae* is an obligate aerobic filamentous fungus frequently used in SSF processes. We have initiated a multi-disciplinary study on molecular mechanisms of *A. oryzae* SSF with wheat kernels to understand the differences observed between SSF and smF and to improve the application of fungi in biotechnological processes.

Filamentous fungal growth on a solid substrate

Wheat kernels are an excellent substrate for use in mixed solid state bioreactors (Nagel et al. 2001). A drawback of using such a complex substrate is that its composition may vary and its pre-treatment may affect the fermentation. An important goal of pre-treatment is to give the wheat kernel an increased amount of water content (Nagel et al. 2001). From our research it is shown that most wheat kernel types showed no difference in respiration pattern (Hoogschagen et al. 2002). However, the pre-treatment may affect water availability for some wheat kernel types, which warrants further studies (Hoogschagen et al. 2002).

Temperature control is one of the main problems in large-scale SSF (Weber et al. 1999, Nagel et al. 2001, Weber et al. 2002), so it is important to know how temperature affects the filamentous fungus. The heat production of the filamentous fungus is proportional to its respiration rate (Weber et al. 1999). Therefore, we measured the respiration rate of *A. oryzae* grown on wheat kernels at different

temperatures. Based on this it is clear that there is a temperature optimum between 30 and 35°C. The oxygen uptake rate (OUR) is a reflection of metabolic activity and also related to heat production. Therefore, a better understanding of oxygen consumption in filamentous fungal SSF might be useful to deal with heat removal problems encountered in SSF. In a conceptual model, we distinguish four layers in the mycelium (Fig. 1). For *Rhizopus oligosporus* and *Coniothyrium minitans*, we found that the OUR during SSF is limited by diffusion of oxygen in the layer of densely packed fungal hyphae (Oostra et al. 2001). For *A. oryzae*, however, we found that the aerial hyphae contribute up to 75% to the OUR. This was demonstrated by suppressing their formation by covering colonies of *A. oryzae* with a gas-permeable polycarbonate membrane (Rahardjo et al. 2001). Measurements with micro-electrodes in the layer of aerial hyphae (1 in Fig. 1) showed no decrease in oxygen concentration, i.e. no diffusion limitation. From these results, it can be concluded that cooling problems in SSF with *A. oryzae* or other aerial mycelium-producing filamentous fungi will be more pronounced than in fermentations with fungi that produce little aerial mycelium, such as *C. minitans*.

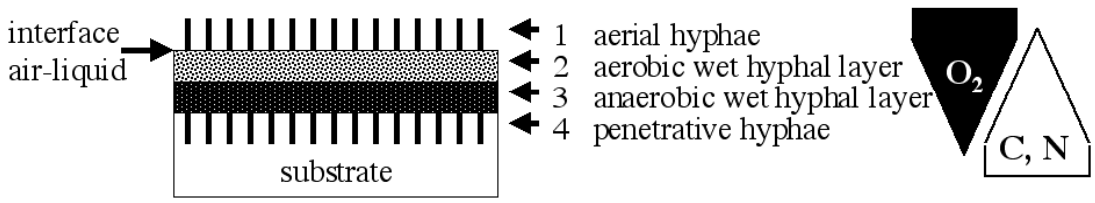


Fig. 1. *Conceptual representation of filamentous fungal growth on a solid substrate. The penetrative hyphae and part of the anaerobic wet hyphal layer are in direct contact with the substrate that serves as a carbon (C) and nitrogen (N) source. The aerial hyphae are mainly responsible for oxygen (O_2) uptake. The polygonal shapes illustrate the gradients in oxygen and substrate concentrations.*

Substrate hydrolysis and product formation

Upon penetration of the wheat kernel the fungal hyphae will first come across a variety of polysaccharides in the kernel coat. The core of the kernel consists largely of starch and hydrolysis by fungal amylases results in release of glucose, which is used as the main carbon and energy source. Accumulation of malto-oligosaccharides is investigated to address the question if these compounds play a role in induction of amylases. Currently, it is believed that growth in the substrate particle is limited by availability of oxygen (Rajagopalan and Modak 1995) and that amylase activity is sufficiently high to hydrolyse starch at a rate that is faster than the rate of utilisation by the fungus. Our data using *A. oryzae* growing on wheat dough are in agreement with this, although glucose accumulation in the substrate varied strongly with culture conditions. For example, the glucose content of wheat dough with an initial water content of 1 kg/kg dry matter increased from an initial 2 g/kg substrate to 2.7 g/kg substrate after 48 h of cultivation. On the other hand, in dough with 0.7 kg water per kg dry matter glucose increased from 2.3 to 16 g/kg substrate in 48 h, which may be related to slower growth and/or higher amylase induction (see later).

In the solid state fermentation process, *A. oryzae* encounters nutrient gradients. Oxygen is mainly or only available at the outside of the kernel, whereas carbon substrates are present only inside the kernel. Since aerial hyphae are not in direct contact with the substrate, they should be provided with a carbon and energy source by transport of one or different carbon compounds through the hyphae. Mannitol is found at a very high concentration in mycelium growing on wheat kernels (Ruijter et al. 2004) and would be a good candidate for a carbon compound that is transported through the hyphae. Polyhydroxy compounds (polyols), such as glycerol, mannitol, erytritol and arabitol, can be accumulated in filamentous fungi to a high

concentration. The composition of the polyol pool is dependent on the growth condition (e.g. in Witteveen and Visser 1995) and polyols therefore apparently play a role in the adaptation of cells to the environmental conditions. One such condition, low water activity (Aw), is usually encountered in SSF and provokes filamentous fungi to accumulate polyols. Glycerol is often reported as the predominant polyol accumulated at low Aw (Blomberg and Adler 1992), but further studies indicate that *A. oryzae* accumulates a mixture of glycerol, erythritol and arabitol at low Aw in SSF (Ruijter et al. 2004).

Since polyols are also secreted to some extent by the mycelium, they form one class of products of fungal metabolism in SSF. Another class of metabolites are organic acids. *A. oryzae* is not a strong acid producer, but a low quantity of citric acid (1g/kg substrate) and traces of oxalic acid were found in wheat dough after 72 h cultivation with *A. oryzae*.

Also production of flavour compounds is characteristic for SSF (reviewed by Feron et al. 1996) and may be related to the limited availability of oxygen inside the kernels resulting in production of 'fermentation' products such as alcohols, aldehyde and keton compounds. Research on metabolism and microbial physiology will be necessary to establish the potential of SSF in production of these biotechnological interesting volatile metabolites.

Protein secretion and gene expression

Our first objective was to identify proteins that are secreted in solid state fermentation of *A. oryzae*. In a first approach we identified secreted proteins in extracts of *A. oryzae* grown on solid substrates and compared this to proteins identified in the growth medium of *A. oryzae* grown in wheat based liquid medium (te

Biesebeke et al. 2004b). In a SDS-PAGE analysis it is shown that the pattern of secreted proteins during wheat kernel cultivation is clearly different from smF. The identity of a few proteins was determined by N-terminal sequence analysis after isolation of protein bands from SDS-PAGE gels. A 50 kD protein from both the smF and SSF SDS-PAGE gel was identified as α-amylase. Three SSF-specific proteins of 35, 28 and 20 kD were isolated from the SSF SDS-PAGE gel and the 28 kD protein could be identified as neutral protease II. We are currently identifying other proteins specifically secreted in wheat kernel SSF by *A. oryzae*.

For a detailed analysis of secretion and localisation of gene expression of amylases and proteases, various molecular genetic tools such as a gene transfer system (de Ruiter-Jacobs et al. 1989) and GFP based reporter proteins (Tsien 1998) have been developed for *A. oryzae*.

Recently, it was shown that the glucoamylase B (*glaB*) is highly induced during rice-koji making solid state fermentation, but not in standard liquid cultivation Hata et al. 1998, Ishida et al. 1998). Expression of *glaB* was also induced by starch or malto-oligosacharides in surface cultures (Hata et al. 1998), but the expression levels obtained were lower than the expression levels found in rice-koji making solid state fermentations. In a further study Ishida et al. (2000) showed that motifs in the promotor region of *glaB* were responsible for starch, low-Aw and high temperature induction. This result suggests that the physical factors that are supposed to be typical for rice-koji making solid state fermentation give rise to the high-level *glaB* induction.

To analyse whether high-level gene expression appears when *A. oryzae* grows on wheat kernels, northern analysis was performed with probes for the *enoA*, *brlA*, *glaB* and *amyA* genes (Fig. 2) and compared to growth of *A. oryzae* in submerged fermentation. The *enoA* probe hybridised equally well under both conditions and was

used as reference for metabolic activity of the fungus. The *amyA* gene, encoding α-amylase was transcribed in both cultivation conditions. The zinc finger transcription factor encoded by the *brlA* gene induces sporulation in *Aspergillus nidulans* (Timberlake 1991) and the induction of sporulation is regulated at the level of the *brlA* gene transcription (Miller 1993, Skromme et al. 1995). The northern analysis suggests that besides the *brlA gene*, also the glucoamylase B encoding gene (*glaB)* is induced only during growth of *A. oryzae* on wheat kernels (Fig. 2). These results support the hypothesis that different control mechanisms are involved in regulation of gene transcription in *A. oryzae* in solid state and submerged fermentation.

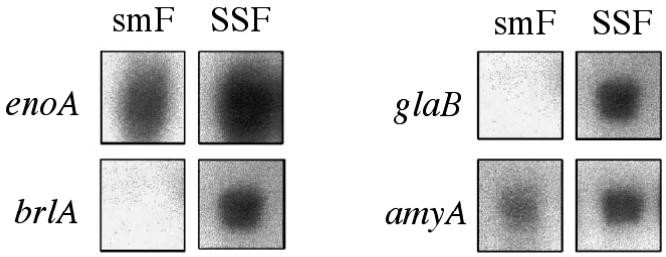


Fig. 2. *Northern analysis of A. oryzae grown in smF and SSF with probes for the enolase (enoA), the zinc-finger transcription factor (brlA), the glucoamylase B (glaB) and α-amylase (amyA) encoding genes. A. oryzae was grown for 5 days on wheat kernels (SSF) and for 48 hours in 1% ground wheat kernels containing liquid medium (smF).*

Conclusion

Many studies on solid state fermentation have focussed on process and fermenter design while the organism has been considered as a black box. The first experimental results with the model organism *A. oryzae* are described during surface growth on wheat kernels and a comparison is made with submerged growth conditions. During SSF the fungus displays a characteristic growth phenotype and has

distinct physiological and molecular characteristics. Physiological studies show that oxygen uptake is mainly performed by the aerial hyphae and that different polyols, potential intracellular carbon source transporters, are accumulated inside the hyphae of the fungus during SSF. Our molecular biological studies with *A. oryzae* show that different proteins are produced and genes are differentially transcribed in submerged and solid state fermentation. These results suggest that gene transcription is regulated by different control mechanisms in *A. oryzae* in solid state and submerged fermentation. With these approaches we aim to elucidate the mechanisms, which lie behind the differences in SSF and SmF and aim to explore the biotechnological application.

CHAPTER 3

Identification of secreted proteins of *Aspergillus oryzae* associated with conversion and penetration of solid cereal substrates

R te Biesebeke, A Boussier, N van Biezen, CAMJJ van den Hondel, PJ Punt (2004) Identification of secreted proteins of *Aspergillus oryzae* associated with conversion and penetration of solid cereal substrates, *submitted for publication.*

ABSTRACT

Filamentous growth of *Aspergillus oryzae* on solid cereal substrates involves secretion of carbohydrate converting enzymes and a substrate specific polarised hyphal growth phenotype. To identify proteins produced under these specific conditions the extracts of *A. oryzae* on cereal substrates were analysed. *A. oryzae* grown on wheat kernels (WK) and rice kernels (RK) showed clearly different growth phenotypes in terms of colonisation, sporulation and aerial hyphae. Also the secreted enzyme concentrations largely differed. These results suggest that conversion of the RK by *A. oryzae* is distinct from conversion of the WK involving different growth phenotypes on both substrates. Zymogram analysis showed that extracts of *A. oryzae* grown on different cereal substrates contain at least 1 active amylase and at least 2 active proteases. Transcription analysis and N-terminal sequence analysis of SDS-PAGE separated proteins indicate that the amylase activities present in extracts of *A. oryzae* grown on WK is derived from α-amylase. One protease was identified as neutral protease II by N-terminal protein sequencing of SDS-PAGE separated proteins. Also transcription analysis of the *nptB* gene encoding neutral protease II confirmed expression of this gene in extracts of *A. oryzae* grown on WK. Further analysis was carried out on the various secreted proteins in extracts of *A. oryzae* grown in both liquid and solid wheat-based medium using N-terminal sequence analysis. In a submerged wheat-based growth medium of *A. oryzae*, besides the α-amylase, also an arabinosidase and xylanase were abundantly produced. In the extracts of *A. oryzae* grown on wheat-based solid substrate besides α-amylase and chitinase, two new proteins of 16 kDa and 27 kDa were identified. All except one of the identified proteins are secreted proteins as suggested on the basis of the presence

of N-terminal signal peptides and pro-sequences in sequences of database entries corresponding to these identified protein sequences. The 16 kDa and 27 kDa hypothetical proteins showed only close homologies to filamentous fungal proteins suggesting filamentous fungal-specific functions.

INTRODUCTION

In their natural environment, filamentous fungi show a polarised hyphal growth phenotype and are known for their secretion of hydrolysing and oxidising enzymes to degrade a wide variety of organic compounds. The major natural substrate consisting of plant cell material is converted by specialised enzymes like pectinases, (hemi)cellulases and xylanases secreted by *A. niger* (de Vries and Visser 2001). The high protein secretion capacity of the so-called koji-molds *Aspergillus oryzae* and *Aspergillus sojae* has been used for more then 1000 years in the traditional Japanese fermentation industry to produce *sake* (rice wine), *shochu* (spirits), *shoyu* (soy sauce) and *miso* (soy bean paste) (Kitamoto 2002, Machida 2002). In other more recently originating applications, the previously mentioned *Aspergillus* species are used as cell factories for the production of homologous and heterologous proteins (Conesa et al. 2001, Archer and Peberdy 1997, Kitamoto 2002, Punt et al. 1994).

Different host vector systems for efficient protein production have been developed and the majority of the industrially proteins secreted by filamentous fungi are produced in large-scale submerged fermentations (smF). An *A. oryzae* transformant with an introduced fusion construct containing the *melO* promoter fused to a glucoamylase encoding gene (*glaB*) produced the glucoamylase B protein up to 3.3 g/l (Ishida et al. 2001) while yields of up to 30 g/l can be obtained in the fermentation of certain *Aspergillus* and *Trichoderma* strains (Conesa et al. 2001).

Although high levels of proteins can be obtained in submerged fermentation with *A. oryzae,* production of proteins by filamentous fungi grown on solid substrates might be advantageous (Pandey et al. 1999, Iwashita et al. 2002, Machida 2002, Holker et al. 2004, te Biesebeke et al. 2002). However, until now, research has mostly focussed on hydrolytic activities of proteins present in extracts of filamentous fungi grown on solid substrates that have direct application in biotechnological processes neglecting the identity of proteins involved.

The availability of the substrate is of considerable importance for filamentous fungal growth on solid substrates. Most substrates used for the production of enzyme activities, are agro-industrial waste products like rice bran, sugar cane bagasse, and wheat bran (Pandey et al. 1999). When inoculated on wheat kernels (WK) one of the first responses of the *A. oryzae* conidiospore is the production of enzymes that enable conversion of the complex polysaccharide kernel wall (te Biesebeke et al. 2002). Emerging hyphal tips penetrate the substrate, and extend to the starch containing WK core. The growth process involves the ongoing formation of novel pionts of growth along the extending hyphae. This will result, not only in the formation of penetrative hyphae, but also in other specialised features like aerial hyphae involved in oxygen uptake (Rahardjo et al. 2001) and surface growing hyphae ultimately leading to colonisation of *A. oryzae* on WK. While the intracellular mechanisms involved in morphogenesis of filamentous fungi is receiving considerable attention (Wendland and Philippsen 2001, Seiler and Plamann 2003) little is known about the involvement of extracellular enzymes in the growth phenotypes of filamentous fungi. The only well-studied filamentous fungal extracellular proteins involved in development of growth phenotype of filamentous fungi grown on a solid substrate are hydrophobins

that are involved in formation of aerial structures, the attachment of hyphae to hydrophobic surfaces and in cell-wall assembly (Wösten 2001).

Due to the complexity of the polysaccharide wall, WK might be an excellent substrate to identify novel carbohydrate hydrolysing enzymes produced by *A. oryzae*. Moreover, also proteins related to the specific growth phenotype may be identified. Therefore, research was started to identify extracellular proteins of *A. oryzae* grown on WK.

MATERIALS AND METHODS

Strains and media. *A. oryzae* ATCC strain 16868 was used throughout this study. Solid state fermentation was performed with rice kernel (RK) (TOKO, Arnhem, the Netherlands), Ritmo WK (ACM, Meppel, the Netherlands) and ground WK. Before inoculation with spores of *A. oryzae* the substrates were pre-treated by mixing substrate (50 g) with H_2O (100 ml) and soaking at 50ºC for 2 h. The excess of water was removed and the substrates were autoclaved at 120ºC for 1 h. Substrates were inoculated with 10^6 conidia/g substrate and incubated up to 6 days and in case of growth on WK up to 9 days at 30°C at 98% water/air (v/v) content in an incubator (VEA-Instruments, Houten, the Netherlands).

Wheat-based medium was prepared with ground WK (te Biesebeke et al. 2004b) by suspending ground WK (5 g) in H_2O (100 ml) resulting in 5% wheat-based liquid medium (5% WLM) that was sterilised for 15 min at 120ºC. Submerged growth was performed in a rotary shaker at 250 rpm at 30ºC after inoculation of 50 ml 5% WLM in 250 ml shake flasks with 10^6 conidia/ml. Surface growth on 5% WSM was performed on 1% agar plates of 5% WLM (25 ml) at 30°C at 98% water/air (v/v)

content in an incubator (VEA-Instruments, Houten, the Netherlands) after inoculating the surface of the 5% WSM growth medium with $2.5X10^7$ spores.

Preparation of extracellular protein samples from *A. oryzae*. The extracts of *A. oryzae* grown on cereal substrates were prepared from biomass (5 g) of *A. oryzae* grown on a cereal substrate that was collected in a 50 ml falcon tube and stored at –20°C. The secreted proteins were extracted basically as described (Aikat and Bhattacharyya 2000). The sample was thawed and after the addition of 25 ml mQ water and mixing on a vortex at maximum speed at 4°C for 12 min the falcon tubes were centrifuged for 5 min at 3500 rpm at 4°C and the supernatants were used for protein activity measurements and zymogram analysis. The extracts of *A. oryzae* grown on 5% WSM were made after careful removal of the filamentous fungal mycelium and freezing of a 5 g substrate sample of the 1% agar plates of 5% WLM. Subsequently the 5 g sample was thawed and after the addition of 25 ml mQ water vortexed as described for the extracts made of *A. oryzae* grown on cereal substrates, centrifuged for 5 min at 3500 rpm and used for identification of extracellular proteins. The growth medium of *A. oryzae* grown in 5% WLM was used for identification of extracellular proteins after centrifugation for 5 min at 3000 rpm. When indicated the extracts or the growth medium were concentrated using Centricon concentrators (10 kDa limit; Millipore, Bedford, Mass.).

Protease activity measurements. The protease activity was measured according to a modified procedure as described by Holm (1980). As a substrate N,N-dimethylcaseine (Sigma, C 9801) was used. 2 µl sample + 13 µl water was mixed with 75 µl reagent (5 g/l N,N-dimethylcaseine in 0.1 M K_2HPO_4 (pH 7)) and incubated at 37°C for 17.5 min. The reaction was stopped by addition of 185 µl 0.1 M $Na_2B_4O_7.10H_2O$/ 4 mM Na_2SO_3 (pH 9.3) and 5 µl starter 2.5% TNBS (2,4,6,-

Trinitrobenzene Sulfonic Acid, Pierce #28997). The absorption at 405 nm was measured after 200 s. A glycine delution range was used as a standard. The procedure was fully automated using a Cobas Mira Plus Autoanalyser (Roche). One unit of protease activity was defined as the amount of enzyme needed to produce 1 μmol of amino acids per min at 37°C at the indicated pH.

Amylase activity measurements. The α-amylase activity was determined in the extracts according to the Megazyme (Wicklow, Ireland) α-amylase assay procedure (Ceralpha method with ICC standard No. 303) using non-reducing-end blocked p-nitrophenyl maltoheptaoside (BPNPG7) as a substrate to avoid hydrolysis by exo-enzymes such as β-amylase, amyloglucosidase and α-glucosidase. One unit of α-amylase activity is defined as the amount of enzyme needed to liberate 1 μmol of p-nitrophenol per min at 37°C at pH 5.5.

Glucoamylase activity measurements. The glucoamylase activity was determined using p-Nitrophenyl-maltoside (Megazyme, Wicklow Ireland) according to the manufacturers amyloglucosidase assay (RAMGR3 11/99). One unit of glucoamylase activity is defined as the amount of enzyme needed to produce 1 μmol of p-nitrophenol per min at 37°C at pH 4.5.

Analysis of glucose and amino acid concentrations. Samples used for determination of glucose and amino acid concentrations are boiled for 5 min at 95ºC and left at RT until use. Glucose and amino acid concentrations are measured as described in te Biesebeke et al. (2004b).

Zymogram analysis. Samples were prepared in native sample buffer and run in a 10% Tris-glycine pre-cast native gel in the mini-PROTEAN 3 ready gel electrophoresis system according to the suppliers instructions (www.bio-rad.com) at 4ºC and 60 V. The gel was rinsed for 1 h at RT in 1.25% Triton X100 (Sigma) in

water and placed for 20 h at 37ºC on either 1% skim milk (Difco-232100) or 1% insoluble starch (Sigma S2760) plates. The plates were prepared by dissolving agar (8 g) and Triton X100 (125 µl) (Sigma) in H_2O (500 ml), sterilisation for 20 min at 120ºC. Thereafter, 1% Glucose, 20 mM $MgSO_4$, 10 ml Aspa-N (13.1 g KCl and 37.4 g KH_2PO_4 in 500 ml H_2O adjusted to pH 5.5) and 1% Skim milk or 1% starch was added.

N-terminal and CNBr protein sequencing and identification of proteins. Extracts of *A. oryzae* grown on WK were separated in 4-15% pre-cast SDS-PAGE in the mini-PROTEAN 3 ready gel electrophoresis system. Extracts of *A. oryzae* grown on 5% WSM and growth medium of *A. oryzae* grown in 5% WLM were separated in a 18% SDS-PAGE with the PROTEAN II Ready Gel System according to the suppliers protocol (www.bio-rad.com) blotted to PVDF membranes (Spectrapor, Spectrumlabs, USA) as described (Deutzmann 2004). A selection of the commassie stained proteins were excised from the PVDF membranes and the N-terminal protein sequence was determined according to the Edman degradation method (Edman 1950) by the Sequencing Center Utrecht (Institute for Biomembranes, Utrecht University, the Netherlands). When indicated, the protein was CNBr cleaved similar to the method as described by Schmid et al. (1997) and the peptide sequence was determined by the Sequencing Center Utrecht. The protein sequences were matched to different databases (*A. oryzae* (nrib.go.jp/ken/EST/db/blast.html), *A. fumigatus* (tigr.org/tdb/e2k1/afu1/), *A. flavus* (genome.ou.edu/flavus_blast.html), EMBL-EBI (ebi.ac.uk/), NCBI (ncbi.nlm.nih.gov/), Fungal Genomes Initiative (broad.mit.edu/annotation/fungi/fgi/), *Saccharomyces* Genome Database (yeastgenome.org/)). Any hypothetical protein sequences identified as matches to our protein sequences were further searched against the public databases using FASTA

(Pearson and Lipman 1988) and BLASTP (Altschul et al. 1990 and 1997) to obtain a possible functional identification. If still no function could be addressed the unknown protein was matched against the Pfam protein families database (sanger.ac.uk/Software/Pfam/search.shtml, Bateman et al. 2002) to search for putative protein domains and matched against the Prosite database (expasy.org/tools/scanprosite/, Biaroch 1991) for the occurrence of patterns, profiles or motifs. The N-terminal protein sorting signal of the obtained protein sequences were predicted (cbs.dtu.dk/services/SignalP/) as described by Nielsen et al. (1997). A predicted prosequence was assigned to the amino acid sequence in between the predicted N-terminal signal sequence and the putative monobasic or dibasic cleavage site directly in front of the experimentally determined N-terminal protein sequence.

Purification of total RNA. Biomass from submerged cultures was collected after filtration through miracloth and biomass from WK was rapidly removed with physiological salt after vigorously shaking collected by centrifugation. Harvested biomass was rapidly frozen in liquid nitrogen, ground in liquid nitrogen and total RNA was isolated with the trizol method (INVITROGEN) according to the manufacturer protocol.

Polymerase Chain Reaction and Northern blot analysis. PCR was used to amplify a 300 bp probe for the *nptB* gene (Tatsumi et al. 1991) with primers (5'-GGCCGCCAAGGTCACCAAGG-3' and 5'-CGCCAGGGGCGTGAGTGAAC-3') and the *amyA* gene (Tsukagoshi et al. 1989) with primers (5'-GGAGCGGGTAGCTCAGTCG-3' and 5'-GTAGTTCGATACCAATGATCC-3') from the *A. oryzae* genome using 40 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C. The probes were purified from a 1 % agarose gel with the Qiaquick DNAeasy columns (Qiagen, UK). Northern blot analysis was performed as described (te

Biesebeke et al. 2004b). The purified probes for the *nptB* or *amyA* genes were ^{32}P labeled (random prime labeling kit, Pharmacia) and hybridised to the fractionated RNA. The hybridisation signals were visualised by exposure of the nitrocellulose membranes to X-OMAT AR film (Kodac, cat. 1651512) and scanned with the Hewlett Packard 6200C Scanjet at 600 dpi.

RESULTS

Growth phenotype of A. *oryzae* grown on WK and RK. WK, RK, and ground WK were inoculated with conidia of *A. oryzae* and incubated at 30°C at 98% water/air (v/v) content while the growth phenotypes on the different substrates were examined macroscopically. The filamentous fungus rapidly colonises on the RK and the ground WK fully covering these substrates with filamentous fungal mycelium after 2 days growth (Table 1). The colonisation on the WK is about 2 times slower compared to the other substrates. Moreover, a clear difference in morphology of *A. oryzae* was observed when grown on the different substrates at the level of sporulation and formation of aerial hyphae (Table 1). *A. oryzae* starts to sporulate after 2 days of cultivation on WK while sporulation appears after more than 4 days on the RK and the ground WK. The aerial hyphae were pronounced when *A. oryzae* was grown on the WK (Table 1). The amount of water present in the WK (39% w/w) and the amount of water present in the RK (41% w/w) is not significantly different and was therefore excluded as a determining factor for the difference in sporulation and colonising capacity. Sporulation has been shown to occur as a response to nutrient starvation, *e.g.* a lack of glucose (Skromne et al. 1995). As sporulation appears after 2 days when *A. oryzae* is grown on WK it is suggested that liberation of glucose from WK and consumption of glucose by *A. oryzae* grown on WK appears slower then that

during growth on RK. This would also explain the relative slow colonising capacity of *A. oryzae* grown on WK. As shown in Fig. 1A, the glucose concentration measured in the extracts of *A. oryzae* grown on RK was more then 30 times higher compared to those grown on WK and ground WK.

SSF Substrate	*Colonisation (day)*	*Sporulation (day)*	*Aerial hyphae (mm)*
WK	4	2	++++
RK	2	>4	++
Ground WK	2	>4	+

Table 1. *The characteristics of the growth phenotype of A. oryzae grown on (ground) wheat kernels (WK) and rice kernels (RK). The characteristics are expressed in terms of time it takes to colonise the substrate completely covering it with filamentous fungal mycelium (Colonisation), starting day of sporulation (>10^3 spores per ml extract) (Sporulation), and aerial hyphae formation (Aerial hyphae; short (+, < 1 mm), long (++++, > 3 mm)).*

Amylase and protease activities. Since α-amylase is the major enzyme involved in starch degradation, its activity was measured in the extracts of *A. oryzae* grown on the different cereal substrates (Fig. 1B). The α-amylase activity was the highest in the extracts of *A. oryzae* grown on RK at day 5.5 (248 U/g). The α-amylase activities in the extracts of *A. oryzae* grown on WK and ground WK remained about 50% lower then those on RK on days 3.5, 4.5 and 5.5. However, on day 1.5, 2 and 2.5 the α-amylase activities measured in the extracts of *A. oryzae* grown on the ground WK were about double compared to those on the WK. Nevertheless, after 9 days, the α-amylase activity measured in the extracts of *A. oryzae* grown on WK (262 U/g) was comparable to that on RK. The glucoamylase activity in the extract of *A. oryzae* grown on WK is only 10 % of that on RK during the first 5.5 days (Not shown).

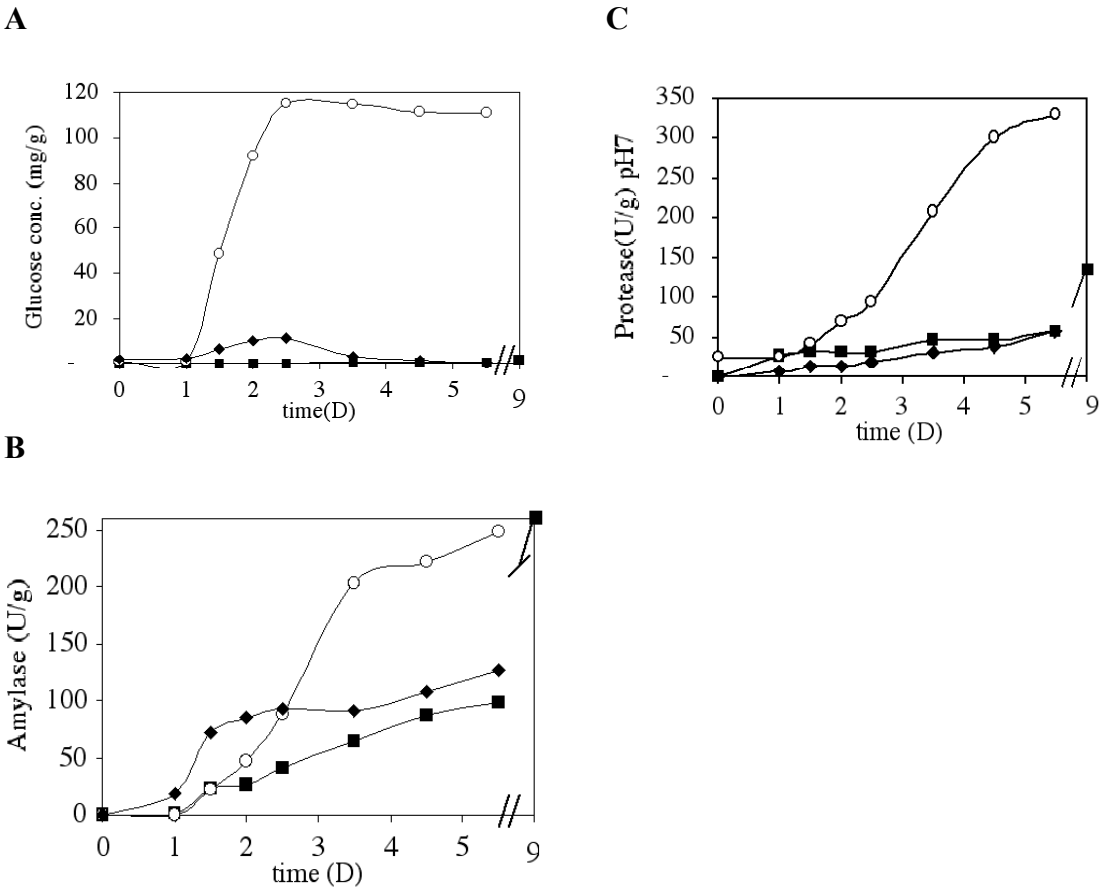


Fig. 1. *The characteristics of the growth phenotype of A. oryzae grown on (ground) wheat kernels (WK) and rice kernels (RK). The different graphs represent glucose concentrations (***A***), α-amylase activities (***B***) and protease activities at pH 7 (***C***) of extracts of A. oryzae grown on WK (■), RK (*O*), and ground WK (♦) per gram wet weight (U/g) solid state fermentation.*

To elaborate further on the hydrolytic activities in the extracts of *A. oryzae* grown on RK and WK the protease activities were measured. Fig. 1C shows the protease activities measured at pH 7 in the extracts of *A. oryzae* grown on WK, RK and ground WK. The highest protease activity was measured in the extracts of *A. oryzae* grown on RK after 5.5 days (335 U/g) and was about 5 times higher in value then those on WK and ground WK (about 60 U/g). The protease activity in the extracts of *A. oryzae* grown on WK increased still after 5.5 days and reached 143 U/g after 9 days.

Identification of α-amylase and neutral protease II. To identify the number of enzymatic activities involved in starch and protein degradation in the extracts of *A. oryzae* grown on WK and RK zymogram analysis was performed with starch and skim milk plates. The extracts of *A. oryzae* grown for 5.5 days on WK and RK were microcon-concentrated (10 times) and separated in two 10% native gels and spread on a starch plate or a skim milk plate. The zymogram showed 1 activity spot on a 1% starch plate and 2 activity spots on a 1% skim milk plate (results not shown) with extracts of *A. oryzae* grown on WK and RK suggesting the presence of at least 1 starch degrading and at least 2 different protease activities in the extracts of *A. oryzae* grown on WK and RK.

To identify the corresponding hydrolytic proteins in the extracts of *A. oryzae* grown on WK, SDS-PAGE was performed with an extract from *A. oryzae* grown for 4.5 days on WK. Fig. 2A shows 4 dominant bands of 28, 35, 50 and 65 kDa in the SDS-PAGE gel. The 28, 35, 50 and 65 kDa bands were isolated and N-terminally sequenced. The N-terminal protein sequencing of the 50 kDa protein revealed 10 amino acids (Table 2) identical to amino acids 32 to 41 of the *A. oryzae* α-amylase precursor (GenBank accession no ALAS1). The N-terminal sequence of the 28 kDa protein consisted of 10 amino acids (Table 2) identical to amino acids 176 to 185 of the *A. oryzae* neutral protease II (GenBank accession no S16547). In agreement with this, transcription analysis revealed expression of the *amyA* gene encoding α-amylase and the *nptB* gene encoding neutral protease II of *A. oryzae* grown on WK (Fig. 2B). The signal intensities after Edman degradation of the 35 and 65 kDa proteins were to low for a reliable protein sequence determination suggesting that the N-terminal sequences may be blocked.

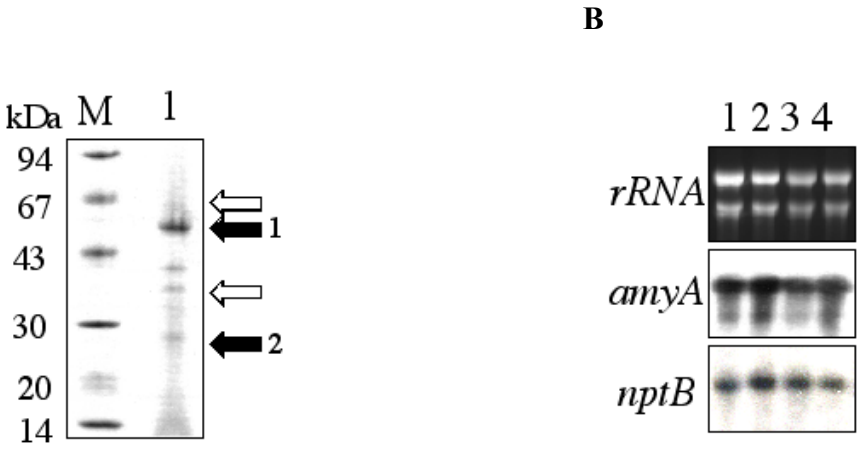


Fig. 2 *(**A**) Proteins extracted from A. oryzae grown for 4.5 days on wheat kernels (WK) separated on SDS-PAGE (lane 1). The arrows indicated the major protein bands that were taken for N-terminal sequencing. Filled and open arrows indicate protein band for which, respectively, successful and unsuccessful N-terminal sequence determination was carried out. M = low molecular weight marker (Biorad). (**B**) Northern analysis of RNA samples obtained from A. oryzae grown for 2.5 (lane 1), 3.5 (lane 2), 4.5 (lane 3), and 5.5 (lane 4) days on WK with probes for the α-amylase (amyA) and neutral protease B (nptB) genes. The ribosomal RNA (rRNA) was used as a loading control.*

Identification of other secreted proteins. To identify other extracellular proteins involved in penetration and conversion of WK, *A. oryzae* was grown on (5%) wheat-based liquid and solid medium. The difference in macroscopic phenotypes of *A. oryzae* grown on 5% WSM and in 5% WLM is clear both in terms of its growth phenotype and formation of aerial hyphae. The major differential expressed proteins of extracts of *A. oryzae* grown on 5% WSM and in the growth medium of *A. oryzae* grown in 5% WLM were identified after separation on 18% SDS-PAGE (Fig. 3) and the N-terminal sequences were determined. The N-terminal sequences were matched to public databases using FASTA (Pearson and Lipman 1988) and BLASTP (Altschul et al. 1990). The results are presented in Table 2. The N-terminal sequences of the 50

kDa proteins was identical to the *A. oryzae* α-amylase sequence, that of the 48 kDa protein was identical to chitinase and that of the 41 kDa protein similar to *A. nidulans* formate dehydrogenase. The N-terminal sequence of the 40 kDA protein was identical to the *A. niger* arabinosidase and that of the 24 kDa protein suggested that the band was made up of 2 proteins since a mixture of amino acids was obtained for the first 16 amino acids. Table 2 shows that one N-terminal sequence from the 24 kDa protein is similar to part of the *A. oryzae* endo-xylanase (Table 2). The remaining amino acids of the N-terminal sequencing results of the 24 kDa matched to AoEST02754. All the obtained protein sequences were compared with database entries from an *A. oryzae* EST library (Machida 2002) to identify the presence of any N-terminal signal sequences. With exception of the protein sequence of *A. oryzae* (AoEST02357) that was homologous to formate dehydrogenase (GenBank accession no Q03134), all protein sequences derived from the EST clones contained a protein sorting signal sequence, as was predicted as described by Nielsen et al. (1997). For about 40% of the excised protein bands no N-terminal sequence was obtained. This may be due to N-terminal blockage as is frequently observed for secreted fungal proteins.

The NCBIest database contained an *A. niger* sequence (GenBank accession no BE759099) of which the derived amino acids sequence corresponded to the 27 kDa N-terminal protein sequence (21 identical amino acids (70%) and 27 similar amino acids (87%). Repeated matching of the derived amino acid sequence of the *A. niger* sequence (GenBank accession no BE759099) in the public available databases revealed the 178 amino acid sequence of the 27 kDA protein of *A. oryzae* (AoEST02339), and its 198 and 180 amino acids homologous counterparts from *A. fumigatus* and *A. nidulans,* respectively (Fig. 4). The identity of the 27 kDa protein of which the complete protein sequence was obtained from the *A. oryzae* database was

confirmed by sequence analysis with the CNBr degradation method (see Fig. 4). The 27 kDa proteins contain a predicted N-terminal signal sequence, 4 conserved cysteine residues suggested to form 2 di-sulfide bridges and 6 conserved proline residues.

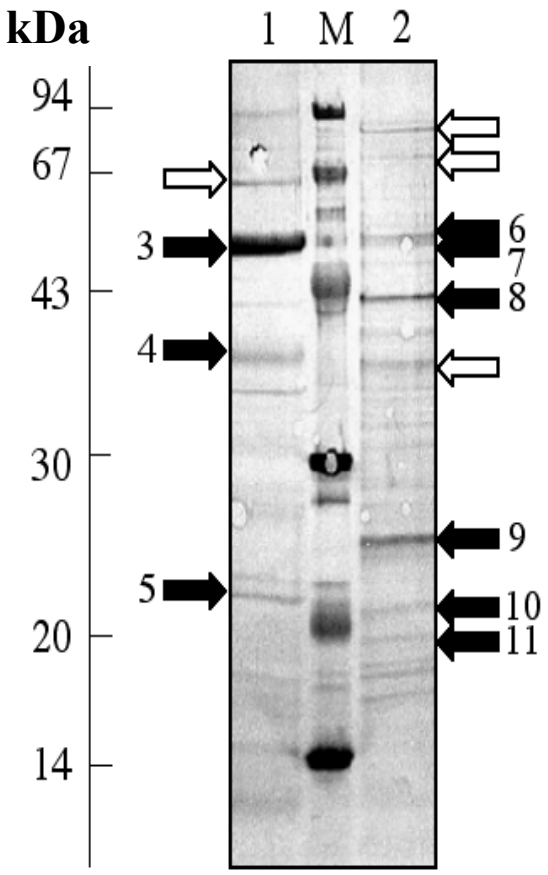


Fig. 3 *Proteins of the growth medium of A. oryzae grown in 5% WLM (lane 1) and in the extracts of A. oryzae grown in 5% WSM separated by SDS-PAGE. Filled and open arrows indicate protein band for which, respectively, successful and unsuccessful N-terminal sequence determination was carried out. M = the low molecular weight marker (Biorad). The numbers correspond to the numbers in Table 2.*

The N-terminal sequence of the 16 kDa protein was identical to AoEST2754 of the *A. oryzae* EST database. Homology searches in the public databases revealed its 123 and 120 amino acids homologous counterparts from *A. flavus* (CA747619) and

Fusarium graminearum. (XM383762.1), respectively. Surprisingly, no homologous proteins were found for *A. fumigatus* or *A. nidulans* or any other fungi for which the full genome sequence is available. The CLUSTALW aligned protein sequences (Fig. 5) showed six conserved cysteine residues that probably form three disulphide bridges. A Pfam search revealed that the 6-cysteine residues of the *A. oryzae* 16 kDa protein matched to the CX module, a domain with unknown function found in several *Ceanorhabditis elegans* proteins. The 124 amino acids *Neurospora crassa* protein Q7S8H0 and the 117 amino acids *Schizosaccharomyces pombe* protein Q9URX2 also contain 6 conserved cysteine residues and a predicted signal sequence suggesting that all these proteins (Fig. 5) belong to a family of fungus specific secreted proteins.

Table 2. *(Next page) Identified proteins with their corresponding GenBank accession number (Genbank no) and observed molecular weight on SDS-PAGE. The numbers (No) correspond to the numbers in Fig. 2A and 3. The N-terminal sequence of the mature protein that has been obtained through N-terminal protein sequencing after Edman degradation is shown double underlined in relation to the complete protein sequence inferred from corresponding A. oryzae EST clones or in case of neutral protease II from the corresponding NCBI database. The dotted underlined sequence represents the signal sequence predicted as described (Nielsen et al. 1997). A putative prosequence was predicted for the neutral protease II sequence (S16547), xylanase and the 16 kDa and 27 kDa proteins. The arrows in the sequence represent the putative cleavage sites of the signal sequence and the prosequence. The putative monobasic or dibasic cleavage sites are shown underlined marking the position where processing of the prosequence may have occurred.*

No	Identity (GenBank no)	MW (kDA)	N-terminal sequence of the identified proteins	AoEST
1, 3, 6	α-amylase (P10529)	**50**	MMVAWWSLFLYGLQVAAPALA↓ATPADWRSQ….	00062, 02370, 03185
2	Neutral protease II (S16547)	**30**	MRVTTLSTALFALTSTAVS↓APTAGSSSPGLEVKLTQIDNTRVKAVVKNTGSEEVSFVHLNFFKDAGPVKKVSVYRGQDE VQFEGIKRRLRSSGITKEAVTSLGAGETLEDEFDIASTSDLASGGPVSIRSHGFVPIVVDGKITGYIPYKSNDLTVNVDG GKAAKVTKALSQLTRR↓TEVTDCKGLA…	-
4	ArabinosidaseA (P42256)	**40**	MLSFLAALSLPLALVNA↓YANPGTCNGNCWAHDPGLWKHDD….	05037
5	Xylanase (JC7577)	**24**	MVSFSSILLACSAAIGAL↓ATPIEPLADHPNEAFNETAFNDLVGRS↓TPSSTGYNNGYYYSFWTDGGGDVTYT….	05492
7	Chitinase (AAK84437)	**48**	MKWKSLALGLLATAQS↓AASLRFVMYIDEYHTQ….	02218
8	Formate dehydrogenase (Q03134)	**41**	MGKILMVLYDGGEHAKQQPGLLGTTENELGL….	02357
9	Unknown	**27**	MIKSIASIALLFSTAIA↓VPTPTELLPR↓ACTTLAPAVINILDAANPNTPYSGQQFTLER….	02339
10	Unknown	**22**	?….GXPGAF(F)YVELALVK….?	-
11	Unknown	**16**	MLFTTILASTLALSMGVSA↓APRP↓APPADTRYVQLRLQ….	02754

Table 2.

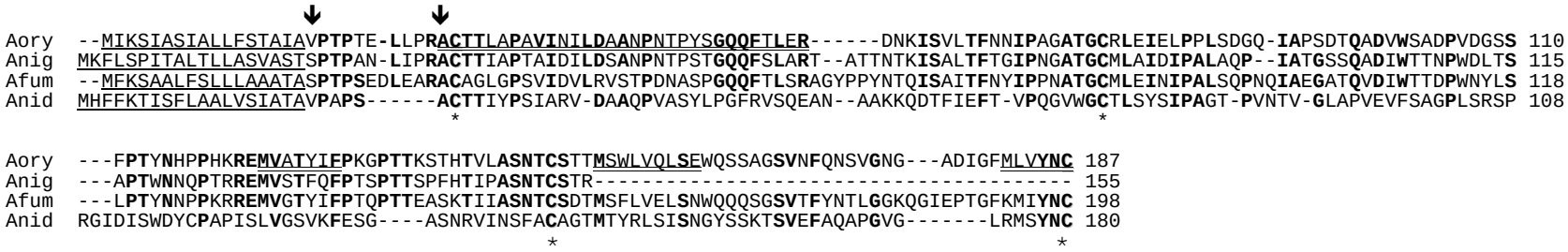


Fig. 4. *CLUSTAL W (1.82) obtained from EMBL-EBI after multiple sequence alignment of the 27 kDa A. oryzae (Aory) protein consisting of 187 amino acids sequence with its homologues from A. niger (Anig, partial 155 amino acids sequence, GenBank accession no BE759099), A. fumigatus (Afum, 198 amino acids, a_fumigatus|chr_0|Sanger.AFGUU35TF|66) and A. nidulans (Anid, 180 amino acids, AN7941.2). Identical amino acids in at least 3 sequences are shown in bold face. The A. niger sequence is very probable incomplete. The A. niger, A. fumigatus, and A. nidulans sequences have 53%, 51% and 29% identity and 68%, 65% and 47% similarity, respectively, to the A. oryzae sequence. Note that the A. nidulans sequence is distinct from the other sequences by the number of identical amino acids and absence of a putative monobasic prosequence cleavage site. The underlined sequences mark the N-terminal protein-sorting signal predicted as described by Nielsen et al. (1997). The conserved cysteine residues are marked with asterisks. The residues that have been resolved with N-terminal sequencing and that have been identified with sequencing after CNBr cleavage of the A. oryzae 27 kDa protein are shown double underlined. The arrows above the sequence represent the putative cleavage sites of the signal sequence and the prosequence.*

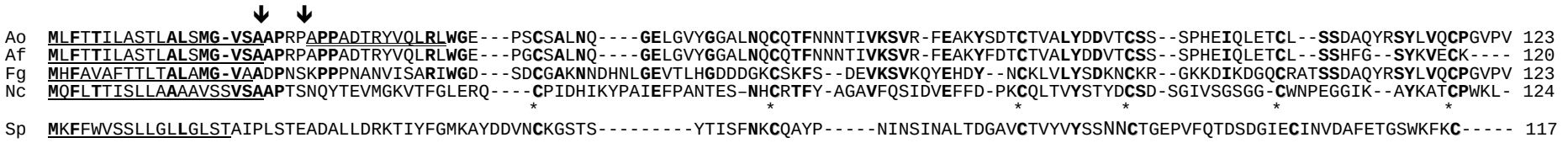


Fig. 5. *CLUSTAL W (1.82) obtained from EMBL-EBI after multiple sequence alignment of the16 kDa A. oryzae (Ao) protein consisting of 123 amino acids sequence with its homologues from A. flavus (GenBank accession no CA747619), F. graminearum (Fg, XM383762.1), and N. crassa (Nc, Q7S8H0). Identical amino acids in at least 3 sequences are shown in bold face. The A. fumigatus, F. graminearum, N. crassa and S. pombe sequences have 91%, 27%, 20% and 11% identity, respectively, to the A. oryzae sequence. The underlined sequences mark the N-terminal protein-sorting signal predicted as described by Nielsen et al. (1997). The cysteine residues that are suggested to be part of the CX module as resolved with a Pfam search are marked with asterisks. The residues of the 16 kDa protein that have been resolved with N-terminal sequencing are shown double underlined. Also shown is the S. pombe sequence (Q9URX2) that shows resemblance in length and position of the cysteine residues compared to the other aligned sequences. The arrows above the sequence represent the putative cleavage sites of the signal sequence and the prosequence.*

DISCUSSION

Filamentous fungi colonise solid substrates while secreting enzymes involved in hydrolysis and penetration of the substrate. In comparison to submerged growth, the filamentous fungal phenotype is different implying that also differences in gene transcription and protein secretion may be expected. In this study different approaches have been used to describe the differences between solid state fermentation with different cereal substrates and the differences between submerged and solid state fermentation. Most of the research related to solid state fermentation has always been focussed on hydrolytic enzymes. In our study we have taken a more generic approach and have tried to determine the identity of the major proteins of *A. oryzae* involved in WK conversion and penetration. The penetrative hyphae of *A. oryzae* encounter complex polysaccharides in the WK wall while growing on cereal substrates before arriving at the starch containing WK core (te Biesebeke et al. 2002). Therefore, differences might be expected in the pattern of secreted proteins when *A. oryzae* is grown in submerged and solid state fermentation.

N-terminal sequencing has identified α-amylase in the extract of *A. oryzae* grown on WK and zymogram analysis showed one spot with starch degrading enzyme activity. The single 50 kDa band, identified as α-amylase in SDS-PAGE analysis, suggests that the α-amylase is responsible for most of the starch degrading activity observed in the zymogram analysis. Although, some *A. oryzae* strains contain at least three nearly identical genes encoding α-amylases (Wirsel et al. 1989, Gines et al. 1989, Tsukagoshi et al. 1989), the obtained N-terminal sequence of α-amylase was too limited to distinguish between the gene products of the different α-amylase genes (Wirsel et al. 1989). The identified α-amylase could account for all the α-amylase activities in the extracts of *A. oryzae* grown on WK.

A. oryzae contains at least 2 genes encoding glucoamylase (Hata et al. 1998), *glaA* and *glaB,* of which the *glaB* gene is markedly expressed in solid state culture (Ishida et al. 2000). As a consequence of this high level expression, high glucoamylase activity levels are measured in extracts of *A. oryzae* grown on RK and it has been suggested that this is due to solid state fermentation specific characteristics (Hata et al. 1998, Ishida et al. 2000). However, glucoamylase levels measured in extracts of *A. oryzae* grown on WK and ground WK are about 10% of those measured in extracts of *A. oryzae* grown on RK. This shows that high glucoamylase activity levels, more than for solid state fermentation on cereal substrates in general, are characteristic for extracts of *A. oryzae* grown on RK.

Zymogram analysis showed that there were at least 2 skim milk degrading enzymes present in the extracts of *A. oryzae* grown on WK, ground WK and RK. The 28 kDa protein, identical to neutral protease II, was suggested to contribute to the skim milk degrading activity measured in the zymogram analysis with extracts of *A. oryzae* grown on WK. Also part of the protease activities measured at pH 7 in the extracts of *A. oryzae* grown on WK were suggested to be due to the neutral protease II protein. Northern analysis sustained our observation that neutral protease II is one of the proteases expressed in *A. oryzae* grown on WK.

Large differences in protein patterns between *A. oryzae* grown on 5% WSM and in 5% WLM can be observed in Fig. 3. α-Amylase, arabinosidase and xylanase are more abundantly secreted by *A. oryzae* grown in 5% WLM while other proteins, were more abundantly secreted during growth of *A. oryzae* on 5% WSM. This difference in identified hydrolytic enzymes suggested a difference in conversion of the WK, dependent on the different cultivation method used. Besides the identification of extracellular hydrolytic enzymes involved in WK conversion, also

another protein related to penetrative growth of *A. oryzae* during growth on 5% WSM was identified. The identified chitinase might be involved in the local maintenance of wall plasticity of *A. oryzae* since branching and cross-linking of polymers in the cell wall of fungi during polarised growth, cell division, and morphogenesis involves a delicate balance of cell wall synthesis and hydrolysis (Adams 2004, Selvaggini et al. 2004).

The extraction method used to obtain the sample for Fig. 3 (lane 2) aimed to extract extracellular proteins of *A. oryzae* grown on 5% WSM and, indeed, all extracted proteins, except the intracellular formate dehydrogenase, carried a predicted signal sequence. Although formate hydrogenase is an intracellular protein its presence seems related to growth of filamentous fungi on solid substrates. This was corroborated by the observation that *N. crassa* transcript levels of the formate dehydrogenase-encoding gene (*fdh*) were only found during conidiation and early germination and were not detectable during mycelial growth (Chow and Rajbhandary 1993).

The *S. pombe* protein Q9URX2 homologous to the 16 kDa *A. oryzae* protein showed also similarity (30/62 identical amino acids (48%)) to the N-terminal part of the Wsc1 protein of *S. cerevisiae*. This domain encodes a cysteine-rich domain conserved in the Wsc1 family of proteins that consist of 4 members. The Wsc1 protein of *S. cerevisiae* is suggested to be a regulator of the stress-activated PKC1-MAP kinase cascade required for the heat shock response and for maintenance of cell wall integrity (Lodder et al. 1999). The N-terminal cysteine domain of Wsc1 is a structural domain suggested to be involved in signal sensing and/or modulation of the activity (Lodder et al. 1999). This finding suggests a putative signaling role for the *A. oryzae* extracellular 16 kDa cysteine rich protein in penetrative growth.

The 27 kDa protein (Fig. 4) showed low similarity to a 153 amino acids protein of *Magnaporthe grisae* (GenBank accession no EAA52090) (not shown). As was observed for the 27 kDa protein, the *M. grisae* proteins is rich in proline residues (12) and has 4 cysteine residues, suggesting that the proteins might perform similar functions. Interestingly, homologous counterparts in the completed genome sequences of *N. crassa*, *F. graminearum*, several yeast species and other basidiomycetous fungi were not found. It is remarkable that no clear function can be assigned to the 27 kDa protein since it is an abundant protein during growth on solid substrates (Fig. 3). The function of the 27 kDa hypothetical protein may, as was suggested for the 16 kDa protein, also be associated to the filamentous fungal growth phenotype of *A. oryzae* on a solid substrate.

CHAPTER 4

Different control mechanisms regulate glucoamylase and protease gene transcription in *Aspergillus oryzae* in solid state and submerged fermentation

R te Biesebeke, N van Biezen, WM de Vos, CAMJJ van den Hondel, PJ Punt (2004) Different control mechanisms regulate glucoamylase and protease gene transcription in Aspergillus oryzae in solid state and submerged fermentation. *Appl Microbiol Biotechnol. Published online 3 dec 2004.*

ABSTRACT

Solid state fermentation (SSF) with *Aspergillus oryzae* results in high levels of secreted protein. However, control mechanisms of gene expression in SSF have been only poorly studied. In this study we show that both glucoamylase (*glaB*) and protease (*alpA, nptB*) genes are highly expressed during surface cultivation on wheat-based solid medium, and even higher during cultivation on wheat kernels. In wheat-based liquid medium, low levels of gene expression are observed. Typical SSF cultivation conditions, such as low water activity and the formation of aerial hyphae, did not contribute to the high-level gene expression on wheat-based solid medium. Analysis of wheat-based solid and liquid cultivations showed differences in carbon and nitrogen utilisation and external pH. The results presented show that the difference in regulation of transcription of the *alpA* and *nptB* genes in wheat-based liquid and solid medium could be pH dependent, involving a pH-dependent transcription regulator. The results obtained suggest that the difference in regulation of transcription of the *glaB* gene in wheat-based liquid and solid medium is caused by a difference in carbohydrate degradation and consumption under the different culture conditions.

INTRODUCTION

Filamentous fungal cultivation on solid substrates differs in many aspects from growth in liquid culture. In particular, the extracellular localisation of secreted proteins differs greatly between liquid and solid cultures. On a solid substrate, the growing hyphae of filamentous fungi penetrate the enzymatically modified solid substrate by extension and branching of the growing hyphal tips (Trinci 1974, Prosner 1994, Dynesen and Nielsen 2003). In liquid fermentation, the secreted proteins are

released into the culture medium, resulting in substrate degradation in the whole culture.

Numerous studies have described the presence of high level hydrolytic enzyme activities in extracts of filamentous fungi grown on different solid substrates (reviewed by Pandey et al. 1999). High protease activities are measured in extracts of wheat and rice kernel solid state food fermentation (SSF) by Aspergillus species (Su et al. 2001, Tunga et al. 2001). Based on research with rice kernel solid state fermentations with *A. oryzae,* it was shown that high levels of glucoamylase B, an enzyme that is clearly different from glucoamylase A (Hata et al. 1998), are due mainly to induction of the glucoamylase B gene (Ishida et al. 1998). Until now, gene expression studies have focussed only on transcriptional regulation of *glaB* (Hata et al. 1998) and *pepA* (Gomi et al. 1993) in surface-grown filamentous fungi like *A. oryzae*. Transcription levels of the acid protease-encoding gene (*pepA*) have been shown to decrease due to high temperatures (>38˚C) when *A. oryzae* was cultured on rice kernels (Kitano et al. 2002). Ishida et al. (2000) found that the promoter region of *glaB* mediates the induction of transcription by starch, high temperature, low Aw (water activity), and physical barriers to hyphal extension. The latter three are SSF-specific environmental conditions and it has been suggested that induction of *glaB* transcription is due mainly to these conditions (Ishida et al. 2000).

N-terminal sequence analysis and enzyme activities of secreted proteins of *Aspergilli* grown on solid substrates were in agreement with preliminary northern analysis and suggested that the alkaline (*alpA*) and neutral (*nptB*) protease encoding genes are also highly expressed during surface growth (Malathi and Chakraborthy 1991, te Biesebeke et al. 2002). However, it is unknown if, like the *glaB* gene, the *alpA* and *nptB* genes are also controlled by SSF-specific environmental conditions. To

gain more insight into the different conditions that play a role in regulation of genes during submerged and surface growth conditions, we analysed the transcription of the *glaB*, *alpA* and *nptB* genes of *A. oryzae* during growth in wheat-based liquid and solid medium.

MATERIALS AND METHODS

Strains and media. The *A. oryzae* strain ATCC16868 was used throughout this study. Ritmo wheat kernels (WK) (ACM, Meppel, the Netherlands) were pre-treated as described in Hoogschagen et al. (2002). Ground WK were prepared by pulverising non-pre-treated WK portions (20 g) with a Moulinex blender (SEB, Ecully, France) for 1 min. Wheat-based liquid medium (WLM) was prepared by shaking 4 g ground WK in 100ml H_2O in shake flasks in a rotary shaker at room temperature for 5 min at 250 rpm. The suspension was poured through Miracloth where 2 g of the ground WK (dry weight) remained in the filter resulting in 2% wheat-based liquid medium (2% WLM), which was sterilised for 15 min at 120°C. The 1% wheat-based liquid medium (1% WLM) was prepared in a similar way using 2 g ground WK. Surface growth on wheat-based solid medium (WSM) was performed on 1% agar plates of 2% WLM (2%WSM).

Cultivation conditions. SSF was performed with 50 g pre-treated WK that was inoculated with 2 ml $5X10^7$ conidia ($1.0X10^6$ conidia/g) in a sterile 1 liter bottle, placed on a roller apparatus (Wheaton modular roller, Millville, N.J.) and kept rolling at maximum speed for 6 h at room temperature. The inoculated WK was transferred to sterile Petri dishes and incubated for 3, 4 or 5 days at 30°C at 98% water/air (v/v) content in a climatic incubator (VEA-Instruments, Houten, the Netherlands). Submerged cultivations were performed with 25 ml 2% WLM inoculated with 10^6

spores/ml and incubated in shake flasks at 30°C in a rotary shaker at 250 rpm. Surface cultivation was performed after inoculation of nitrocellulose (NC) membranes (3 μm pore size; Millipore, Bedford, Mass.) placed on 2% WSM (25ml), with $2.5X10^7$ spores (10^6 spores/ml 2% WSM) followed by an incubation at 30°C at a relative humidity of 98% in a climatic incubator (VEA-Instruments). Liquid (LTC) and membrane (MTC) biomass transfer cultivations were performed as described by Ishida et al (1998) after production of biomass in wheat-based medium (17 h in 2% WLM and 48 h in 2% WSM). After transfer to fresh wheat-based medium (2% WLM and 2% WSM, respectively) samples taken at time points 0, 2, 6, 8, 10 and 12 h in the case of MTC and 0, 0.5, 1, 6, 8 and 24 h in the case of LTC, were used for transcription analysis. Low Aw in the culture medium was achieved by supplementing 2% WSM with maltose (5%, 10% and 40%) or 5 M NaCl, resulting in Aw values of 0.98, 0.96, 0.93 and 0.93, respectively, measured using an electric hydrometer (Type EK 84/3H/63T, sensor type BSK-4; Novasina, Pfäffikon, Switzerland). The maltose- or NaCl-supplemented 2% WSM media were used in a 6 h MTC. A biomass layer without aerial hyphae-the so-called sandwich cultivation (SWC)-was prepared by covering the biomass on the NC membrane with another NC membrane. The SWC was transferred for 6 h to 2% WSM. Transfer experiments for 6 h to 1% WLM with and without 40% maltose were performed to determine the effects of maltose, pH and extracellular glucose on gene transcription.

Purification of total RNA and DNA. *A. oryzae* grown on WK was separated from the WK after 1 min vigorous shaking with a 0.7% NaCl solution, collected after filtration through Miracloth and rapidly frozen in liquid nitrogen. *A. oryzae* grown on NC membranes that placed on WSM and *A. oryzae* grown in WLM (collected after filtration through Miracloth) were rapidly frozen in liquid nitrogen. The collected *A.*

oryzae biomass was ground in liquid nitrogen and total RNA extracted using TRIzol™ reagent (Invitrogen, Carlsbad, Calif.) according to the supplier's instructions. Chromosomal DNA was isolated as described by Kolar et al. (1988) from *Aspergillus oryzae* after 30 h growth in 2% WLM.

Polymerase Chain Reaction and purification of probes. PCR was used to amplify probes for the *enoA*, *glaB*, *alpA* and the *nptB* genes from *A. oryzae* chromosomal DNA using 40 cycles of 30s at 94°C, 30s at 55°C, 30s at 72°C. Table 1 shows the primers used to obtain probes for the genes indicated. Probes were purified from 1% agarose gels using Qiaquick DNAeasy columns (Qiagen Hilden, Germany).

Gene	*Primers*	*Probe Size (bp)*	*Reference*
EnoA	5'-CGCTGCCCCCTCTTTCTCCG-3' 5'-CCAACAATCTGGAAGTCAG-3'	420	Machida et al. 1996
GlaB	5'-GAGCAATTCATCGGGGGA-3' 5'-CCCTGATAGGTGTCCTCTGCG-3'	740	Gomi et al. 1993
AlpA	5'-GAGCGCAACTACAAGATCAA-3' 5'-CGGAATTCAGCCCAGTTGAAGCCGTC-3'	500	Cheevahanarak et al. 1991
NptB	5'-GGCCGCCAAGGTCACCAAGG-3' 5'-CGCCAGGGGCGTGAGTGAAC-3'	300	Tatsumi et al. 1991

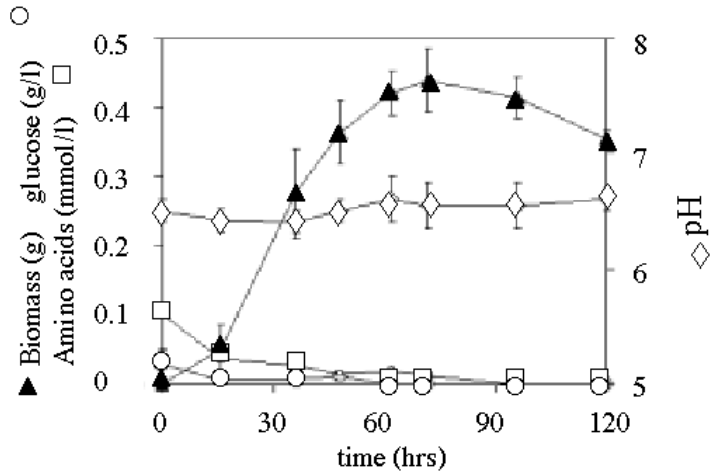
Table 1. *Primers used to obtain the probes for the enoA, glaB, alpA and nptB genes.*

Southern and northern blot analysis. Southern blot analysis was performed according to standard procedures (Sambrook et al. 1989) after cutting chromosomal DNA with *Eco*RI and *Bam*HI. For northern analysis total RNA (10 μg) was fractionated by formaldehyde-agarose (1%) gel electrophoresis and transferred to a Hybond-N nylon membrane (Amersham Pharmacia, Little Chalfont, UK). Southern and northern blots were hybridised with ^{32}P-labeled (Random Prime Labelling Kit, Pharmacia) PCR-amplified probes for the *enoA*, *glaB*, *alpA* and *nptB* genes (see Table 1) at 62˚C in standard hybridisation buffer (Sambrook et al. 1989). Hybridisation signals were visualised after exposure to X-OMAT AR film (Kodac, cat. 1651512;

Eastman Kodak, Rochester, N.Y.) and scanned with a Hewlett Packard 6200C Scanjet at 600 dpi (Hewlett Packard, Waldbronn, Germany). The signal intensities on the northern blots were quantified with gene tools from Syngene (Synoptics, Cambridge, UK) using the signal for *enoA* as a loading control. In Southern analysis, the probes for the *enoA*, *glaB* and *alpA* genes all showed single specific hybridising bands and the probe for *nptB* showed two hybridising bands.

Biomass, pH, glucose and amino acids determination. The wet weight biomass of *A. oryzae* grown in 2% WLM was determined after filtering the biomass through Miracloth. The wet weight biomass of *Aspergillus oryzae* grown on 2% WSM was determined after weighing the biomass grown on the filter. The extracellular growth medium (1 ml) of *Aspergillus oryzae* grown in 2% WLM was boiled for 5 min and used for determination of glucose and amino acid concentrations. Extracts of the growth medium of *A. oryzae* grown on 2% WSM were obtained after removing the membrane with biomass and freezing the 2% WSM at –20°C. Subsequently, the 2% WSM was thawed and, after addition of 10 ml milliQ water, vortexed for 2 min, centrifuged for 5 min at 3,500 rpm, boiled for 5 min and used for determination of glucose and amino acid concentrations. Glucose was analyzed enzymatically using the glucose HK 125 method (ABX Diagnostics, Montpellier, France; cat. no. A11A00116) from (Burrin and Price 1985). Amino acids were analysed using the TNBS (trinitrobenzenesulfonic acid) method described by Adler-Nissen (1979). The pH of the medium of samples of *A. oryzae* grown in 2% WLM and the extracts of *A. oryzae* grown in 2% WSM were measured with a PHM83 autocal pH meter (Radiometer, Copenhagen, Denmark) at the time points indicated.

A



B

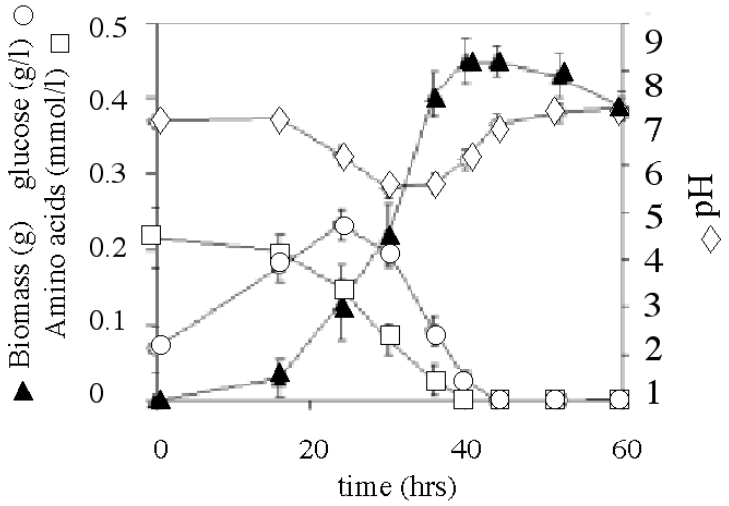


Fig. 1. *Parameters measured in time (h) in surface (***A***) and submerged (***B***) cultivation of A. oryzae grown in 2% wheat-based solid medium (WSM) and 2% wheat-based liquid medium (WLM), respectively.* ▲*Wet weight biomass (g),* O *glucose (g/l),* □ *amino acids (mmol/l),* ◊ *pH measured in the growth medium of submerged cultivation and in the extracts of surface cultivation at the time points indicated.*

RESULTS

Substrate conversion, biomass increase and external pH during surface and submerged cultivations. Surface and submerged cultivation in 2% WSM and 2% WLM, respectively, was carried out to determine biomass, glucose, amino acid concentrations, and pH, over time (Fig. 1). The final wet weight biomass produced under both cultivation conditions was equal but during surface cultivation it took around twice as long to produce the maximum amount of biomass on 2% WSM. During surface cultivation, glucose and amino acid concentrations liberated from the substrate were lower than 0.05 g/l and 0.05 mmol/l, respectively. In submerged cultivations, the glucose and amino acids concentrations were maximal (0.23 g/L and 0.22 mmol/L, respectively) in the beginning of the logarithmic phase and decreased towards the stationary phase. Apparently, during submerged cultivation, an excess of glucose was liberated from the substrate, whereas the glucose concentrations in surface cultivation remained low at all time points. The pH of the 2% WSM extract remained around 6.5 at all time points. In 2% WLM, the pH reached a minimum of about 5.5 after 30 h submerged cultivation. These results show that surface and submerged cultivations of *A. oryzae* in 2% WSM and 2% WLM differ in at least three environmental parameters.

Transcription of *enoA*, *glaB*, *alpA* and *nptB* genes. To examine the transcription of *enoA*, *glaB, alpA* and *nptB* under different cultivation conditions, northern blot analysis was performed. Total RNA was isolated from the mycelium after 17, 24, 30 and 48 h submerged cultivation in 2% WLM, after 2 and 3 days surface cultivation in 2% WSM and after 3, 4 and 5 days SSF with WK. The results of the northern hybridisations with the labelled probes for the *enoA*, *glaB, alpA* and *nptB* genes are shown in Fig. 2. The probe for the enolase (*enoA*) gene (Machida et al.

1996) was used as a loading control as its transcript levels are equal during cultivation on different carbon sources (Nakajima et al. 2000). The *glaB* gene was transcribed to a very low level at 17 h in 2% WLM (lane 1) compared to that on 2% WSM (lanes 5-8). Transcription of the *alpA* and *nptB* genes occurs on 2% WSM (lanes 5-8) and in 2% WLM at 17 h, and at lower transcript levels at 24 h (lanes 1, 2). Moreover, the transcript levels of the *glaB*, *alpA* and *nptB* genes after 3 days on WK was about four times higher then on 2% WSM after 3 days as determined after quantification of signal intensity (lanes 9, 10). The transcriptional induction of the *glaB*, *alpA* and *nptB* genes during growth of *A. oryzae* was further investigated to understand the observed differences in transcript levels in submerged and surface cultivation.

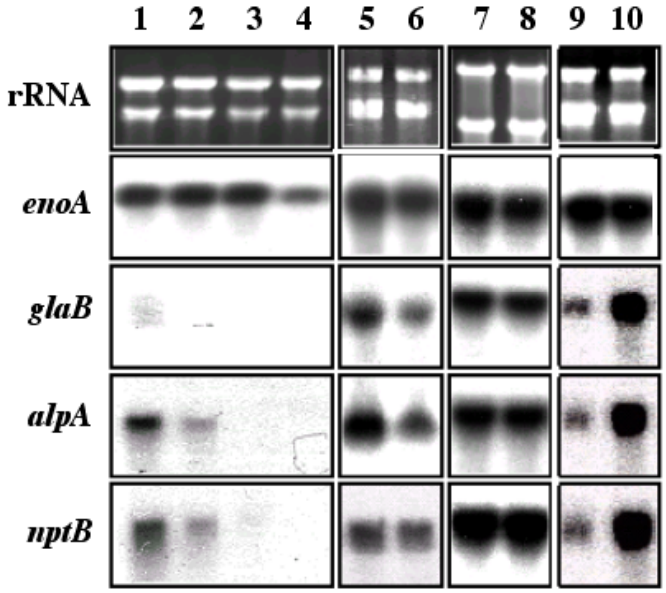


Fig. 2. *Hybridisation of the probes for the enoA, glaB, alpA and nptB genes with RNA samples isolated from solid state fermentations (SSF) on wheat kernels (WK) and surface cultivation on 2% WSM, and submerged cultivation in 2% WLM in northern analysis. Lanes: 1 17 h WLM, 2 24 h WLM, 3 30 h WLM, 4 48 h WLM, 5 2 days WSM, 6 3 days WSM, 7 4 days WK, 8 5 days WK, 9 3 days WSM, 10 3 days WK.*

***glaB, alpA* and *nptB* transcription in LTC and MTC**. To examine in more detail the observed differences in transcriptional regulation, we performed liquid and membrane biomass transfer assays (LTC and MTC, respectively) to 2% wheat-based medium after 17 h growth in 2% WLM and 48 h growth on 2% WSM, respectively. Similar to the results shown in Fig. 1, the concentration of glucose and amino acids is low over time during MTC (Fig. 3A). The pH remained between 7,1 and 6,7 (Fig. 3B). The transcription of the *glaB, alpA*, and *nptB* genes was induced at all time points in MTC (Fig. 3C; lane 1-6) although transcript levels decreased more then 2 times over 12 h MTC (cf. lane 1 and 6). In LTC, the amino acid concentrations decreased from 4 mmol/l to low levels in the first 6 h and the maximum level of glucose was liberated from the substrate at 5.5 h (Fig. 3A). The presence of increasing amounts of glucose liberated from 2% WLM (Fig. 3A) during the first 5 h LTC suggested that within that time frame, sufficient levels of starch degradation products are available. In LTC, transcripts of the *glaB* gene disappeared at 0.5 h when glucose levels increased, and re-appeared at 6 h (Fig. 3C lanes 7-12).

The transcript level of the *alpA* and *nptB* genes decreased within the 1st h of LTC (Fig. 3C, lane 7-9) suggesting an overall degradation. Apparently, transcription of the *alpA* and *nptB* genes is not induced directly upon transfer. The results in Fig. 3 (A, C) show that transcription of *alpA* and *nptB* genes occurs when glucose concentrations are low, suggesting that glucose could act as a repressor for *alpA* and *nptB* transcriptional induction. However, the observation that transcription of the *alpA* and *nptB* genes occurs at 17 and 24 h of submerged cultivation, when glucose concentrations are still high, suggests otherwise (Figs. 1B; 2, lane 1-4). As shown in Fig. 3 (B, C), the pH of the submerged cultivation also changed during fermentation. Directly after transfer of the growing *A. oryzae* to 2% WLM, the growth medium

started to acidify (Fig. 3B), and at 6 and 8 h LTC the growth medium started to recover from acidity. At these time points, the *alpA* and *nptB* gene transcript levels also increased (Fig. 3C; lanes 10, 11). However, besides pH effects, specific induction at 6-8 h may also play a role as at 24 h LTC no transcription was observed although the pH was neutral.

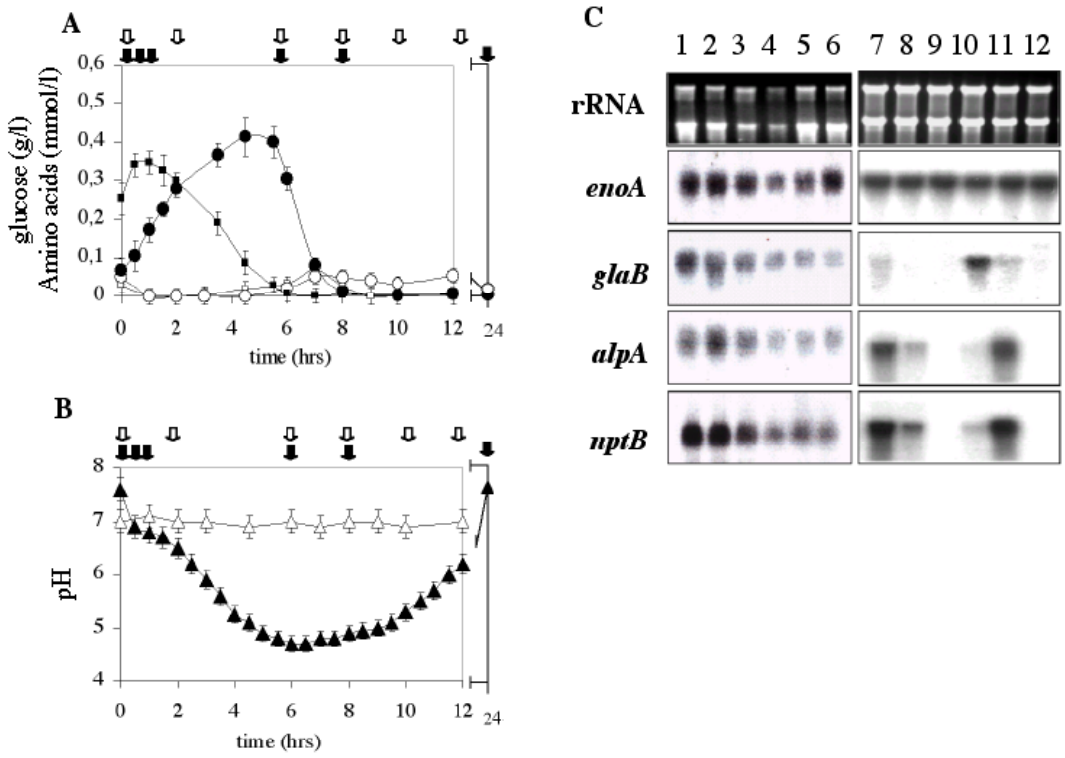


Fig. 3. A *Glucose (●○; g/l) and amino acid (▪▫; mmol/l) concentrations, and* **B** *pH (Δ▲) during membrane biomass transfer (MTC, open symbols) or liquid biomass transfer (LTC, closed symbols) over time. Open and closed arrows indicate the MTC and LTC samples, respectively, taken for C.* **C** *northern hybridisations with probes for the enoA, glaB, alpA and nptB genes with RNA time samples isolated from MTC and LTC. Lanes 1-6: MTC, 7-12 LTC, 1 0 h, 2 2 h, 3 6 h, 4 8 h, 5 10 h, 6 12 h, 7 0 h, 8 0.5 h, 9 1 h, 10 6 h, 11 8 h, 12 24 h.*

Effect of low-Aw, maltose and pH on *glaB*, *alpA* and *nptB* transcription. Low-Aw is a characteristic of SSF (Ishida et al. 1998). It was postulated that

transcriptional induction of the *glaB* gene is enhanced by low-Aw (Ishida et al. 2000). As not only the *glaB* gene but also the *alpA* and *nptB* genes were transcribed strongly during surface growth on 2% WSM, we examined the effect of low-Aw on transcription of these genes using MTC. We chose to study the effect of low-Aw at 6 h MTC, as transcript levels of the *glaB*, *alpA* and *nptB* genes after 6 h MTC were representative of induction during surface growth on 2% WSM (Fig. 3C). Low-Aw was achieved by supplementing 2% WSM with 5%, 10% and 40% maltose or 5M NaCl, resulting in Aw values of 0.98, 0.96, 0.93 and 0.93, respectively. The results of the MTCs are presented in Fig. 4. The *glaB* transcript level was about 2 and 4 times higher in 2% WSM with 5% (lane 2) and 10% (lane 3) maltose, respectively, and the *glaB* transcript level in 2%WSM with 5 M NaCl (lane 5) was the same in unsupplemented 2% WSM (lane 1) as determined after quantification of transcript levels. From these results we concluded that the *glaB* gene was induced not by the Aw lowering effect of maltose during surface cultivation, but by maltose directly. We cannot formally exclude that the lack of effect of NaCL on gene expression is due to a combination of counteracting effects, including Aw. Transcription of the *alpA* and *nptB* genes was not induced by 5% and 10% maltose in MTC. Surprisingly, MTC 2% WSM with 40% maltose showed equal transcript levels for the *glaB* gene, and five and six times lower transcript levels of the *alpA* and *nptB* genes (cf. lanes 3, 4). These lower *alpA* and *nptB* transcript levels were not due to the effects of low Aw (cf. lanes 1, 4, 5).

Further analysis of the environmental conditions of the maltose-supplemented surface cultivation revealed that the amount of sugar available in the 5%, 10 % and 40% maltose was high, and that in the 40% maltose cultures the pH was clearly lower than in the other cultures (Fig. 4). To analyse whether the observed decrease in the

alpA and *nptB* gene transcript level was due to pH regulation, LTC to 1% WLM was also carried out in the presence of 40% maltose. As shown in Fig. 4 (lanes 7, 8) similar effects were observed also in liquid cultivation regarding increased *glaB* and decreased *alpA* and *nptB* transcript levels, suggesting that *glaB* is induced by maltose while induction of the *alpA* and *nptB* genes is pH-mediated.

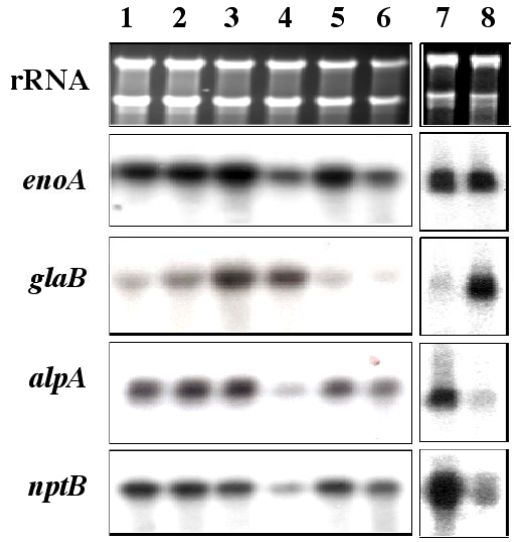


Fig. 4. *Effect of pH, external glucose, absence of aerial hyphae, and low water activity (Aw) on transcription of the enoA, glaB, alpA and nptB genes after 6 h MTC or LTC. Addition of maltose or NaCl resulted in low Aw. Lane: 1 MTC to 2% WSM (Aw = 0.99), 2 MTC to 2% WSM + 5% maltose (Aw = 0.98), 3 MTC to 2 %WSM + 10% maltose (Aw = 0.96), 4 MTC to 2% WSM + 40% maltose (Aw = 0,93), 5 MTC to 2% WSM + 5M NaCl (Aw = 0.93), 6 MTC with sandwich cultivation (SWC-without aerial hyphae) to 2% WSM, 7 LTC to 1% WLM, 8 LTC 1% WLM + 40% maltose.*

Effect of aerial hyphae on *glaB, alpA* and *nptB* transcription. Filamentous fungal morphology during surface cultivation is different from that in submerged cultivation. A strongly characteristic feature is the presence of aerial hyphae during surface cultivation. The role of the aerial hyphae in gene transcription during surface cultivation was investigated by suppression of aerial hyphae grown with the aid of an

oxygen permeable membrane in a 6 h MTC with sandwich cultivation (SWC). Northern analysis with mRNA samples from 6 h SWC showed no difference in transcript levels of the *glaB*, *alpA* and *nptB* genes compared to 6 h MTC (cf. lanes 1 and 6 in Fig. 4), indicating that the presence of aerial hyphae does not affect *glaB*, *alpA* and *nptB* gene transcription during surface cultivation.

DISCUSSION

In solid state and submerged fermentation, the environmental conditions for growth of *A. oryzae* differ in many aspects. In contrast to submerged fermentation, SSF is characterised by gradients of nutrients, oxygen, temperature and water availability (Iwashita 2002, te Biesebeke et al. 2002) and, as a consequence, differences in transcriptional regulation are expected. The only well studied example of a gene that shows high transcription in rice kernel SSF is the glucoamylase B encoding gene (Ishida et al. 2000). In the study presented here, we have carried out northern analysis showing that the transcription of the *A. oryzae* genes encoding *glaB*, alkaline protease (*alpA*) and neutral protease (*nptB*) is induced during growth in 2% WSM, and that the level of transcription is 4-fold higher in *A. oryzae* during WK SSF. In submerged cultivation with 2% WLM the transcription of the *alpA* and *nptB* genes were only transiently transcribed after 17 and 20 h growth. We have investigated some possible mechanisms for transcription of the *glaB, alpA* and *nptB* genes during surface cultivation on 2% WSM by comparing it to transcriptional induction in submerged cultivation in 2% WLM.

Southern analysis with a probe for the *nptB* gene (Table 1) showed 2 bands. Neutral proteases I (GenBank accession no AF099904), II (E05048) and III (AB041338) are isolated from *A. oryzae*. The sequence of a single gene fragment

from the *nptB* gene obtained by PCR with the primers shown in Table 1 showed 100% identity to neutral protease II, while gene sequence similarities to the neutral protease I and III genes were low (results not shown). A high degree of identity was observed between the neutral protease II protein (E05048) and a recently identified fourth neutral protease-like protein (CAE11758) of *A. oryzae* (E-value E-98, results not shown) indicating that 2 closely related copies of the neutral protease II gene could be present in the genome of our *A. oryzae* strain. Northern results obtained with a probe for the *nptB* gene showed one hybridising band in all analysis.

The glucose and amino acid concentrations available during surface cultivation in 2% WSM are low in comparison to those measured in submerged cultivation in 2% WLM (Fig. 1, 3A). An explanation for this difference could be that secreted enzymes are immobilised in the substrate during surface cultivation. Consequently, their hydrolytic activity is limited to substrate in the close vicinity of the penetrating hyphae. The low levels of glucose and amino acids liberated from 2% WSM are probably directly consumed by the filamentous fungal mycelium, also explaining the slower biomass formation in surface cultivation, as seen in Fig. 1. The absence of free glucose in 2% WSM may result in increased levels of gene expression due to the absence of carbon catabolite repression. In contrast to *glaB*, the transcriptional induction of *alpA* and *nptB* was observed even in the presence of high concentrations of sugar (Fig. 2, 4) suggesting that the *alpA* and *nptB* genes are not regulated by carbon catabolite repression like that mediated by the negatively-acting transcriptional regulator CREA (Dowzer et al. 1991).

Ishida et al. (2000) postulated that *glaB* transcription is induced by the low Aw that results from addition of maltose to MTC. The Northern analysis (Fig. 4) confirmed that transcription of *glaB* is also induced in fungal mycelium during MTC

to 2% WSM supplemented with 5%, 10% and 40% maltose (Aw = 0.98, 0.96 and 0.93, respectively) compared to MTC to 2% WSM (Aw = 0.99). Although 2% WSM with 40% maltose has the same low Aw as 2% WSM with 5M NaCl, *glaB* transcript level in MTC to 2% WSM with 5M NaCl were equal to those in MTC to 2% WSM. Therefore, we conclude that transcriptional induction of *glaB* in the fungal mycelium during MTC to 2% WSM supplemented with maltose is due to an effect of maltose other than the induction of low-Aw.

Small oligosaccharides, such as maltose, isomaltose, maltotriose and dextrin, have been shown to induce *glaB* gene transcription (Ishida et al. 1998). As expected, the transcription of the *glaB* gene was induced in LTC to 1% WLM with 40% maltose (Fig. 4, lane 8) and in MTC to 2% WSM with 40% maltose (Fig. 4, lane 4), at glucose concentrations of 1 and 2,2 g/l, respectively. In agreement with a deletion analysis of the *glaB* promoter (Ishida et al. 2000), these results show that glucose repression did not overrule maltose induction (Fig. 4, lanes 2-4, 8). The results obtained with LTC to 2% WLM suggest that starch can not induce *glaB* transcription (Fig. 3C, lane 7-9) but that the breakdown products of starch that appeared in the culture medium after 6 h of LTC (Fig. 3C, lane 10) induced *glaB* gene expression. This proposed induction of *glaB* transcription by starch breakdown products suggests that the induction of *glaB* gene expression appears in surface cultivation on 2%WSM (Fig. 3, lanes 1-6) is due to the continuous presence of inducer. Therefore, we propose that the processes of carbohydrate breakdown and consumption differ in 2% WLM submerged and 2% WSM surface cultivation.

As described previously, the promoter region of the *glaB* gene contains cis-acting elements mediating maltose induction, and two heat shock elements (f-HSE) that have been shown to be essential for the high level transcriptional induction in rice

kernel SSF. Interestingly, in the promoter region of the *A. oryzae alpA* gene (D10062), 3 potential f-HSE motifs (Mary et al. 1994) in the stretch from –149 to –123 were also found, suggesting that the high level transcriptional induction of the *glaB* and *alpA* genes during wheat kernel SSF is mediated by these potential f-HSE motifs.

Under aerobic conditions, the cellular metabolism of filamentous fungi involves a net intracellular production of protons that takes place by the formation of tricarboxylic acid cycle acids, CO_2/H_2CO_3 and protein synthesis (Sanders et al. 1982). Consequently, proton fluxes occur across cellular membranes to maintain intracellular pH homeostasis (Hesse et al. 2002). Upon transfer of growing *A. oryzae* biomass to 2% WLM, the intracellular pH decreases due to metabolic activity and, as a consequence of proton fluxes across the extra cellular membrane, the growth medium started to acidify. The extracellular medium no longer acidifies at 6 h LTC when glucose concentrations are low, and subsequently recovers from acidity. In surface cultivation (Fig. 1A, 3A, B), less sugar is available, *A. oryzae* grows more slowly and the pH of the growth medium remains between 6.5 and 7.1, indicating a balance between growth and metabolism.

Transcription of the *alpA* and *nptB* genes was induced in surface cultivation when the extracellular pH was about 6,8 and in LTC when the pH recovered from acidity. This suggested that *alpA* and *nptB* gene transcription is regulated by pH. As described previously, pH regulation is mediated by the pH-responsive wide domain regulator PACC (Penalva et al. 2002). The possible involvement of PACC is sustained by the presence of a potential PACC binding site (GCCAAG) at position –685 in the promoter sequence of the *alpA* gene (GenBank accession no D10062). Unfortunately, the sequence upstream of *nptB* is not available. The pH regulatory

mechanism of PACC (Penalva et al. 2002) in *alpA* and possibly *nptB* transcriptional induction may explain in part the observed differences in transcript levels in submerged and surface cultivation in wheat based medium presented in this paper.

CHAPTER 5

Identification of growth phenotype related genes of *Aspergillus oryzae* by using heterologous macroarray and suppression subtractive hybridization

R te Biesebeke, A Levin, C Sagt, J Bartels, T Goosen, A Ram, CAMJJ van den Hondel, PJ Punt (2005) Identification of growth phenotype related genes of *Aspergillus oryzae* by using heterologous macroarray and suppression subtractive hybridisation. *Mol Genet Genomics. Published online 28 feb 2005.*

ABSTRACT

Aspergillus oryzae requires polarised growth for colonisation of solid substrates, and this growth phenotype differs from that seen in liquid medium. Various experimental approaches were used to identify genes that are differentially expressed when *A. oryzae* is grown on wheat kernels (WK) and in a wheat-based liquid medium. Hybridisation of *A. oryzae* RNAs to a macroarray, bearing cDNAs isolated from a library representing at least 16% of the total number of *A. niger* genes, identified 14 differentially expressed cDNA clones. These results show that a heterologous macroarray analysis with an *A. niger* cDNA library can be used to identify regulated gene transcripts in the related species *A. oryzae*. Moreover, northern analysis with a selection of eight probes for *A. niger* genes encoding proteins involved in morphological development and cell wall biosynthesis identified five more differentially expressed genes. A suppression subtractive hybridisation procedure revealed another 12 differentially expressed genes. The results presented show that, of the 29 identified genes which are expressed at higher levels during growth on WK, six encode proteins that are functionally related to polarised growth, four encode products known to be involved in morphogenesis, three code for proteins related to cell wall composition, and nine of the cDNA clones encode novel proteins. These findings pinpoint genes associated with the changes in cellular morphogenesis seen in *A. oryzae* grown on WK as opposed to wheat-based liquid medium.

INTRODUCTION

Aspergillus oryzae has a fast rate of tip growth (>60 μm/h; Müller et al. 2002) and a high productive capacity with respect to polarised secretion of enzymes (Machida 2002). Owing to these properties *A. oryzae* can easily colonise solid

substrates by the extension of existing hyphae and the formation of new branches on pre-existing hyphae (Trinci 1974; Prosser 1994; Dynesen and Nielsen 2003). Nutrients that are essential for growth are liberated from the substrate during invasion by the penetrating hyphae. Oxygen is taken up from the air mainly by the aerial hyphae (Rahardjo et al. 2001). In contrast to the case during growth in liquid media, enzymes are trapped in the solid substrate and nutrients are only liberated in close proximity to growing penetrative hyphae. Due to these differences in the availability of nutrients for growth, the growth phenotype of *A. oryzae* on solid substrates clearly differs from that in liquid medium. Comparisons of the transcript profiles expressed under these different cultivation conditions will allow the identification of genes associated with these differences in the morphology of growing *A. oryzae* cells.

The growth phenotype is the result of a number of cellular processes, including polarised growth, cell differentiation, signal transduction, cell wall synthesis, polarised secretion and maintenance of cell shape, that are controlled by finely tuned molecular mechanisms that involve many gene products (Seiler and Plamann 2003). Previous studies of *A. oryzae* have identified genes for proteins involved in substrate degradation, metabolism, transport and cell wall synthesis associated with surface growth (Akao et al. 2002; te Biesebeke et al. 2004a). Since little is known about the microbial growth phenotype during solid state fermentation (te Biesebeke et al. 2002; Holker et al. 2004), we were interested in identifying genes associated with the growth of *A. oryzae* on a solid substrate. We have used a number of different experimental approaches, including macroarray hybridisation, to identify genes expressed differentially in *A. oryzae* grown on wheat kernels (WK) and in a wheat-based liquid medium. One of these approaches involved the use of a cDNA library

from *A. niger* to perform a heterologous macroarray analysis, since no *A. oryzae* cDNA library was available.

MATERIALS AND METHODS

Strains and media. *Aspergillus oryzae* ATCC16868 was used in this study. Solid state fermentation (SSF) was performed using Ritmo wheat kernels (WK) (ACM, Meppel, The Netherlands) pretreated as described by Hoogschagen et al. (2001). A 50 g sample of pretreated WK was inoculated with 2 ml of a conidial suspension ($5X10^7$ conidia/ml, giving a starting density of $1.0X10^6$ conidia/g of substrate) in a sterile 1-l bottle, which was placed on a roller apparatus (Wheaton Modular Roller; Wheaton Science Products, Millville, N.J.) and rotated at maximum speed for 6 h at room temperature. The inoculated WK were then transferred to sterile petri dishes and incubated for 2, 2.5 or 3 days at 30°C and 98% humidity in a climate incubator (VEA-Instruments, Houten, The Netherlands). Wheat-based Liquid Medium (WLM; 2%) was prepared as described by te Biesebeke et al. (2004a). Submerged fermentations (smF) were performed using 2% WLM inoculated with $1X10^5$ conidia/ml medium and incubated for 24, 30 and 35 h at 30°C on a rotary shaker (250 rpm).

Purification of total RNA. Total RNA from *A. oryzae* grown on WK and in 2% WLM was isolated as described by te Biesebeke et al. (2004a).

Synthesis of ^{32}P labeled first-strand cDNA. First-strand cDNAs (probes) were synthesised from isolated total RNA with Superscript II H- Reverse Transcriptase (Invitrogen, Carlsbad, Calif.) according to a modification of the manufacturers protocol. An aliquot (25–30 μg) of total RNA was mixed with μl of (dT)15 mix (containing (dT)15, (dT)15 A, (dT)15 G and (dT)15 C, each at 1 mg/ml)

in 11 µl of distilled water. The mixture was heated to 70°C for 10 min and cooled on ice. On ice, 3 µl of (lowC)dNTP mix (containing 2.5 mM dATP, 2.5 mM dGTP, 2.5 mM dTTP and 16.6 µM dCTP) and 6 µl of 5X first-strand buffer was added. The reaction mixture was incubated for 2 h at 43°C after the addition of 5 µl of [α ^{32}P]dCTP (~50 µCi) and 2 µl of Superscript II enzyme. Then RNase was added (to a final concentration of 10 mg/ml), and incubation continued for 15 min at 37°C. Probes were purified by passage over Sephadex G50 columns (Amersham Biosciences, Little Chalfont, Bucks., UK), denatured for 2 min at 95°C and used for hybridization.

DNA macroarray. The library of *Aspergillus niger* cDNAs used here was constructed in the yeast/ *Escherichia coli* shuttle vector pEMBLyex4 (Goldman et al. 1992) as described by Veldhuisen et al. (1997). Transformed *E. coli* DH5 α (Sambrook et al. 1989) colonies containing the *A. niger* cDNAs (Veldhuisen et al. 1997) were picked at random from selective L-medium plates and distributed into twelve 384-well plates containing L-medium and ampicillin (100 µg/ml) and grown overnight at 30°C. In all, 4608 cDNA clones were inoculated onto a Hybond-N+ membrane with a 96-pin tool containing array spotting robot from Engineering Services Inc. (Toronto, Ont., Canada). The membrane was placed on an OMNI-TRAY (Nunc, Wiesbaden, Germany) containing 50 ml of selective L-medium agar and incubated overnight at 30°C. The resulting colonies were lysed by alkaline lysis using a modification of the method of Clak et al. (1999). Membranes were placed on a sheet of Whatman 3MM paper moistened with 0.5 M NaOH/1.5 M NaCl for 4 min at room temperature, followed by a 4-min incubation over a steaming (85°C) water bath. The membranes were neutralised for 4 min with 1 M TRIS-HCl (pH 7.5)/1.5 M NaCl at room temperature, and transferred to a buffer containing 50 mM TRIS-HCl

(pH 7.5), 50 mM EDTA, 100 mM NaCl, 1% Sarkosyl and 250 µl/ml Proteinase K, and incubated for 20 min at 37°C. The cDNAs released from the cells were covalently bound to the membrane by exposure to a UV Crosslinker (Stratagene, La Jolla, Calif.). After hybridization with ^{32}P labeled first-strand cDNA at 60°C in phosphate-buffered hybridization solution (20% SDS, 200 mM NaCl, 5% BSA, pH 7), the membranes were washed twice at 65°C with 2XSSC and 0.5% SDS (Sambrook et al. 1989) for 30 min, and twice at 65°C with 0.2XSSC and 0.5% SDS for 1 h. The signals on the membranes were visualised after exposure to a phosphoimager screen for 5, 10, 15, 20, 25 and 30 min using the Cyclone (Packard Instruments Division, Applied Biosystems, Foster City, Calif.) storage phosphoimager system and the ImaGene 4.2 software from BioDiscovery (Marina del Rey, Calif.). The membranes that showed equal signal intensities for *A. niger gpdA* cDNAs at 50% of the maximum spot intensity as quantified with the ImaGene 4.2 software were compared. *A. niger* cDNAs that showed differential hybridisation intensities (>50% difference) with probes synthesised from total RNA isolated from *A. oryzae* grown on WK and in 2% WLM were selected for further analysis.

Spot-blot analysis. The cDNA clones selected from the macroarray analysis were isolated with the Miniprep plasmid isolation kit (Qiagen, Hilden, Germany), and a 2 µl aliquot of each plasmid preparation was spotted onto Hybond-N+ membranes and covalently bound to the membranes by irradiation with the UV-cross-linker (Stratagene). The membranes were subsequently used for hybridisation experiments (under the same conditions as used for the DNA macroarray) with ^{32}P labeled first-strand cDNA. The blots were exposed to X-OMAT AR films (Kodak, Rochester, N.Y.) for 2–48 h. The developed films were scanned with the Hewlett Packard 6200C Scanjet at 600 dpi. Films showing the *gpdA* signals equivalent to 50% of the

maximum spot intensity, as quantified with Gene Tools from Syngene (Synoptics, Cambridge, UK), were used for the analysis of differential transcription. The signal intensity of the *gpdA* spot was set at 100 and the signal intensities of the other spots on the same blot were normalised with respect to the *gpdA* signal. Furthermore, ratios of spot intensities of cDNAs were determined. The ratio smF for a given cDNA was obtained by dividing the signal intensity obtained with a probe from *A. oryzae* grown in 2% WLM by that observed after hybridisation with a probe originating from *A. oryzae* grown on WK. The ratio SSF is the converse ratio.

Construction of a subtraction cDNA library. cDNA was synthesised from total RNA with the SMART PCR cDNA synthesis Kit (Clontech, Palo Alto, Calif.) according to the suppliers protocol. Starting from two populations of total RNA obtained from *A. oryzae*, a cDNA subtraction library was constructed using the PCR-select cDNA subtraction kit (Clontech), which is based on the suppression subtractive hybridization method of Diatchenko et al. (1996), according to the suppliers protocol. For the forward subtraction protocol cDNA produced from total RNA isolated from *A. oryzae* grown for 3 days on WK was used as the tester together with driver cDNA produced from total RNA from *A. oryzae* grown for 30 h in 2% WLM. The forward subtraction protocol is designed to enrich for cDNAs derived from transcripts that are expressed at higher levels in *A. oryzae* grown on WK. The cDNAs were cloned in pGemT-easy (Promega, Madison, Wis.) and used to transform *E. coli* DH5α (Sambrook et al. 1989). Individual *A. oryzae* cDNA clones (380) were randomly picked from selective L-medium agar plates, and four individual *A. niger gpdA* cDNA clones were taken from the ordered *A. niger* cDNA library. These clones were transferred to a 384-well plate containing selective L-medium and grown overnight at 37°C. The 384 cDNA clones were then inoculated manually onto a Hybond-N+

membrane, incubated overnight at 30°C, and lysed as described above. Hybridisation with ^{32}P-labelled first-strand cDNA and washing of the membranes was performed as for the macroarray membranes, except that both steps were carried out at 68°C. The signal intensities of the spots were quantified and compared after equalising the signal intensities of the *A. niger gpdA* cDNAs on the different membranes as described earlier. In all, 79 of the cDNAs showed detectable hybridization with ^{32}P-labeled probes. cDNAs that showed three-fold higher hybridisation with probes originating from *A. oryzae* grown on WK compared to probes originating from *A. oryzae* grown in 2% WLM were selected for further analysis.

PCR and DNA sequencing. PCR was used to amplify the cDNA inserts from the clones. PCR was performed with the primers MBL1589 (5'-GGATCAATTCGAGCTCGGTA-3') and MBL1588 (5'-ATTGGATCATAAGCTTGC-3') for the *A. niger* cDNA clones, and the primers M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3') for the *A. oryzae* cDNA clones, using 40 cycles of 30 s at 94°C, 1 min at 45°C and 30 s at 72°C. Probes for the cDNA clones were purified from 1% agarose gels after electrophoresis, using Qiaquick DNAeasy columns (Qiagen). Sequencing was performed with primer MBL1589 or primer MBL1588 to obtain the *A. niger* cDNA sequences or with primer M13F or primer M13R to obtain the *A. oryzae* cDNA sequences. The Cycle Sequencing kit from Amersham Pharmacia was used according to the manufacturers protocol, and sequences were determined using the ABI Prism 310 Genetic Analyzer from Applied Biosystems (Perkin-Elmer Division). The sequences obtained were used for Blast searches in the databases at NCBI (http://www.ncbi.nlm.nih.gov), the *A. oryzae* EST site (http://www.nrib.go.jp/ken/EST/db/blast.html), the Broad Institute

(http://www.broad.mit.edu/annotation/fungi/fgi) or the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). GenBank Accession Nos were obtained after submission of the sequences to the EMBL-EBI (http://www.ebi.ac.uk/Submissions/) or NCBI database.

Northern analysis. Total RNA (10 μg) was fractionated by electrophoresis on formaldehyde-agarose (1%) gels, and transferred to Hybond-N+ membranes (Amersham Bioscience). The fractionated RNA was hybridised at 60°C (in the hybridisation and wash buffers used for the macroarray analysis) with [α^{32}P]dCTP (~50 μCi)-labeled probes, synthesised using the Random Prime labeling kit (Amersham Pharmacia). Blots were exposed to X-OMAT AR film and the exposed films were scanned as described above. The signal intensities were quantified with Gene Tools from Syngene (Synoptics) after adjustment to the *gpdA* signal intensity and the ratio smF and ratio SSF were determined as described above.

Probes for *A. niger* genes. Fragments of the genes for α-1,3-glucan synthases (*agsA*, *agsB*) and β-1,3-glucan synthase (*fksA*) were isolated by PCR using degenerate primers that were based on conserved amino acid sequences (R. A. Damveld and A. F. J. Ram, unpublished). Fragments of putative MAP kinase genes were isolated by PCR using degenerate primers based on conserved amino acid sequences of the HOG family of MAP kinases (*hogA* and *hogB*) (M. Arentshorst and A. F. J. Ram, unpublished). Fragments of two genes for Rho-related small GTPases were isolated using degenerate primers based on conserved amino acid sequences (*racA*, Punt et al. 2001; *cftA*, A. F. J. Ram, unpublished). A gene encoding a putative GPI-anchored cell wall mannoprotein was identified in a collection of *A. niger* ESTs deposited in GenBank (Database accession number BE759683, Tsang and Storm, unpublished). Primers based on this sequence were used to clone a fragment of this gene (*cwpA*, R.

A. Damveld and A. F. J. Ram, unpublished). All DNA fragments were amplified from *A. niger* genomic DNA, with the exception of *cftA* and *cwpA*, which were isolated from the *A. niger* cDNA library (Veldhuisen et al. 1997). All the DNA fragments were cloned in pGemT easy (Promega) and sequenced to confirm their identity.

RESULTS

Macroarray analysis. To determine the complexity of an ordered collection of 4608 *E. coli* DH5α clones containing *A. niger* cDNAs (Veldhuisen et al. 1997), 50 of the clones were randomly chosen and their inserts were sequenced. Analysis of the sequencing data revealed that 29 cDNA sequences appeared only once among the 50 cDNA clones. Of cDNA sequences that were present more than once, six cDNAs corresponded to *A. niger* ribosomal RNA and two to the *A. niger gpdA* gene. Although these results indicate that the library includes a number of redundant clones, they imply that it contains 2950 (±600, for a confidence interval of 95%) unique cDNAs. More than 14,000 ORFs have been identified during the *A. niger* Genome Sequencing Project (http://www.aspergillus.man.ac.uk), suggesting that our collection represents 16–25% of the protein-coding genes present in *A. niger*. We considered this amount sufficient to justify its use as a heterologous macroarray for the identification of growth phenotype-specific genes in *A. oryzae*.

The 4608 *A. niger* cDNAs were spotted on several Hybond-N+ membranes. To further evaluate the complexity of the cDNA library, probes for two highly expressed genes, the *A. niger gpdA* and *bipA* genes, were used to screen the cDNAs. Of the 4608 cDNAs screened, 4% (188 cDNAs) hybridised with the *gpdA* probe and 0.3% (13 cDNAs) with the *bipA* probe, confirming the presence of a fraction of redundant clones in the library.

The membranes were then used to identify differentially expressed genes in *A. oryzae* grown for 3 days on WK and for 30 h in 2% WLM. cDNA robes synthesised from the total RNA of *A. oryzae* grown under these two conditions were hybridised to the *A. niger* library on the membranes. In *A. oryzae* housekeeping genes, like the glyceraldehyde-3-phosphate dehydrogenase encoding gene *gpdA*, are among the most highly expressed genes (Machida 2002). Since the transcript levels of the *gpdA* gene of *A. oryzae* grown on different carbon sources are similar (Nakajima et al. 2000), the intensity of the signals for *A. niger gpdA* clones was used to adjust spot intensities on the different membranes. Furthermore, the *A. oryzae gpdA* cDNA (GenBan accession no AB032274) shows 88% identity to the nucleotide sequence of the *A. niger gpdA* gene (X99652), which guarantees hybridisation under the chosen conditions. Based on inspection of the membrane images, 46 *A. niger* cDNA clones were selected, of which 34 showed a >50% higher signal after hybridisation with cDNA probes from *A. oryzae* grown on wheat kernels than with cDNA from liquid cultures. The other 12 clones showed >50% higher spot intensities after hybridisation with cDNA probes from *A. oryzae* grown in 2% WLM than with probes from solid state cultures.

Spot-blot, Northern and sequence analyses. To determine if the 46 selected *A. niger* cDNAs also hybridised differentially with probes originating from *A. oryzae* grown on WK and in 2% WLM at different time points during culture, the selected clones and a *gpdA* cDNA clone were spotted on Hybond-N+ membranes. The spot blots were then hybridised with probes synthesised from total RNA isolated from *A. oryzae* grown for 2, 2.5 and 3 days on WK or for 24, 30 and 35 h in 2% WLM. Comparison of the signal intensities showed that, of the 46 selected cDNAs, 15 cDNAs showed about equal signal intensities and 17 cDNAs showed differences in signal intensities, but the patterns were inconsistent when the various signal intensities

were compared over the time course. Of the remaining 14 cDNAs, 12 showed higher signal intensities with all probes from *A. oryzae* grown on WK and two cDNAs showed higher signal intensities with probes from cultures grown in 2% WLM. To verify the results obtained for the macroarray and spot-blot analysis, northern hybridisation analysis was performed with total RNA from *A. oryzae* grown on WK for 3 days or in 2% WLM for 30 h, using the 14 identified cDNAs from *A. niger* as probes. The results presented in Fig. 1A are in agreement with the results obtained with the macroarray and the spot-blot analysis. These 14 cDNAs were sequenced and subjected to BLAST analysis. The results are presented in Table 1. Northern analysis with the probes for the 4,5-dihydroxyphthalate decarboxylase (SSF10) and the DNAJ domain (SSF12) encoding genes (Fig. 1A) detected two bands of different sizes indicating that these genes express different mRNA products.

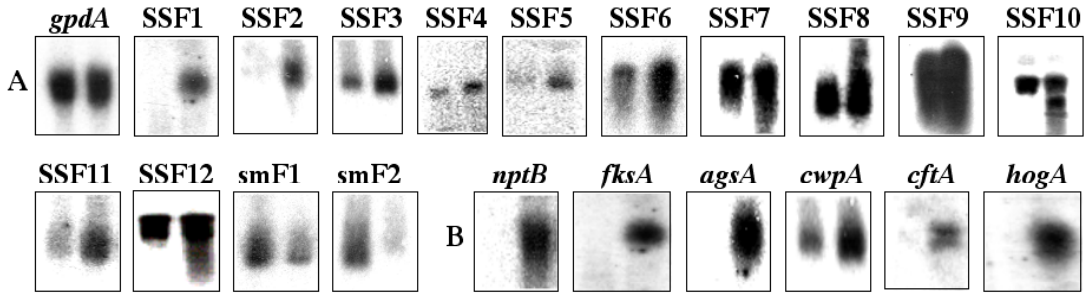


Fig. 1. *Differential hybridisation of A. niger probes with total RNA from A. oryzae grown for 3 days on wheat kernels (WK) (right lane in each panel) or for 30 h in 2% WLM (left lane in each panel). (***A)** *Northern analysis with probes for A. niger cDNAs (SSF1–12, smF1, smF2). A probe for the A. niger glyceraldehyde-3-phosphate dehydrogenase gene was used as a reference.* **(B)** *Northern analysis with probes for the A. niger genes for β-1,3-glucan synthase (fksA), α-1,3-glucan synthase (agsA), cell wall mannoprotein (cwpA), Cdc42 rho-related small GTPase (cftA) and HOG-family MAP kinase (hogA). The probe for the A. oryzae nptB gene, which was known to be differentially expressed under these conditions (te Biesebeke et al. 2004b), was included as a reference.*

Clone name	Length (bp)	GenBank no	First HIT Blast ID [A]	Positives (%) [B]	Blastp ID with First hit Blast protein [C]	E-value	Ratio SSF [D]	Ratio SmF [D]	AoEst [E]	BlastN [F] Positive
gpdA	349	CAA67966	Glyceraldehyde-3-phosphate dehydrogenase CAA67966 *(A. niger)*	110/110 (100 %)	Glyceraldehyde-3-phosphate dehydrogenase CAA67966 *(A. niger)*	0.0	1	1	-	-
SSF1	540	CK769166	Hypothetical protein EAA48840 *(Magnaporthe grisae)*	58/88 (65%)	DNA helicase AAA7040.1 *(Homo sapiens)*	e^{-100}	>5	-	-	-
SSF2	508	CK769167	Hypothetical protein EAA63566 *(Aspergillus nidulans)*	31/51 (60%)	Actin cytoskeleton organization and biogenesis pin3 protein NP595286.1 *(Saccharomyces cerevisiae)*	$4e^{-7}$	>5	-	00576	68/76
SSF3	311	CK769168	Unknown gene	-	-	-	1.7	-	02571	219/225
SSF4	329	CK769169	Unknown gene	-	-	-	2	-	-	-
SSF5	260	CK769170	Unknown gene	-	-	-	2.5	-	-	-
SSF6	233	CK769171	Hypothetical protein EAA62187.1 *(A. nidulans)*	44/46 (95%)	Putative senescence associated protein BAB33421.1 *(Pisum sativum)*	$1e^{-4}$	3	-	02787	271/275
SSF7	333	AJ632136	Hypothetical protein EAA60158.1 *(A. nidulans)*	78/135 (58%)	40S ribosomal protein CAA21965 *(Candida albicans)*	$6e^{-97}$	1.5	-	03131 & 00803	127/158 & 127/158
SSF8	469	AJ632137	Hypothetical protein EAA 56294.1 (*M. grisae)*	29/50 (58%)	Prephenate Dehydratase ZP00065118 *(Microbulferdegradans)*	$1e^{-24}$	1.6	-	-	-
SSF9	304	AJ627189	Hypothetical protein EAA66371.1 *(A. nidulans)*	54/72 (75%)	Eukaryotic translation elongation factor 1 BAB64568 *(Carassius auratus)*	$5e^{-36}$	1.5	-	-	-
SSF10	455	AJ632138	4,5-dihydroxyphthalate decarboxylase BBA03974.1 *(Comamonas testosterone)*	34/102 (53%)	4,5-dihydroxyphthalate decarboxylase BBA03974.1 *(Comamonas testosterone)*	0.0	>5	-	-	-
SSF11	304	AJ632139	orf403 NP 074914 *(Podospora anserine)*	55/92 (59%)	Endonuclease NP 570153.1 *(Hypocrea jecorina)*	$1e^{-88}$	1.5	-	-	-
SSF12	483	AJ632140	Hypothetical protein XP 322593.1 *(Neurospora crassa)*	52/87 (59%)	Tail-anchored ER membrane protein, similar to the DNAJ protein; Hlj1p. NP 013884 *(S. cerevisiae)*	$1e^{-19}$	1.8	-	-	-
smF1	647	CK769172	Unknown gene	-	-	-	-	3	-	-
smF2	522	CK769173	Hypothetical protein EAA65208.1 *(A. nidulans)*	72/87 (83%)	Unknown protein		-	>5	03162 & 00225	181/220 & 145/173

Table 1. *Identified A. niger cDNAs that were selected in the heterologous macroarray analysis.* [A]*Best result obtained by BLASTX analysis using the cDNA sequence as a query in the GenBank at NCBI.* [B]*Number of (percentage) amino acid sequence identities to the best BLAST hit in the segment compared.* [C]*cases where a hypothetical protein emerged as the best BLAST hit, the full-length hypothetical protein was used as the query in a BLASTP search to determine its best hit. The e-value refers to this comparison.* [D]*SSF is the ratio of the expression level under solid state growth conditions to that in liquid medium; smF is the converse ratio. These data are based on the results of Northern analysis (see Materials and methods).* [E]*Accession No(s). of homologous EST(s) in the A. oryzae EST database (http://www.nrib.go.jp/ken/EST/db/blast.html).* [F]*Nucleotide sequence identities to EST(s) from A. oryzae, based on BLASTN analysis.*

Northern analysis with probes for genes encoding proteins involved in cell morphology and cell wall biosynthesis. The growth phenotype of *A. oryzae* on WK differs markedly from that of cultures grown in 2% WLM. To gain more insight into the type of genes that are expressed in each situation we performed a heterologous northern analysis with a selection of probes prepared from genes previously isolated (Table 2). Probes for genes encoding MAP kinases (*hogA* and *hogB*) and two Rho-related small GTPases (*cftA* and *racA*) were selected, as their protein products are assumed to be involved in morphological development of *A. oryzae*. Probes for the genes encoding β-1,3-glucan synthase (*fksA*), a cell wall mannoprotein (*cwpA*), and two α-glucan synthases (*agsA* and *agsB*) are involved in the maintenance of cell wall integrity. The labelled probes were hybridised to northern blots loaded with total RNA from *A. oryzae* grown for 3 days on WK or for 30 h in 2% WLM. The results are shown in Fig. 1B. Transcripts of the genes *hogA*, *fksA*, *agsA* and *cftA* could only be detected in *A. oryzae* grown on WK, and the level of the *cwpA* transcript was higher in *A. oryzae* grown on WK. The transcripts of the *racA*, *hogB*, and *agsB* genes were below the limit of detection (data not shown).

Differentially transcribed *A. oryzae* genes. Northern analysis of total RNA from *A. oryzae* with selected cDNA probes from *A. niger* (Fig. 1) revealed differences in hybridisation. It seems probable that these differences reflect differential expression of the corresponding genes in *A. oryzae*. To support this, the nucleotide sequences of the *A. niger* cDNAs were used in a homology search (BLASTN) of an *A. oryzae* EST library (http://www.nrib.go.jp/ken/EST/db/blast.html). The results presented in Tables 1 and 2 show that in about 50% of the cases the *A. niger* cDNAs have sequences similar to an EST sequence from *A. oryzae*. Based on the level of sequence identity

between the *A. niger* cDNA and the *A. oryzae* EST, hybridisation of these sequences is indeed expected under the conditions used in our screens (Howley et al. 1979).

Gene	Length (bp)	GenBank no	Gene ID [A]	Ratio [B] SSF	Ratio [B] SmF	AoEst [C]	BlastN [D] Positives
FksA	365	AY533027	Beta 1,3-glucan synthase	> 5	-	01813	185/218
AgsA	678	AY530786	Alpha 1,3-glucan synthase	> 5	-	06191	40/45
AgsB	762	AY530789	Alpha 1,3-glucan synthase	-	-	-	-
CwpA	1155	AY540627	Cell wall mannoprotein	3	-	02990	129/132
CftA	756	AY540628	Rho-related small GTPases	> 5	-	01954	278/340
RacA	968	AY540629	Rho-related small GTPases	-	-	01170	77/86
HogA	547	AY540624	Hog family of MAP kinases	> 5	-	04179	169/196
HogB	509	AY540625	Hog family of MAP kinases	-	-	-	-

Table 2. *A. niger cDNAs used as probes for heterologous northern analysis of A. oryzae RNA.* [A]*All A. niger cDNA and DNA fragment sequences were used in a BLASTN search of the A. oryzae EST database (http://www.nrib.go.jp/ken/EST/db/blast.html). The probes were chosen on the basis of sequence identity to A. oryzae ESTs indicating that cross-hybridization of these sequences can be expected under our experimental condition (Howley et al. 1979).* [B]*The ratios SSF and smF were calculated from the northern analysis results as described in materials and methods.* [C]*Accession No. of A. oryzae EST.* [D]*Degree of nucleotide sequence identity between A. niger cDNA/DNA and A. oryzae EST*

Suppression Subtraction Hybridisation. We also used a different strategy to identify differentially expressed genes associated with the growth phenotype of *A. oryzae*. In this second approach, a cDNA library was constructed that was enriched for cDNAs synthesised from total RNA isolated from *A. oryzae* grown for 3 days on WK by using the suppression subtractive hybridisation method (Diatchenko et al. 1996) with cDNAs synthesized from *A. oryzae* grown in 2% WLM for 30 h. The method combines subtraction and normalisation in a single procedure. The *A. oryzae* subtraction cDNA library, consisting of 380 *A. oryzae* cDNA clones, was spotted together with four *A. niger gpdA* cDNA clones onto several Hybond-N+ membranes. A control hybridisation with the labelled *A. niger gpdA* probe showed that only the

four *A. niger* cDNAs hybridised, suggesting that subtraction of the *A. oryzae gpdA* cDNAs had been successful. Hybridisation of the membranes with probes synthesised from total RNA isolated from *A. oryzae* grown for 3 days on WK or for 30 h in 2% WLM revealed 79 spots. To identify genes that were most relevant for the difference in growth phenotype, 12 spots with an SSF ratio of more than 3 were selected, and the corresponding cDNAs were sequenced. The results are presented in Table 3.

Clone Name	Length (bp)	GenBank no	First HIT Blast ID [A]	Positives (%) [B]	Blastp ID with First hit Blast protein [C]	E-value	Ratio SSF [D]	AoEst [E]
SSF13	208	CV066918	Hypothetical protein EAA59495.1 *(A. nidulans)*	59/61 (96%)	COPI-coated vesicle related protein YJL123C *(S. cerevisiae)*	2e-19	>3	00954
SSF14	370	CV066919	Hypothetical protein EAA53697.1 *(M. grisea)*	38/79 (48%)	CsgA protein AAA25391.2 *(Myxococcus xanthus)*	3e-29	>3	-
SSF15	571	CV066920	Hydrophobin CoH1 CAA71652.1 *(Coprinopsis cinerea)*	44/101 (43%)	Hydrophobin CoH1 CAA71652.1 *(Coprinopsis cinerea)*	0.0	>3	02217
SSF16	323	CV066921	Ca2+-binding actin-bundling protein Fimbrin NP_990678.1 *(Gallus gallus)*	45/78 (57%)	SAC6 fimbrin homolog *(S. cerevisiae)*	2e-113	>3	02824
SSF17	322	CV066922	Hypothetical protein XP_409823.1| *(A. nidulans)*	82/106 (77%)	*TPM*2 tropomyosine *(S. cerevisiae)*	3e-25	>3	02851
SSF18	199	CV066923	Septin B AAB41233.1 *(A. nidulans)*	65/66 (98%)	Septin B AAB41233.1 *(A. nidulans)*	0.0	>3	07110
SSF19	296	CV066924	Hypothetical protein AN5422.2 *(A. nidulans)*	28/33 (84%)	Putative esterase NP_945813.1 (*Rhodopseudomonas palustris*)	3e-48	>3	01498
SSF20	416	CV066925	Hypothetical protein EAA54143.1 *(M. grisea)*	89/112 (79%)	Cyclopentanone 1,2-monooxygenase CAD10798 (*Comamonas testosteroni)*	7e-79	>3	04908
SSF21	280	CV066926	Hypothetical protein XP_383324.1 *(Gibberella zeae)*	40/62 (64%)	Unknown protein	-	>3	02544
SSF22	300	CV066927	Unknown	-	Unknown protein	-	>3	-
SSF23	344	CV066928	Hypothetical protein XP_411922.1 *(A. nidulans)*	156/226 (69%)	Unknown protein	-	>3	03077
SSF24	346	CV066929	Hypothetical protein XP_406323.1 *(A. nidulans)*	44/81 (54%)	Unknown protein	-	>3	06676

Table 3. *Identity of the Aspergillus oryzae cDNAs selected from the cDNA subtraction library.* [A] *Best result obtained by BLASTX analysis using the cDNA sequence as a query in the nr database at NCBI.* [B] *Number of (percentage) amino acid sequence identities to the best BLAST hit in the segment compared.* [C] *In cases where a hypothetical protein emerged as the best BLAST hit, the full-length*

hypothetical protein was used as the query in a BLASTP search to determine its best hit. The e-value refers to this comparison. [D] *The SSF ratio was calculated from the results of spotblot analysis as described in materials and methods.* [E] *Accession No(s). of homologous EST(s) in the A. oryzae EST database (http://www.nrib.go.jp/ken/EST/db/blast.html).*

DISCUSSION

The growth phenotype of *A. oryzae* during culture on WK differs from that adopted during growth in 2% WLM, suggesting a difference in cellular morphogenesis. Our results show that, of the genes of known function that were more highly expressed during growth on WK, about half encode proteins that are involved in determining cell morphology or in related processes.

During germination of spores, determination of the site of polarisation is one of the first events in hyphal growth. Thereafter, various events regulate, determine and maintain polarised cell growth and cell shape. These events include signal transduction involving cAMP and MAP kinase pathways, the re-organization of the actin cytoskeleton, cell wall formation and polarised secretion (see Seiler and Plamann 2003 , and references therein). The maintenance of a highly polarised actin cytoskeleton is required to direct polarised growth (Cali et al. 1998; Bretscher 2003; Seiler and Plamann 2003). Interestingly, six of the products identified as showing enhanced expression during growth on WK in our analyses are related to, or involved in, the organisation of the actin cytoskeleton and polarised growth. The identification of the *A. oryzae* homologues (SSF16 and SSF17; Table 3) of the *S. cerevisiae* genes *SAC6* and *TPM2* suggests that the morphological differences between *A. oryzae* cells grown on WK and those grown in 2% WLM could be a consequence of a difference

in actin cytoskeleton organisation. Sac6 is also known as fimbrin, a calponin (Ca2+-binding) domain-containing protein involved in bundling actin filaments (Goodman et al. 2003) and Tpm2 is one of the two tropomyosins in *S. cerevisiae* required for the formation and stability of actin cables in vivo (Korman and Tobacman 1999). A change in the organisation of the cytoskeleton during solid state growth was further corroborated by the identification of a homologue of the *S. pombe* gene *mok1+* (*Aspergillus agsA*; Table 2), that codes for a α-glucan synthase involved in morphogenesis (Hochstenbach et al. 1998). This protein requires the actin cytoskeleton for localisation of the sites of growth (Katayama et al. 1999). An *A. oryzae* homologue of *PIN3* (SSF2; Table 1) was also recovered. The product of this *S. cerevisiae* gene is suggested to be involved in actin cytoskeleton organisation and biogenesis (Goffeau et al. 1997; Madania et al. 1999). In agreement with the macroscopically observed difference in growth phenotype, the differential expression of the *A. nidulans hogA* and CDC42 homologues (*Aspergillus hogA* and *cftA*; Table 2) suggests a difference in polarised growth between *A. oryzae* grown on WK and in 2% WLM. The *A. nidulans hogA* gene product is a MAP kinase involved in the maintenance of polar growth (Han and Prade 2002). A *cdc-42* mutant of *Neurospora crassa* was found to perturbed in cell polarisation during growth (Seiler and Plamann 2003), which is compatible with the finding that the Rho-family GTP-binding protein Cdc42 and several actin-binding proteins direct the assembly of actin (Stamnes 2002) and thus control microtubular dynamics.

The actin cytoskeleton also plays an important role in the intracellular distribution of several organelles (e.g. vesicles) and in polarised secretion of proteins (Stamnes 2002; Bretscher 2003). Therefore, the suggested difference in the organisation of the actin cytoskeleton during growth of *A. oryzae* on WK and in 2%

WLM could have an impact on the polarised secretion of proteins. In this context it is interesting to note that a homologue of the *S. cerevisiae* ORF *yjl123c* was identified as being differentially expressed in in A. oryzae (SSF13, Table 1). The product of ORF *yjl123c* gene has been localised to the COPI-coated vesicles (Huh et al. 2003) and is therefore suggested to be involved in Golgi to endoplasmic reticulum (retrograde) vesicle transport, and possibly in intra-Golgi transport. The correlation between the suggested polarised organisation of the actin cytoskeleton and the protein secretion capacity of *A. oryzae* needs to be elucidated further. However, both amylase and protease activities were more than 10 times higher in extracts of *A. oryzae* grown for 2 days on WK (expressed in U per g per h) than those measured (in U per ml per h) in the growth medium after 24 h growth in 2% WLM (R. te Biesebeke, unpublished results).

In addition to the previously identified *brlA* gene (te Biesebeke et al. 2002), which codes for a zinc-finger transcription factor that induces sporulation (Timberlake 1991), other *A. oryzae* gene homologues encoding proteins suggested to be involved in differentiation and/or morphogenesis were identified as induced under solid state growth conditions. The *A. nidulans aspB* homologue of *A. oryzae* SSF18 encodes a septin involved in cell division and the organisation of branching and conidiation (Westfall and Momany 2002). The *Myxococcus xanthus csgA* homologue of *A. oryzae* SSF14 encodes a C-signal cell surface protein (Hagen and Shimkets 1990) with conserved residues in the NAD(P)+ binding pocket domain and a short-chain alcohol dehydrogenase (SCAD) catalytic domain (Lee et al. 1995). The C-signal protein is involved in the co-ordination of multicellular fruiting body morphogenesis and sporulation in *M. xanthus* (Kruse et al. 2001; Li and Shimkets 1992). The *Coprinopsis cinerea* CoH1 homologue of *Aspergillus oryzae* SSF15 encodes a hydrophobin (Table

3) containing eight conserved cysteine residues that allow formation of four disulphide bridges that are essential for hydrophobin I function (Wösten and de Vocht 2000). Self-assembling hydrophobins are involved in the formation of aerial structures and in the attachment of hyphae to hydrophobic surfaces, while monomeric hydrophobins have been implicated in cell-wall assembly (Wösten 2001).

The probes for the *A. niger agsA*, *fksA* and *cwpA* genes (Table 2) detected higher levels of transcripts in *A. oryzae* grown for 3 days on WK. These results may reflect differences in cell wall composition relative to cells grown for 30 h in 2% WLM.

The results presented in Tables 1 and 3 are similar to those of a study by Akao et al. (2000) in showing that about half of the differentially expressed genes are novel genes. The number of novel genes identified in this study reflects the potential of the solid state growth system for the identification of novel protein functions in *A. oryzae* that might be involved in the control of cellular morphogenesis.

Traditionally, macroarray analysis is performed with cDNA libraries constructed from nucleic acids derived from the investigated species. However, in many cases where such homologous libraries are not available, it would be desirable to utilise heterologous cDNA libraries. We have shown here that genes that are differentially expressed in *A. oryzae* can be identified using an ordered *A. niger* cDNA library. Therefore, the availability of heterologous macroarray analysis for the identification of differentially expressed fungal genes should allow genome-wide expression analysis of fungal species for which cDNA libraries are not available.

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CHAPTER 6

Branching mutants of *Aspergillus oryzae* with improved amylase and protease production on solid substrates

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ABSTRACT

To study the relation between the number of hyphal tips and protein secretion during growth on a solid substrate we have constructed two mutant strains of *A. oryzae* with increased hyphal branching. We have analysed hydrolytic enzyme activities during growth on wheat kernels (WK) of *A. oryzae* strains carrying the disrupted allele of the *pclA* gene encoding a secretion pathway specific (KEX2-like) endo-protease and the disrupted allele of the *pg/pi-tp* gene encoding a phosphatidylglycerol/phosphatidylinositol transfer protein. The biomass levels produced by the *pclA* and *pg/pi-tp* disrupted strains on wheat-based solid media were similar as found for the wild-type strain. However, the *pclA* disrupted strain showed much more compact colony morphology than the other two strains. Sporulation of the *pclA* and *pg/pi-tp* disrupted strains occurred respectively 2 and 1 days later compared to the wild type during fermentation on ground WK. During surface growth, microscopic analysis revealed that the hyphal growth unit length (L_{hgu}) of the *pclA* and *pg/pi-tp* disrupted strains were on average 50% and 74% of that of the wild-type strain. This implies that in both mutant strains a higher branching frequency occurs than in the wild-type strain. Compared to the wild-type strain, the *pclA* and *pg/pi-tp* disrupted strains produced at least 50% more amylase, at least 100% more glucoamylase and at least 90% more protease activity levels after growth on WK. These results support the hypothesis that branching mutants with an increased branching frequency can improve the solid state fermentation process by secreting higher levels of hydrolytic enzyme activity in their growth environment.

INTRODUCTION

Aspergillus species are known for their capacity to secrete high levels of enzymes in their growth environment. Several of these secreted enzymes, produced in large scale submerged fermentation, have been widely used by the food and beverage industry for decades. The enzymes secreted during solid state fermentation by the so-called koji-molds like *A. oryzae* and *A. sojae* have been used for an even longer period for modification of rice, wheat kernels (WK) and soybean substrates for the production of fermented foods and alcoholic drinks. Only recently, solid state fermentation with filamentous fungi has gained increasing attention for production of specific enzymes (Pandey et al. 1999, te Biesebeke et al. 2002, Holker et al. 2004).

Although there has been some controversy, most studies suggest that protein secretion in filamentous fungi occurs around the apical and sub-apical region of the advancing hyphal tip (Gordon et al. 2000, Wösten et al. 1991, Müller et al. 2002, Archer and Pederby 1997). In *A. oryzae,* a correlation between the number of growing hyphal tips and protein secretion levels has been suggested (Amanullah et al. 2002, Muller et al. 2002). However, conflicting evidence about this correlation in other filamentous fungal cultivations has been presented (Bocking et al. 1999, McIntyre et al. 2002, Wessels 1993). To date only morphological mutants with unknown genotypes and different, often pleiotropic, mutations altering hyphal morphology have been investigated (See for references Conesa et al. 2001) while only specific gene disruptions that affect the formation of apical branches would ultimately establish whether protein secretion capacity is correlated to the number of growing tips.

In different studies it has been shown that filamentous fungi grown on a solid substrate can secrete high titres of enzymes and in some cases even higher enzyme

titres compared to growth in a liquid medium (Holker et al. 2004, Machida 2002, Pandy et al. 1999 and references therein). Growth of *A. oryzae* on a solid substrate involves modification of the substrate by secreted enzymes and subsequently allows the growing hyphae to penetrate the substrate. Therefor, to study the correlation between the number of hyphal tips and protein secretion, solid state fermentation with *A. oryzae* appears to be an excellent cultivation method. In this paper we describe two mutants, constructed by site-directed gene disruption, that are altered in the number of hyphal tips. Both mutant *A. oryzae* strains were analysed for branch frequency and amylase, glucoamylase and protease expression during growth on WK.

MATERIALS AND METHODS

Strains and media. *A. oryzae* 16868 was used for total RNA preparation and isolation of samples for amylase and protease activities measurements. Wheat kernels (WK) were pre-treated and inoculated as described in Hoogschagen et al. (2001). Ground WK were prepared and pretreated as described by te Biesebeke et al. (2004b). The wheat-based solid medium (2% WSM) supplemented with 10 mM uridine was prepared as described (te Biesebeke et al. 2004b) and used to determine the amount of biomass formed during growth at 30°C at 98% humidity in an climate controlled incubator (VEA-Instruments, Houten, the Netherlands). The 4% wheat-based liquid medium (4% WLM) was prepared by suspending ground WK (4 g) in H_2O (100 ml) that was sterilised for 15 min at 120ºC. Surface growth was performed for determination of the radial extension rate on 1% agar plates of 4% WLM supplemented with 10 mM uridine at 30°C at 98% humidity in an incubator (VEA-Instruments, Houten, the Netherlands).

Extraction of *A. oryzae* cultures grown on solid substrates and measurements of enzyme activities. Extracts of *A. oryzae* grown on WK and ground WK were prepared as described by te Biesebeke et al. (2004a). The α-amylase and glucoamylase activities were measured in the extracts as described by te Biesebeke et al. (2004a). Protease activities were measured as described (te Biesebeke et al. 2004a) except that the 2 µl sample + 13 µl water was mixed with 75 µl reagent (5 g/l N,N-dimethylcaseine in 0.1 M NaAc/Hac (pH 5.5), 0.1 M K_2HPO_4 (pH 7.0) or 0.1 M $Na_2B_4O_7.10H_2O$ (pH 8.5) to measure the protease activities at pH 5.5, pH 7 and pH 8.5, respectively.

Construction of the *pclA* and *pg/pi-tp* disruption vectors. The (KEX2-like) proprotein convertase-encoding gene (*pclA*) of *A. sojae*, a strain that is closely related to *A. oryzae*, was isolated by functional complementation of an *A. niger pclA* disruption strain which was described by Punt et al. (2003), with an *A. sojae* cosmid library (Heerikhuisen et al. 2004). A genomic clone was identified to complement the hyper branching phenotype of the *A. niger pclA* strain, resulting in wild-type growth characteristics. PCR and sequence analysis confirmed the presence of the homologous *pclA* gene on this genomic clone. A 5.2kb *Xba*I/*Bam*HI fragment was sub-cloned, resulting in the vector pAS2-4. Subsequently, the *pclA* disruption vector pAS2-4pcl was constructed by cloning the repeat-flanked *A. niger pyrG* gene from pAB4-1rep as a *Sma*I fragment into the unique *Eco*RV site of the *pclA* gene of pAS2-4 (Heerikhuisen et al. 2004).

The *pg/pi-tp* gene (and a tandemly arranged thiolase gene) was amplified from genomic DNA from *A. oryzae* ATCC 16968 using primers (5'CGGTTGTCGTCATAGTGAGC3') and (5'CGATCGGAATCTAGAGAGACGG3') (Record et al. 2001). The total DNA sequence of 4279 bp was cloned in pGEM-T easy vector (Promega) and verified by sequencing.

The pGEM-T vector containing the *pg/pi-tp* gene was digested with *Avr*II and *Eco47*III to excise a 723bp fragment from the *pg/pi-tp* gene. This fragment was replaced with the 2.8 kb *Spe*I-*Sma*I fragment from vector pAB4-1rep carrying the *A. niger pyrG* selection marker (Heerikhuisen et al. 2004), resulting in vector pG-PLTPdel.

Southern and Northern blot analysis. Southern blot analysis was performed according to the standard procedure (Sambrook et al. 1989) after cutting the chromosomal DNA with *Eco*RI and *Bam*HI. Southern blots were hybridised with ^{32}P labelled (Random Prime Labeling Kit, Pharmacia) PCR amplified probes for the *pclA* and *pg/pi-tp* genes and hybridised at 68˚C in standard hybridisation buffer (Sambrook et al. 1989). Hybridisation signals were visualised after exposure to X-OMAT AR films (Kodak, cat. 1651512) and scanned with the Hewlett Packard 6200C Scanjet at 600 dpi. A probe (401 nucleotides) for the *pclA* gene was PCR amplified in 35 cycles of 1 min at 94˚C, 1 min at 55˚C, 3 min at 68˚C using primers (5'CCTGATGATAGAGACCGATTTCCCG3') and (5'GCCTTTGCATTATCTGACCGCCGCGC3') and vector pAS2-4 as template. A probe (361 nucleotides) for the *pg/pi-tp* gene was PCR amplified in 40 cycles of 1 min at 94˚C, 1 min at 30˚C, 2 min at 72˚C using primers (5'ACCCTCTGGAGTATTGTAATG3') and (5'TTCAGGCAAGTGATATGCTC3') and chromosomal DNA of *A. oryzae* as template. In Southern analysis, the probes for the *pclA* and *pg/pi-tp* genes both showed single specific hybridising bands in the wild-type *A. oryzae* strain. Disruption of the *pclA* and *pg/pi-tp* genes was confirmed in the respective *pclA* and *pg/pi-tp* disrupted strains. Northern blot analysis was performed with isolated total RNA and PCR amplified probes for the α-amylase, glucoamlyase B, alkaline protease A and neutral protease B genes as described (te Biesebeke et al. 2004a and 2004b)

Microscopy and hyphal measurements. To obtain the number of spores per ml extract, spores were counted microscopically in 25 ml H_2O extract of *A. oryzae* strain grown on WK (5 g). To obtain a constant and reliable value for our measurement of hyphal branching, expressed as the hyphal growth unit length ($L_{hgu,}$ Prosser 1994), germination and subsequent branching was observed of colonies originating from single spores of *A. oryzae* grown on 2% WSM. From the moment that hyphal tips appeared, every 10 min the length of the hyphal elements were determined and the number of tips on each hyphal element were counted. For determination of L_{hgu}, of *A. oryzae* wildtype, *pclA* and *pg/pi-tp* disrupted strains were grown on 2%WSM and pictures were taken of hyphal elements having 5-12 hyphal tips using a Zeiss microscope (AXIOLAB) with a 10 X objective. Pictures were taken with the Sony 3CCD camera and imported and saved as TIFF-files using the Paintshop Pro software. The pictures were analysed on a Macintosh (iMAC, Macintosh incorporate, USA) computer using the IMAGEJ software (www.imageJ.com) according to the manufacturer protocol. The L_{hgu} was determined by dividing the total hyphal length of the growing hyphal element (Ltot) by the number of tips (Nt) present on the growing hyphal element. L_{hgu} was expressed in hyphal length per hyphal tip (millimeter*tip^{-1}) and is an indication for the frequency at which hyphal branching occurs on a hyphal element (branch frequency). For all experiments 40-50 hyphal elements were analysed.

RESULTS

Construction of the *pclA* and *pg/pi-tp* disrupted strains. A previous study has shown that disruption of the (KEX2-like) proprotein convertase-encoding gene (*pclA*) in *A. niger* resulted in a hyperbranching phenotype (Punt et al. 2003). Mycelial

morphology is also influenced by the concentration of the cell membrane phospholipid, phosphatidyl choline, apparently by controlling branch initiation (Markham et al. 1993). The phospholipid transfer protein encoded by the *pg/pi-tp* gene of *A. oryzae* transferred besides phosphatidylglycerol and phosphatidylinositol also phosphatidylcholine (Record et al. 1995). Moreover, a correlation was shown between growth and phospholipid transfer activity of *A. oryzae* under specified conditions (Record et al. 2001). Therefore, also an *A. oryzae* mutant strains with a disruption in the *pg/pi-tp* gene may have a different growth phenotype compared to the wild-type.

A transformation system for the *A. oryzae* ATCC16868 strain based on the orotidine-5'-phosphate decarboxylase gene (*pyr*G) gene as selection marker was developed as described previously (Mattern et al. 1987). An ATCC16868*pyr*G mutant strain was used for the construction of the *pcl*A and *pg/pi-tp* disruption strains. For the disruption of the *pcl*A gene in ATCC16868*pyrG*, the *pcl*A disruption vector pAS2-4Δpcl (Heerikhuisen et al. 2004) was used. From this vector, a 8.2 kb *Not*I/*Asc*I fragment, containing the *pcl*A gene interrupted by the *pyrG* gene, was used to transform ATCC16868*pyrG*. Of the Pyr+ transformants, 12 out of 15 showed a compact growth morphology as was observed for the *pcl*A disrupted strain in *A. niger* (Punt et al. 2003). Southern blot analysis revealed that 2 of the compact Pyr+ transformants showed a shift in band size with the probe for the *pcl*A gene compared to the wild-type as a consequence of the insertion of the 8.2kb *Not*I/*Asc*I fragment at the *pcl*A locus (results not shown). To disrupt the *pg/pi-tp* gene a 6.3kB *Eco*RI fragment from pG-PLTPdel was used to transform ATCC16868*pyr*G. In 2 of 3 of the Pyr+ transformants analysed the *pg/pi-tp* gene was shown to be disrupted using Southern analysis with a probe for the *pg/pi-tp* gene (results not shown). Two strains

for which Southern analysis confirmed the disruption of the *pcl*A or the *pg/pi-tp* gene were selected for further analysis.

Strain	*Disrupted protein*	*Radial extension Rate (mm*hr^{-1})*	*Aerial hyphea*	*Sporulation (D)*	*L_{hgu} (mm*tip^{-1})*
ATCC16868	-	0.23 (0.01)	++++	2	ND
ATCC16868 pyrG	Orotidine-5'-phosphate decarboxylase	0.22 (0.02)	++++	2	104 (11)
ATCC16868 pclA	Endoprotease	0.16 (0.02)	++	4	51 (13)
ATCC16868 pg/pi-tp	PG/PI transfer protein	0.23 (0.01)	++++	3	77 (14)

Table 1. *Growth phenotype of ATCC16868, ATCC16868pyrG, pclA and pg/pi-tp disruption strains. The radial extension rate (Trinci 1971) and standard deviation (SD) was determined after growth on 4% WSM. The mean length of the aerial hyphae was measured after 3 days of growth on ground wheat kernels (WK) (++++: >3mm, ++: 1-2 mm). The time in days (D) it takes before sporulation (>10^3 spores*ml^{-1} extract) occurs after growth on WK. The hyphal growth unit lengths (L_{hgu}) with the standard deviation (SD) were determined after growth on 2% WSM. ND = not determined.*

Growth phenotypes of the *pclA* and *pg/pi-tp* disrupted strains. To determine the effects of the disruption of the *pclA* or *pg/pi-tp* gene in ATCC16868*pyrG* the growth phenotype was compared to ATCC16868*pyrG* during growth on WK. The amount of biomass that was formed during surface growth on 2% WSM was equal for all strains and equal as previously described for the *A. oryzae* ATCC16868 strain (te Biesebeke et al. 2004b). Although the obtained biomass was the same, the *pcl*A disrupted strain clearly showed a more compact morphological phenotype compared to the ATCC16868*pyrG* and the *pg/pi-tp* disrupted strain. The

radial extension rate is a reliable method to determine growth of a fungal colony (Trinci 1971). After spotting 10 µl containing 10^8 conidia on 4% WSM only minor differences in radial extension rate were observed between the ATCC16868*pyrG* and the *pg/pi-tp* disrupted strain while that of the *pclA* disrupted strain was about 70% of ATCC16868*pyrG* (Table 1). After 3 days of growth on 4% WSM, ATCC16868*pyrG* started to sporulate in the center of the colony while sporulation of the *pclA* and *pg/pi-tp* disrupted strains appeared after 4 days. After 3 days of growth on ground WK the aerial hyphae of the mycelial layer of these two strains were longer (>3 mm) compared to the *pclA* disrupted strain (1-2 mm) (Table 1). The colonisation on WK in terms of time that it takes to fully cover the substrate with filamentous fungal mycelium was equal for all strains (4 days). However, sporulation of the *pclA* or *pg/pi-tp* disrupted strains occurred 2 and 1 days, respectively, later compared to ATCC16868*pyrG* (Table 1). There were no differences observed between ATCC16868 wild-type and the ATCC16868*pyrG* strain under these cultivation conditions (Table 1).

Microscopical phenotype of the *pcl*A and *pg/pi-tp* disrupted strains. To determine the effect of the disrupted genes on hyphal branching, the *pcl*A or *pg/pi-tp* disrupted strains and ATCC16868*pyr*G were grown for up to 24 h at 37°C in a 1-2 mm layer of 2% WSM at a density of 20 conidiospores/ml^{-1}. The conidiospores were observed microscopically with an amplification of 100X. During germination of the conidiospores, the *pcl*A or *pg/pi-tp* disrupted strains showed clearly a higher number of hyphal tips compared to ATCC16868*pyr*G. In order to define this process in a quantitative way the hyphal growth unit length (L_{hgu}) was determined as a measure to describe branching (Dynesen and Nielsen 2003, Christiansen et al. 1999, Prosser 1994, Trinci 1974). For the successful use of this parameter a similar approach was used as described by Dynesen and Nielsen (2003). First, it was determined for the

ATCC16868*pyr*G strain that at least 6 branches should be formed on 2% WSM before L_{hgu} approached a constant value (Fig. 1A). To determine branch frequency of the *pcl*A or *pg/pi-tp* disrupted strains only germinating conidiospores with hyphal elements possessing 5-12 hyphal tips were analysed. Fig. 1B shows that L_{hgu} is clearly distinct in the different strains, whereas for all strains, L_{hgu} is almost constant after formation of at least 7 hyphal tips. Table 1 shows that the *pcl*A or *pg/pi-tp* disrupted strains show an average value for L_{hgu} (calculated with the 7 to 12 tips L_{hgu} values shown in Fig. 1B) that is respectively 50% and 74% compared to that of the wild-type. Since more hyphal tips occurred in both *pcl*A and *pg/pi-tp* disrupted strains during growth on 2% WSM compared to ATCC16868*pyr*G also more hyphal tips were assumed for the *pcl*A and *pg/pi-tp* disrupted strains grown on WK and ground WK compared to ATCC16868*pyr*.

Expression of amylase and protease by the *pcl*A and *pg/pi-tp* disrupted strains. To study the correlation between the number of hyphal tips and protein secretion, the activity of amylases, glucoamylases and proteases was studied in the extracts of *A. oryzae* ATCC16868*pyr*G and the *pcl*A and *pg/pi-tp* disrupted strains grown on WK. In a former study it was found that after 5 and 6 days of growth of *A. oryzae* on the WK and ground WK considerable enzyme activities were present (te Biesebeke et al. 2004a). After 6 days of growth, the α-amylase activities measured in the extracts of the *pcl*A and *pg/pi-tp* disrupted strains grown on WK and ground WK were all more then 50% higher compared to those of ATCC16868*pyr*G and the wild-type ATCC16868 strain (Table 2). The glucoamylase activities measured in the extracts of the *pcl*A and *pg/pi-tp* disrupted strains grown for 6 days on WK and ground WK were more then 100% higher compared to those of ATCC16868*pyr*G (Table 2). The proteases activities measured at pH 5.5, pH 7 and pH 8.5 in the extracts of the *pcl*A and *pg/pi-tp* disrupted strains grown on WK were all more then 90% higher compared to those of ATCC16868*pyr*G (Table 2). In contrast to this, all

protease activities measured at pH 5.5, pH 7 and pH 8.5 in the extracts of the *pcl*A and *pg/pi-tp* disrupted strains grown on ground WK were all about equal (see Table 2) compared to those of ATCC16868*pyr*G.

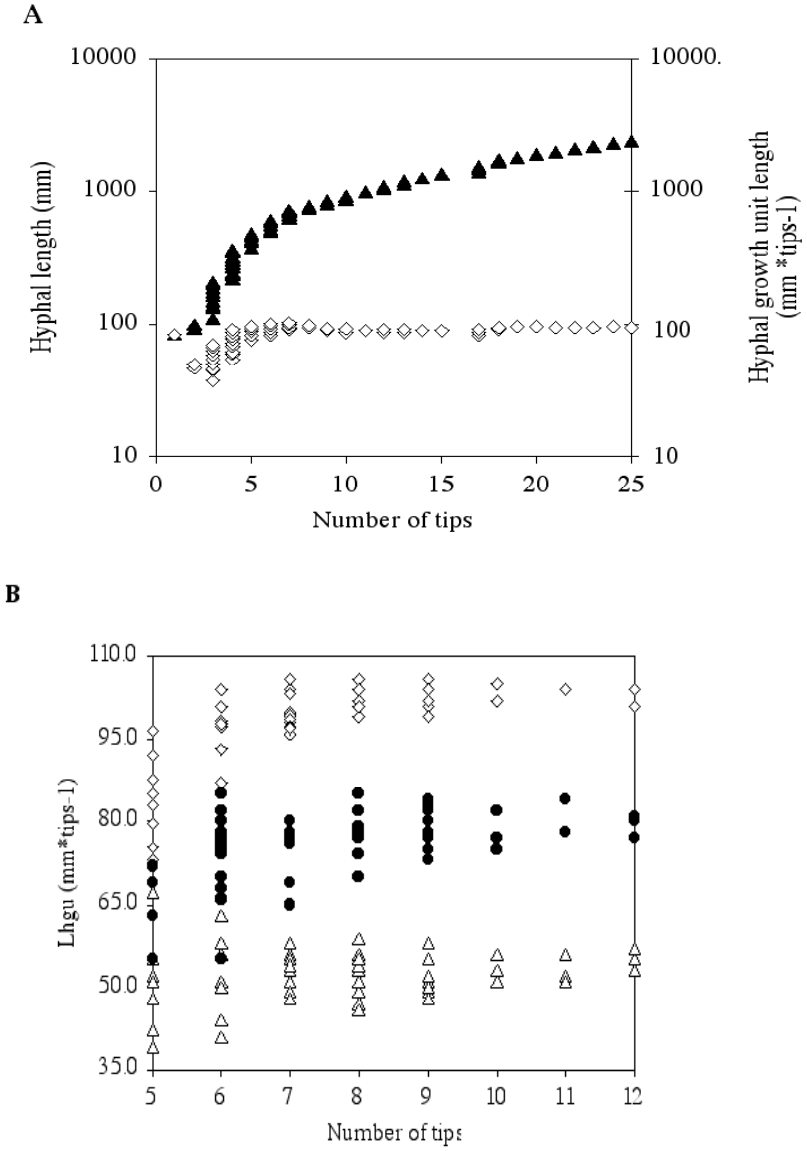


Fig. 1. (A)*The hyphal growth unit length (L_{hgu} in mm*tip-1) of the ATCC16868pyrG strain grown on 2% WSM. The hyphal length (mm) (▲) and the number of tips were determined from hyphal elements derived from germinating conidiospores. The L_{hgu} (◊) was calculated from the hyphal length and the number of tips. (***B)*** The hyphal growth unit length (L_{hgu} in mm*tip^{-1}) of the ATCC16868pyrG strain (◊) and the pclA*

(Δ) and pg/pi-tp (●) disrupted strains grown on 2% WSM. Of every strain 40-50 hyphal elements with 5-12 hyphal tips were analysed.

Enzyme Measurement	Substrate	Time (D)	ATCC16868	ATCC16868 *pyr*G	ATCC16868 *pcl*A	ATCC16868 *pg/pi-tp*
α-amylase (U/g)	Wheat kernel	5 6	61 70	64 75	94 115	97 122
	GWK	5 6	79 112	82 113	109 191	108 171
Glucoamylase (U/g)	Wheat Kernel	5 6	ND	1 1	3 4	4 7
	GWK	5 6	ND	5 10	10 24	12 19
Protease pH 5.5 (U/g)	Wheat Kernel	5 6	ND	21 24	44 51	60 61
	GWK	5 6	ND	46 53	34 54	34 45
Protease pH7 (U/g)	Wheat Kernel	5 6	ND	5 5	10 15	13 19
	GWK	5 6	ND	12 18	13 24	16 27
Protease pH8.5 (U/g)	Wheat kernel	5 6	ND	3 5	8 14	10 13
	GWK	5 6	ND	18 24	14 22	14 25

Table 2. *α-Amylase, glucoamlyase activities and protease activities at pH 5.5, pH 7 and pH 8.5 in extracts of the wild-type ATCC16868, ATCC16868 pyrG and the pclA and pg/pi-tp disrupted strains grown for 5 and 6 days (D) on wheat kernels (WK) or ground wheat kernels (GWK). Activities were calculated and expressed in Unit per gram solid state fermentation sample (U/g). The values are the average of 2 independently performed experiments. ND = not determined.*

Transcriptional analysis of the *pcl*A and *pg/pi-tp* disrupted strains

Northern analysis was performed to determine the transcription levels of a selection of genes encoding hydrolytic enzymes of ATCC16868*pyr*G and the *pcl*A and *pg/pi-tp* disrupted strains. The strains were grown for 6 days on WK and mRNA samples were isolated and analysed for transcription of the α-amylase, the

glucoamylase, the alkaline and neutral protease genes (Fig. 2). The *pcl*A and *pg/pi-tp* disrupted strains showed equal transcription levels for the α-amylase, and the alkaline and neutral protease genes compared to ATCC16868*pyr*G. However, the transcription level of the glucoamylase B gene in the *pcl*A and *pg/pi-tp* disrupted strains was clearly higher compared to ATCC16868*pyr*G (Fig 2) at 6 days growth on WK.

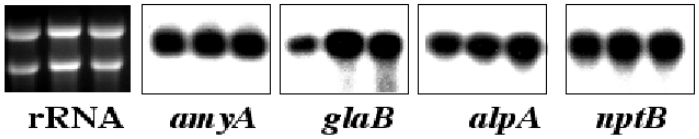


Fig 2 *Northern analysis of the ATCC16868pyrG strain (left lane of each panel) and the pclA (middle lane of each panel) and pg/pi-tp (right lane of each panel) disrupted strains grown for 6 days on wheat kernels (WK) with probes for the α-amylase (amyA), glucoamylase B (glaB), alkaline protease (alpA) and neutral protease II (nptB) genes. As a loading control the ribosomal RNA (rRNA) was used.*

DISCUSSION

Filamentous fungal growth of *A. oryzae* on WK solid substrate was used to study protein secretion. In solid state cultivation penetrative hyphae secrete hydrolysing enzymes to degrade the solid substrate and subsequently penetrate the substrate. To study whether secretion of proteins by *A. oryzae* depends on the number of hyphal tips, two site-directed gene-disrupted mutants were constructed and analysed during growth on wheat kernels.

The *pcl*A disrupted strain of *A. oryzae* showed two-fold lower hyphal growth unit length corresponding to a two-fold higher branch frequency during surface growth on 2% WSM compared to ATCC16868*pyr*G. The *A. oryzae pclA* gene encodes an homologue of the *A. niger* endoproteolytic proprotein processing enzyme

kexB that processes dibasic cleavage sites (KR) in target proteins that are transported through the secretion pathway (Jalving et al. 2000, Punt et al. 2003). The hyperbranching phenotype of the *pcl*A disrupted strain is apparently the result of at least one unprocessed proprotein. Interestingly, chitin synthase B (GenBank accession no AAK31732) encoded by the *chs*B gene of *A. oryzae* contains putative mono and dibasic processing sites for PclA. As an abnormal branching phenotype has been observed for a strain with a disrupted *chs*B gene in *A. oryzae* (Müller et al. 2002) an unprocessed dysfunctional chitin synthase B might contribute to the observed phenotype of the *pcl*A disrupted strain. This is in agreement with a recent study suggesting disordered cell integrity signalling in response to perturbation of the cell wall (Mizutani et al. 2004). Alternatively, PclA might be required for processing of sensor proteins in the cell integrity pathway (Mizutani et al. 2004).

The *pg/pi-tp* disrupted strain showed 1.5 times lower hyphal growth unit length on 2% WSM compared to ATCC16868*pyr*G. The amino acid sequence of the phosphatidylglycerol/phosphatidylinositol transfer protein (GenBank accession no AAG13652) suggests that it is an outer cell membrane targeted protein (Record et al. 1999). The purified phospholipid transfer protein (AAG13652), a putative homologue of human NPC2 whose deficiency causes Niemann-Pick type C2 disease (Ikonen and Holtta-Vuori 2004), was found to transfer besides phosphatidylglycerol and phosphatidylinositol also phosphatidylcholine (Record et al. 1995). Therefore, the phospholipid transfer protein may be involved in integrity of the lipid content of the cell membrane. Choline appears as phosphatidyl choline (lecithin) in the outer cell membrane and reduced concentrations of choline result in multiple tip-formation of *Fusarium graminearum* (Markham et al. 1993). The phenotype of the *pg/pi-tp* strain might therefore be a consequence of a reduced phosphatidylcholine transfer activity.

The resulting lipid imbalance in *A. oryzae* cell membranes is suggested to result in a higher branch frequency.

As shown both branching mutants showed increased levels of amylase and protease production. The option that increased biomass yields cause increased protein production seems unlikely as biomass yields for the mutant strains were not higher than the wild-type strain during growth on 2% WSM (results not shown). Only for glucoamlyase, the increased production may be due to increased gene expression (Fig. 2). The *glaB* gene is induced by maltose (Ishida et al. 1998, 2000) that is liberated from the WK (te Biesebeke et al. 2004b). Our results could indicate that the maltose induction is increased in the *pcl*A and *pg/pi-tp* disrupted strains. Compared to the wild-type, the growth phenotype of both mutant strains on WK could imply more penetrative hyphae involved in uptake of inducer for *glaB* gene transcription. Also the retarded sporulation in the *pcl*A and *pg/pi-tp* disrupted strains may indicate improved nutrient uptake compared to the wild-type, as nutrient state is one of the regulatory cues for sporulation (Adams et al. 1990, Skromme et al. 1995). For amylase and protease production, we suggest that a higher number of WK penetrating hyphae per hyphal growth unit may explain the higher secretion of enzyme activities of the mutant strains. Our results underline the important contribution of hyphal tips to the production of proteins during growth of *A. oryzae* on a solid substrate.

CHAPTER 7

An *Aspergillus oryzae* gene expressed during hyphal growth is a member of an unusual family of fungal flavohemoglobins

R. te Biesebeke, A. Boussier, C.A.M.J.J. van den Hondel, P.J. Punt (2004) An *Aspergillus oryzae* gene expressed during hyphal growth is a member of an unusual family of fungal flavohemoglobins. *Submitted for publication.*

ABSTRACT

The *fhbA* gene encoding a putative flavohemoglobin (FHb) from *A. niger* was isolated. Comparison of the deduced amino acid sequence of the *A. niger fhbA* gene and other putative filamentous fungal FHb-encoding genes to that of *Alcaligenes eutrophus* shows conserved catalytic residues. Several yeast and filamentous fungi have been found to contain small FHb gene families. Transcriptional analysis with *A. oryzae* suggests that the regulation of the filamentous fungal FHb-encoding gene is different compared to that from the bacterial FHb-encoding genes. Different experimental approaches show that *fhbA* transcript levels appear during hyphal growth, independent of the amount of fungal biomass. Moreover, in a *pclA*-disrupted strain with a hyperbranching growth phenotype, the transcript levels of the *fhbA* gene were 2-4 times higher compared to the wild-type. These results suggest that FHb from filamentous fungi have a function that is correlated to the hyphal growth phenotype.

INTRODUCTION

Flavohemoglobin proteins (FHb's) consist of an N-terminal hemoglobin domain that reversibly binds oxygen and a carboxy-terminal redox active domain with potential binding sites for FAD and NAD(P)H. They have been described for a number of bacterial taxons and several fungal species like *Saccharomyces cerevisiae* (Zhu and Riggs 1992), *Fusarium oxysporum* (Takaya et al. 1997), *Candida norvegensis* (Kobayashi et al. 2002) and *Cryptococcus neoformans* (Jesus-Berrios et al. 2003). FHb's appear to provide protection to nitrosative (NO) stress in bacteria (reviewed by Frey and Kallio 2003). Also in fungi, the involvement in protection to nitrosative stress is suggested. After deletion of the *S. cerevisiae* FHb-encoding gene (YHB1) and exposure to an artificial NO donor, higher levels of nitrosylation of high

molecular mass molecules were measured compared to the wild-type (Liu et al. 2002). *C. neoformans,* an established human fungal pathogen that replicates in macrophages, has a FHb that protects against nitrosative stress and is necessary for full pathogenesis (Jesus-Berrios et al. 2003). Other studies have suggested a role of the *S. cerevisiae* FHb in protection against oxidative stress (Zhao et al. 1996, Buisson and Labbe-Bois 1998). In contrast to bacterial FHb's, the high affinity of oxygen binding of *Candida norvegensis* FHb led Kobayashi et al. (2002) to suggest that yeast FHb could also serve as an oxygen storage protein.

Oxygen allows aerobic cellular metabolism to function at optimal levels with respect to substrate utilization and energy yield (Frey and Kallio 2003). In yeast, the heme-activated proteins respond to intracellular heme levels and regulate the transcription of target genes of which the gene products are involved in respiratory growth (Zitomer and Lowry 1992). It has been shown that the yeast YHB1 gene is positively regulated by the transcription factors heme-activated proteins HAP 1 and HAP 2/3/4 (Crawford et al. 1995). However, oxygen is only partly (70%) responsible for regulation of YHB1 gene expression (Crawford et al. 1995, Zhao et al. 1996). Repression of YHB1 occurs with increasing cell density involving a gene regulatory mechanism that probably overrules the oxygen control of the YHB1 gene (Crawford et al. 1995).

During growth of filamentous fungi on solid substrates oxygen metabolism becomes often limited in the hyphal elements that have penetrated the solid substrate (Oostra 2001, Rahardjo et al. 2001). To gain further insight in the function of FHb in filamentous fungi we performed gene expression studies in the obligate aerobe filamentous fungus *A. oryzae* under different growth conditions. This study describes the isolation of the *fhbA* gene encoding FHb from *A. niger* and compares its deduced

amino acid sequences with putative FHb sequences from other filamentous fungi. Northern analysis was used to analyse the expression of the FHb-encoding genes in *A. oryzae* in order to provide clues for the role of FHb's of fungi.

MATERIALS AND METHODS

Strains and media. *A. oryzae* ATCC16168 was used throughout this study and *A. niger* N402 was used to isolate the *fhbA* gene encoding flavohemoglobin (FHb). The *pclA* disrupted *A. oryzae* strains were constructed as described (te Biesebeke et al 2004d). Growth on wheat kernels (WK), in 2% wheat-based liquid medium (2% WLM) and on 2% wheat-based solid medium (2% WSM) was performed as described (te Biesebeke et al 2004a, 2004b). The fungal biomass transfer to 2% WLM, 2% WSM or water agar medium (WAM) was performed as described (te Biesebeke et al. 2004b). WAM is 2% agar-solidified H_2O (100 ml) sterilised for 15 min at 120°C. For surface growth on 2% WLM, 10^6 *A. oryzae* conidia/ml were inoculated in a shake flask (250 ml) containing 2% WLM (100 ml) and incubated without shaking at 30°C.

Isolation of the *fhbA* gene of *A. niger*. In an heterologous macroarray analysis similar as described in te Biesebeke et al. (2004c) a cDNA clone was identified that showed a differential hybridisation intensity with labelled first-strand cDNA of total RNA from *A. oryzae* grown in 2% WSM compared to that grown on WK. The complete cDNA was PCR amplified with primers MBL1588 and MBL1589 (te Biesebeke et al. 2004c) using 40 cycles of 30 s at 94°C, 1 min at 45°C, 30 s at 72°C. The DNA fragment was purified from 1 % agarose gel electrophoresis with the Qiaquick DNAeasy columns (Qiagen, UK) and cloned in pGEM-T easy vectors (PROMEGA) and sequenced. Sequencing was performed with the Cycle Sequencing

Kit from Pharmacia according to the manufacturer protocol. Sequence data were obtained with the ABI Prism 310 Genetic Analyser from Applied Biosystems (Perkin-Elmer division). The complete cDNA was isolated from the *A. niger* cDNA library (Veldhuisen et al. 1997), sequenced and the cDNA sequence and the deduced amino acid sequence was deposited at the EMBL database under the respective GenBank accession no AJ627189 and CAF25490.1.

Blast searches, Homology, ClustalW. The cDNA sequence was matched to different databases as described (te Biesebeke et al. 2004c) in blast searches (Altschul et al. 1997) to obtain homologous sequences of other fungi. Homology between *Aspergillus* DNA sequences was determined with blast 2 sequences (Tatusova and Madden 1999). Homologous protein sequences were submitted for ClustalW 1.82 (Thompson et al. 1994) alignment at EMBL-EBI (www.ebi.ac.uk).

Southern and northern analysis. Southern and northern analysis was performed as described (te Biesebeke et al. 2004b). Northern analysis was performed with ^{32}P labelled (Random Prime Labeling Kit, Pharmacia) *A. niger* probe for the *fhbA* gene that was PCR amplified from the above mentioned pGEM-T vector containing the FHb cDNA sequence (AJ627189). The probe were purified from 1% agarose gel with Qiaquick DNAeasy columns (Qiagen, UK).

RESULTS

Isolation of a putative FHb-encoding gene *fhbA* from *A. niger*. In a previous study (te Biesebeke et al. 2004c) a heterologous macroarray analysis was used to identify genes associated with the growth phenotype of *A. oryzae*. The complete cDNA of one clone was sequenced (CAF254990.01) and its deduced amino acid sequence was identified as a protein homologous to the FHb of *Alcaligenes*

eutrophus (Ermler et al. 1995). Based on the cDNA sequence, primers were designed to PCR amplify and sequence the genomic copy of the *A. niger fhbA* gene. Based on the sequence of the PCR fragment, the *A. niger fhbA* gene did not contain any introns.

Fig. 1. *Schematic drawing of the three domains of flavohemoglobin (FHb) (Ermler et al. 1995, Ilari et al. 2002). The N-terminal hemoglobin domain has a high affinity for oxygen, the middle domain for FAD, and the C-terminal domain for NAD(P)H. The dotted line illustrates the variable length at the N-terminus of some species.*

Other fungal FHb's. The FHb's contain a N-terminal hemoglobin domain, a central FAD-binding domain and a C-terminal FAD binding domain (Fig. 1; Ermler et al. 1995, Ilari et al. 2002). Comparison of the *A. niger* FHb sequence with several publicly available sequence databases revealed a number of related FHb sequences. Remarkably, several fungal species of which the full genomes are available in public databases (*Aspergillus nidulans*, *Neurospora crassa*, *Gibberella zea* (*Fusarium graminearum*), *Debaryomyces hansenii* (*Candida famata*), *Podospora anserina, Yarrowia lipolytica* have 2 genes encoding putative FHb's in their genome (Table 1). *Candida albicans* has 3 FHb-encoding genes (Ullman et al. 2004), whereas *Aspergillus fumigatus*, *Magnaporthe grisae, Phanerochaete chrysosporium, Crypotococcus neoformans, S. cerevisiae* and *S. pombe* have only a single FHb-encoding gene in their genomes (Table 1).

Interestingly, the overall sequence identity of the *A. niger* FHb compared to other fungal FHb sequences but also to the *A. eutropus* and *E. coli* FHb sequences is

in the range of 25-45%, with exception of the *A. fumigatus* and one of the *A. nidulans* sequences (Table 1). A clearly different feature of the *A. niger* FHb compared to that of most other fungal FHb's is the N-terminal extension with 43 amino acid residues. Only *P. chrysosporium*, *M. grisae*, *C. neoformans* and *S. pombe* have N-terminal extensions of respectively 15, 24, 79 and 83 amino acid residues (not shown). In general, Table 1 shows that the different fungal and yeast FHb's are unusually divergent in sequence.

Organism	Abbreviation	GenBank no	Identity (%)
Aspergillus niger	Anr	CAF25490.1	100
Aspergillus fumigatus	Afu	TIGR_5085contig5277	65
Aspergillus nidulans	Ans1	EAA59083.1	58
Podospora anserina	Pan1	Contig430	46
Podospora anserina	Pan2	Contig132	46
Gibberella zeae	Gze2	EAA70711.1	45
Aspergillus nidulans	Ans2	EAA61421.1	45
Gibberella zeae	Gze1	EAA73242.1	44
Neurospora crassa	Ncr1	EAA34752.1	43
Neurospora crassa	Ncr2	EAA28703.1	43
Cryptococcus neoformans	Cne	EAL22289.1	43
Fusarium oxysporum	Fox	BGA33011.1	43
Magnaporthe grisae	Mgr	EAA48540.1	43
Phanerochaete chrysosporium	Pch	AADS01000126.1	42
Alcaligenus eutrophus	Aeu	A53396	42
Eschericia coli	Eco	BAA16460	37
Saccharomyces cerevisiae	Sce	NC_001139.2	37
Candida glabrata	Cgl	CAG62036.1	35
Kluyveromyces lactis	Kla	CAH02568.1	35
Deboramyces hansenii	Dha1	CAG91139.1	33
Schizosaccharomyces pombe	Spo	NC_003424.1	32
Deboramyces hansenii	Dha2	CAG91152.1	32
Yarrowia lipolytica	Yli1	CAG81069.1	30
Candida albicans	Cal3	EAK91821.1	29
Pichia Norvegensis	Pno	S26964	29
Candida albicans	Cal2	EAK91824.1	27
Candida albicans	Cal1	EAL00511.1	26
Yarrowia lipolytica	Yli2	CAG83795.1	22

Table 1. *Species origin of flavohemoglobin protein (FHb) sequences used in this study. The indicated identity (%) is determined in comparison to the A. niger FHb sequence. GenBank accession numbers were obtained from NCBI (www.ncbi.nlm.nih.gov/) or in case of Afu, Pan (1, 2) and Pch from (//www.tigr.org), (//podospora.igmors.u-psud.fr/) and (//www.jgi.doe.gov/), respectively.*

Conserved amino acids of filamentous fungal FHb's. Table 1 suggests low sequence identity between the putative filamentous fungal FHb's. To determine whether the different filamentous fungal FHb sequences share homology in functionally relevant residues, the amino acid sequences were aligned to the FHb sequence of *A. eutrophus* of which the three-dimensional structures has been elucidated and functional relevant residues have been determined (Elmer et al. 1995). The FHb of *A. eutrophus* is made up of a hemoglobin, FAD and NAD(P)H binding domain (Fig. 1, 2). The globin domain ranging from residue 1 to 147 (*A. eutropus*), consists of 6 α-helices (A, B, C, E, F, G, H) and holds the heme molecule that is embedded in a hydrophobic crevice formed by 6 α-helices (Weber and Vinogradov 2001, Ilari et al. 2002, Frey and Kallio 2003). A number of residues in the globin domains are invariant according to all known FHb sequences. The Tyr-B10 and Gln-E7 have been suggested to be involved in stabilisation of the heme bound dioxygen (Frey and Kallio 2003) and are conserved in the globin domain of the filamentous fungal FHb's. His-F8 in α-helix F, Tyr-G5 in helix G and Glu-H23 in helix H, suggested to form the catalytic triad at the proximal site by modulating redox properties of the heme-iron atom (Frey and Kallio 2003), are also conserved in the globin domain of the filamentous fungal FHb's. The FAD and NAD(P)H binding domain ranges from the respective residues 153 to 266 and residue 267 to 397 in the *A. eutrophus* sequence (Ermler et al. 1995). The FAD binding domain consists of a six-stranded antiparallel β-barrel (Fβ1-6) capped by a helix (Fα1) (Erlmer et al. 1995). The residues 206-209 (*A. eutrophus)* in the loop between sheet Fβ4 and Fβ5 are involved in FAD binding (Frey and Kallio 2003) and are conserved in the suggested FAD domain of the filamentous fungal flavoHb proteins. The NAD(P)H binding domain is built up of a five-stranded parallel β-sheet flanked by 2 helices

(Nα1, Nα2) on one side and by one helix (Nα4) at the other side (Erlmer et al. 1995). The conserved Lys-F7 in α-helix F and Glu-394 in sheet Nβ5 are amongst other residues, considered to be essential for transport of electrons from FAD to the heme iron (Frey and Kallio 2003).

***A. oryzae fhbA* gene transcription.** An *A. oryzae* FHb-encoding gene is unknown and we could not isolate a full length *A. oryzae fhbA* gene by PCR. Therefore, we decided to use a PCR amplified probe from the *A. niger fhbA* gene to study transcriptional regulation of the *A. oryzae fhbA* gene. Sequence similarity between these two species suggests specific hybridisation under the chosen conditions (te Biesebeke et al. 2004c). Moreover, heterologous Southern analysis with the *A. niger fhbA* probe and chromosomal DNA of *A. oryzae* revealed a single hybridising band showing that this probe is specific for a single copy FHb-encoding gene from *A. oryzae*. Therefore, the *A. niger fhbA* probe was used to detect transcript levels of the *A. oryzae fhbA* gene.

To determine the growth conditions under which transcription of the *fhbA* gene occurs, *A. oryzae* was grown in 2% WLM, on 2% WSM and on WK. Northern analysis showed that the *A. oryzae fhbA* gene transcript level is highest in the exponential growth phase in 2% WLM at 17 and 24 h (Fig. 3 lane 1 and 2) and on 2% WSM after 48 h of growth (Fig. 3 lane 5). The correlation between the growth phase and *fhbA* gene transcription was further corroborated in northern analysis with total RNA of *A. oryzae* grown for 3, 4 and 5 days on WK (Fig. 3 lanes 8-10). Although a continuous increase in biomass can not be determined accurately under these cultivation conditions, the fact that oxygen uptake rate is still increasing (Rahardjo et al. 2004), suggests that *A. oryzae* on WK continues to grow in the indicated timeframe.

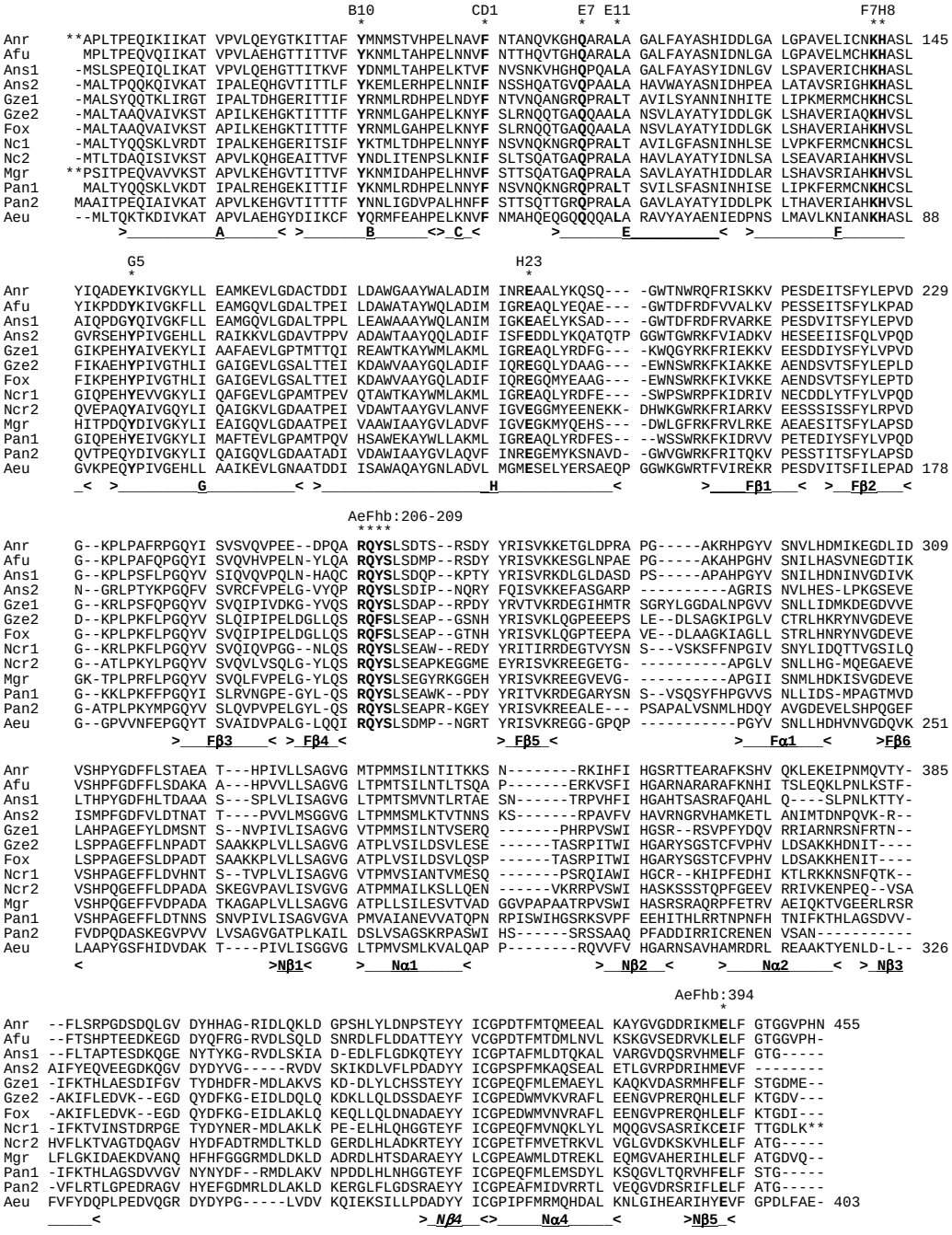

B10 CD1 E7 E11 F7H8
Anr **APLTPEQIKIIKAT VPVLQEYGTKITTAF YMNMSTVHPELNAVF NTANQVKGHQARALA GALFAYASHIDDLGA LGPAVELICNKHASL 145
Afu MPLTPEQVQIIKAT VPVLAEHGTTITTVF YKNMLTAHPELNNVF NTTHQVTGHQARALA GALFAYASNIDNLGA LGPAVELMCHKHASL
Ans1 -MSLSPEQIQLIKAT VPVLQEHGTTITKVF YDNMLTAHPELKTVF NVSNKVHGHQPQALA GALFAYASYIDNLGV LSPAVERICHKHASL
Ans2 -MALTPQQKQIVKAT IPALEQHGVTITTLF YKEMLERHPELNNIF NSSHQATGVQPAALA HAVWAYASNIDHPEA LATAVSRIGHKHASL
Gze1 -MALSYQQTKLIRGT IPALTDHGERITTIF YRNMLRDHPELNDYF NTVNQANGRQPRALT AVILSYANNINHITE LIPKMERMCHKHCSL
Gze2 -MALTAAQVAIVKST APILKEHGKTITTTF YRNMLGAHPELKNYF SLRNQQTGAQQAALA NSVLAYATYIDDLGK LSHAVERIAQKHVSL
Fox -MALTAAQVAIVKST APILKEHGKTITTTF YRNMLGAHPELKNYF SLRNQQTGAQQAALA NSVLAYATYIDDLGK LSHAVERIAHKHVSL
Nc1 -MALTYQQSKLVRDT IPALKEHGERITSIF YKTMLTDHPELNNYF NSVNQKNGRQPRALT AVILGFASNINHLSE LVPKFERMCNKHCSL
Nc2 -MTLTDAQISIVKST APVLKQHGEAITTVF YNDLITENPSLKNIF SLTSQATGAQPRALA HAVLAYATYIDNLSA LSEAVARIAHKHVSL
Mgr **PSITPEQVAVVKST APVLKEHGVTITTVF YKNMIDAHPELHNVF STTSQATGAQPRALA SAVLAYATHIDDLAR LSHAVSRIAHKHVSL
Pan1 MALTYQQSKLVKDT IPALREHGEKITTIF YKNMLRDHPELNNYF NSVNQKNGRQPRALT SVILSFASNINHISE LIPKFERMCNKHCSL
Pan2 MAAITPEQIAIVKAT APVLKEHGVTITTTF YNNLIGDVPALHNFF STTSQTTGRQPRALA GAVLAYATYIDDLPK LTHAVERIAHKHVSL
Aeu --MLTQKTKDIVKAT APVLAEHGYDIIKCF YQRMFEAHPELKNVF NMAHQEQGQQQQALA RAVYAYAENIEDPNS LMAVLKNIANKHASL 88
A B C E F
G5 H23
Anr YIQADEYKIVGKYLL EAMKEVLGDACTDDI LDAWGAAYWALADIM INREAALYKQSQ--- -GWTNWRQFRISKKV PESDEITSFYLEPVD 229
Afu YIKPDDYKIVGKFLL EAMGQVLGDALTPEI LDAWATAYWQLADIM IGREAQLYEQAE--- -GWTDFRDFVVALKV PESSEITSFYLKPAD
Ans1 AIQPDGYQIVGKFLL EAMGQVLGDALTPPL LEAWAAAYWQLANIM IGKEAELYKSAD--- -GWTDFRDFRVARKE PESDVITSFYLEPVD
Ans2 GVRSEHYPIVGEHLL RAIKKVLGDAVTPPV ADAWTAAYQQLADIF ISFEDDLYKQATQTP GGWTGWRKFVIADKV HESEEIISFQLVPQD
Gze1 GIKPEHYAIVEKYLI AAFAEVLGPTMTTQI REAWTKAYWMLAKML IGREAQLYRDFG--- -KWQGYRKFRIEKKV EESDDIYSFYLVPVD
Gze2 FIKAEHYPIVGTHLI GAIGEVLGSALTTEI KDAWVAAYGQLADIF IQREGQLYDAAG--- -EWNSWRKFKIAKKE AENDSVTSFYLEPLD
Fox FIKPEHYPIVGTHLI GAIGEVLGSALTTEI KDAWVAAYGQLADIF IQREGQMYEAAG--- -EWNSWRKFKIVKKE AENDSVTSFYLEPTD
Ncr1 GIQPEHYEVVGKYLI QAFGEVLGPAMTPEV QTAWTKAYWMLAKML IGREAQLYRDFE--- -SWPSWRPFKIDRIV NECDDLYTFYLVPQD
Ncr2 QVEPAQYAIVGQYLI QAIGKVLGDAATPEI VDAWTAAYGVLANVF IGVEGGMYEENEKK- DHWKGWRKFRIARKV EESSSISSFYLRPVD
Mgr HITPDQYDIVGKYLI EAIGQVLGDAATPEI VAAWIAAYGVLADVF IGVEGKMYQEHS--- -DWLGFRKFRVLRKE AEAESITSFYLAPSD
Pan1 GIQPEHYEIVGKYLI MAFTEVLGPAMTPQV HSAWEKAYWLLAKML IGREAQLYRDFES-- --WSSWRKFKIDRVV PETEDIYSFYLVPQD
Pan2 QVTPEQYDIVGKYLI QAIGQVLGDAATADI VDAWIAAYGVLAQVF INREGEMYKSNAVD- -GWVGWRKFRITQKV PESSTITSFYLAPSD
Aeu GVKPEQYPIVGEHLL AAIKEVLGNAATDDI ISAWAQAYGNLADVL MGMESELYERSAEQP GGWKGWRTFVIREKR PESDVITSFILEPAD 178
G H F
AeFhb:206-209
Anr G--KPLPAFRPGQYI SVSVQVPEE--DPQA RQYSLSDTS--RSDY YRISVKKETGLDPRA PG-----AKRHPGYV SNVLHDMIKEGDLID 309
Afu G--KPLPAFQPGQYI SVQVHVPELN-YLQA RQYSLSDMP--RSDY YRISVKKESGLNPAE PG-----AKAHPGHV SNILHASVNEGDTIK
Ans1 G--KPLPSFLPGQYV SIQVQVPQLN-HAQC RQYSLSDQP--KPTY YRISVRKDLGLDASD PS-----APAHPGYV SNILHDNINVGDIVK
Ans2 N--GRLPTYKPGQFV SVRCFVPELG-VYQP RQYSLSDIP--NQRY FQISVKKEFASGARP ----------AGRIS NVLHES-LPKGSEVE
Gze1 G--KRLPSFQPGQYV SVQIPIVDKG-YVQS RQYSLSDAP--RPDY YRVTVKRDEGIHMTR SGRYLGGDALNPGVV SNLLIDMKDEGDVVE
Gze2 D--KPLPKFLPGQYV SLQIPIPELDGLLQS RQFSLSEAP--GSNH YRISVKLQGPEEEPS LE--DLSAGKIPGLV CTRLHKRYNVGDEVE
Fox G--KPLPKFLPGQYV SVQIPIPELDGLLQS RQFSLSEAP--GTNH YRISVKLQGPTEEPA VE--DLAAGKIAGLL STRLHNRYNVGDEVE
Ncr1 G--KRLPKFLPGQYV SVQIQVPGG--NLQS RQYSLSEAW--REDY YRITIRRDEGTVYSN S---VSKSFFNPGIV SNYLIDQTTVGSILQ
Ncr2 G--ATLPKYLPGQYV SVQVLVSQLG-YLQS RQYSLSEAPKEGGME EYRISVKREEGETG- ----------APGLV SNLLHG-MQEGAEVE
Mgr GK-TPLPRFLPGQYV SVQLFVPELG-YLQS RQYSLSEGYRKGGEH YRISVKREEGVEVG- ----------APGII SNMLHDKISVGDEVE
Pan1 G--KKLPKFFPGQYI SLRVNGPE-GYL-QS RQYSLSEAWK--PDY YRITVKRDEGARYSN S--VSQSYFHPGVVS NLLIDS-MPAGTMVD
Pan2 G-ATPLPKYMPGQYV SLQVPVPELGYL-QS RQYSLSEAPR-KGEY YRISVKREEALE--- PSAPALVSNMLHDQY AVGDEVELSHPQGEF
Aeu G--GPVVNFEPGQYT SVAIDVPALG-LQQI RQYSLSDMP--NGRT YRISVKREGG-GPQP ----------PGYV SNLLHDHVNVGDQVK 251
F β3 β4 β5 α1 β6
Anr VSHPYGDFFLSTAEA T---HPIVLLSAGVG MTPMMSILNTITKKS N--------RKIHFI HGSRTTEARAFKSHV QKLEKEIPNMQVTY- 385
Afu VSHPFGDFFLSDAKA A---HPVVLLSAGVG LTPMTSILNTLTSQA P-------ERKVSFI HGARNARARAFKNHI TSLEQKLPNLKSTF-
Ans1 LTHPYGDFHLTDAAA S---SPLVLISAGVG LTPMTSMVNTLRTAE SN------TRPVHFI HGAHTSASRAFQAHL Q----SLPNLKTTY-
Ans2 ISMPFGDFVLDTNAT T----PVVLMSGGVG LTPMMSMLKTVTNNS KS-------RPAVFV HAVRNGRVHAMKETL ANIMTDNPQVK-R--
Gze1 LAHPAGEFYLDMSNT S--NVPIVLISAGVG VTPMMSILNTVSERQ -------PHRPVSWI HGSR--RSVPFYDQV RRIARNRSNFRTN--
Gze2 LSPPAGEFFLNPADT SAAKKPLVLLSAGVG ATPLVSILDSVLESE ------TASRPITWI HGARYSGSTCFVPHV LDSAKKHDNIT----
Fox LSPPAGEFSLDPADT SAAKKPLVLLSAGVG ATPLVSILDSVLQSP ------TASRPITWI HGARYSGSTCFVPHV LDSAKKHENIT----
Ncr1 VSHPAGEFFLDVHNT S--TVPLVLISAGVG VTPMVSIANTVMESQ -------PSRQIAWI HGCR--KHIPFEDHI KTLRKKNSNFQTK--
Ncr2 VSHPQGEFFLDPADA SKEGVPAVLISVGVG ATPMMAILKSLLQEN ------VKRRPVSWI HASKSSSTQPFGEEV RRIVKENPEQ--VSA
Mgr VSHPQGEFFVDPADA TKAGAPLVLLSAGVG ATPLLSILESVTVAD GGVPAPAATRPVSWI HASRSRAQRPFETRV AEIQKTVGEERLRSR
Pan1 VSHPAGEFFLDTNNS SNVPIVLISAGVGVA PMVAIANEVVATQPN RPISWIHGSRKSVPF EEHITHLRRTNPNFH TNIFKTHLAGSDVV-
Pan2 FVDPQDASKEGVPVV LVSAGVGATPLKAIL DSLVSAGSKRPASWI HS------SRSSAAQ PFADDIRRICRENEN VSAN----------
Aeu LAAPYGSFHIDVDAK T----PIVLISGGVG LTPMVSMLKVALQAP P--------RQVVFV HGARNSAVHAMRDRL REAAKTYENLD-L-- 326
β1 α1 β2 α2 β3
AeFhb:394
Anr --FLSRPGDSDQLGV DYHHAG-RIDLQKLD GPSHLYLDNPSTEYY ICGPDTFMTQMEEAL KAYGVGDDRIKMELF GTGGVPHN 455
Afu --FTSHPTEEDKEGD DYQFRG-RVDLSQLD SNRDLFLDDATTEYY VCGPDTFMTDMLNVL KSKGVSEDRVKLELF GTGGVPH-
Ans1 --FLTAPTESDKQGE NYTYKG-RVDLSKIA D-EDLFLGDKQTEYY ICGPTAFMLDTQKAL VARGVDQSRVHMELF GTG-----
Ans2 AIFYEQVEEGDKQGV DYDYVG-----RVDV SKIKDLVFLPDADYY ICGPSPFMKAQSEAL ETLGVRPDRIHMEVF --------
Gze1 -IFKTHLAESDIFGV TYDHDFR-MDLAKVS KD-DLYLCHSSTEYY ICGPEQFMLEMAEYL KAQKVDASRMHFELF STGDME--
Gze2 -AKIFLEDVK--EGD QYDFKG-EIDLDQLQ KDKLLQLDSSDAEYF ICGPEDWMVKVRAFL EENGVPRERQHLELF KTGDV---
Fox -AKIFLEDVK--EGD QYDFKG-EIDLAKLQ KEQLLQLDNADAEYY ICGPEDWMVNVRAFL EENGVPRERQHLELF KTGDI---
Ncr1 -IFKTVINSTDRPGE TYDYNER-MDLAKLK PE-ELHLQHGGTEYF ICGPEQFMVNQKLYL MQQGVSASRIKCEIF TTGDLK**
Ncr2 HVFLKTVAGTDQAGV HYDFADTRMDLTKLD GERDLHLADKRTEYY ICGPETFMVETRKVL VGLGVDKSKVHLELF ATG-----
Mgr LFLGKIDAEKDVANQ HFHFGGGRMDLDKLD ADRDLHTSDARAEYY LCGPEAWMLDTREKL EQMGVAHERIHLELF ATGDVQ--
Pan1 -IFKTHLAGSDVVGV NYNYDF--RMDLAKV NPDDLHLNHGGTEYF ICGPEQFMLEMSDYL KSQGVLTQRVHFELF STG-----
Pan2 -VFLRTLGPEDRAGV HYEFGDMRLDLAKLD KERGLFLGDSRAEYY ICGPEAFMIDVRRTL VEQGVDRSRIFLELF ATG-----
Aeu FVFYDQPLPEDVQGR DYDYPG-----LVDV KQIEKSILLPDADYY ICGPIPFMRMQHDAL KNLGIHEARIHYEVF GPDLFAE- 403
α4 β4 α4 β5

Fig. 2. (*previous page*) *Amino acid alignment of N-terminal truncated (**) FHb of A. niger, the N-terminal truncated (**) FHb's and non-truncated FHb's of the Pezizomycotina, to the A. eutropus (Ermler et al. 1995) FHb. The abbreviations are as shown in Table 1. Bold-faced residues marked with an asterisk represent important residues (see text for details). The 6 α-helices of the hemoglobin domain are marked (A, B, C, E, F, G, H) as well as the different secundary structures in the FAD binding domain (Fα/β) and the NAD(P)H binding domain (Nα/β), and flanked by > <.*

The results in Fig. 3 (lanes 1-10) are in agreement with the results shown for *S. cerevisiae* that the YHB1 gene is induced during exponential growth (Crawford et al. 1995). To analyse if, as suggested to be the case in *S. cerevisiae* (Crawford et al. 1995), the amount of *A. oryzae* biomass affects *fhbA* transcript levels, cultures were grown for 53 h until the maximum amount of biomass was produced in 25 ml 2% WLM. Subsequently, biomass was harvested after filtration through miracloth and transferred to 25 ml 2% WLM and the *fhbA* gene transcription was analysed. The transcript levels of the *A. oryzae fhbA* gene re-appeared after 4, 6 and 8 h transfer (Fig. 3 lane 11-16) and disappeared again after 30 h. These results show that the absence of transcript at 53 h is not the effect of the amount of biomass produced because increase in biomass formation after transfer to fresh medium resulted in renewed *fhbA* gene transcription.

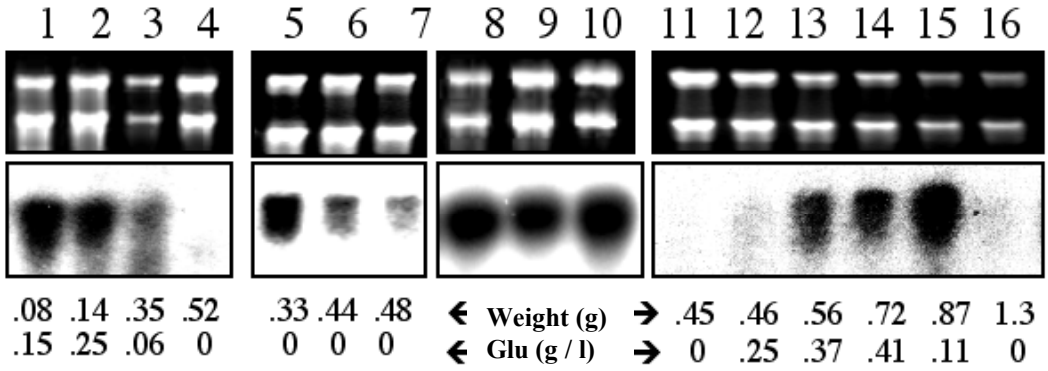


Fig. 3. *Transcript levels of the fhbA gene during growth of A. oryzae in 2% WLM (lane 1: 17 h, 2: 24 h, 3 42 h, 4: 53 h), 2% WSM (lane 5: 2 days, 6: 3days, 7: 4 days), wheat kernels (WK) (lane 8: 3 days, 9: 4 days, 10: 5 days). Alternatively, A. oryzae was grown for 53 h in 2% WLM and transferred to 2% WLM for either 0 (lane 11), 2 (lane 12), 4 (lane 13), 6 (lane 14), 8 (lane 15) or 30 (lane 16) h. The Biomass (weight (g)) and glucose concentration (Glu (g/L)) in the growth medium or the extracts of the growth medium were determined as described in (te Biesebeke et al. 2004a).*

***fhbA* gene transcription during hyphal growth.** To eleborate further on the correlation between *fhbA* gene transcription and growth, two experimental approaches were performed. *A. oryzae* grown for 4 days on 2% WSM on a membrane was transferred to fresh 2% WSM and to an agar plate with only water (WAM). There was no difference in biomass observed after 6 and 9 h transfer to either 2% WSM and WAM. However, newly formed penetrative hyphae were observed and transcript levels of the *fhbA* gene were detected only in 2% WSM after 6 and 9 h transfer (Fig. 4 lane 1-4). In another approach, shake flasks with 2% WLM were inoculated and incubated without shaking. Compared to 48 h of growth, at 72 h biomass increased and formation of aerial hyphae was observed (See schematic drawing Fig. 4). At 120 h no biomass and macroscopic difference was observed compared to that at 96 h. Fig. 4 shows that transcript levels of the *A. oryzae fhbA* gene were detected during

submerged biomass formation (lane 5), surface growth and aerial hyphae formation (lane 6, 7) and disappeared when cells entered stationary growth phase (lane 4). As suggested before for *S. cerevisiae* (Gasch et al. 2000) and *C. albicans* (Nantel et al. 2002) these results (Fig. 3 and Fig. 4 lane 1-4 and 5-8) sustain a suggested relation between *fhbA* gene transcription and growth.

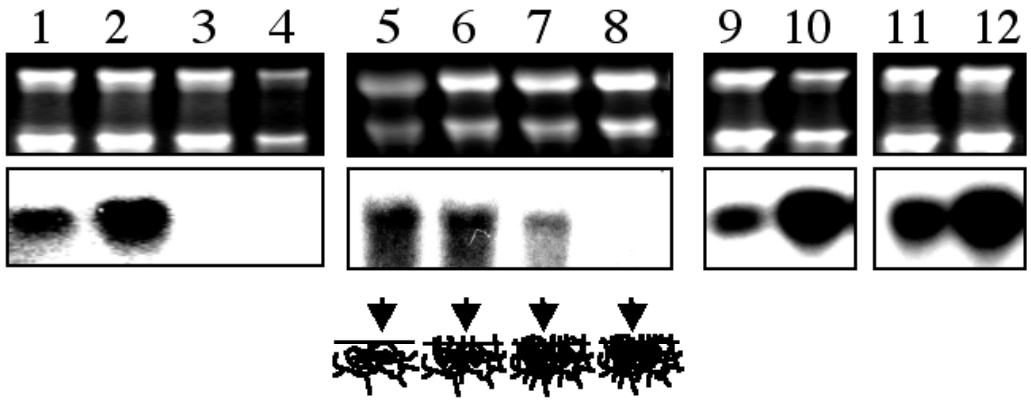


Fig. 4. *Transcription of the fhbA gene during growth of A. oryzae and the hyperbranching pclA disrupted strain. Northern analysis after transfer of 72 h grown A. oryzae in 2% WSM to fresh 2% WSM for 6 (lane 1) or 9 h (lane 2) or transfer to agar medium (WAM) for 6 (lane 3) or 9 h (lane 4). Transcriptional analysis during growth of A. oryzae in 2% WLM without shaking after 48 (lane 5), 72 (lane 6), 96 (lane 7) and 120 (lane 8) h. The drawings represent a schematic progress of the observed filamentous fungal growth. The horizontal line in the drawing represents the 2% WSM/air interface. The wild-type (lane 9) and pclA disrupted (lane 10) strains were grown for 72 h and transferred for 6 h to fresh 2% WSM. Moreover, the wild-type (lane 11) and pclA disrupted (lane 12) A. oryzae strains were grown for 3 days on WK and fhbA gene transcription was analysed.*

***fhbA* gene transcription in a hyperbranching strain.** Disruption of the *pclA* gene in *A. oryzae* results in a hyperbranching growth phenotype (Mizutani et al. 2004,

te Biesebeke et al. 2004d), in higher transcript levels for the *mpkA* gene and constitutive increased levels of phosphorylated MpkA compared to the wild-type (Mizutani et al. 2004). Northern analysis was performed with total RNA isolated from the wild-type and *pclA* disrupted strain after 6 h of membrane transfer assay performed as described (te Biesebeke et al. 2004b). Under these conditions, the *pclA* disrupted strain showed high transcript levels of the *mpkA* gene compared the wild-type (not shown). Fig. 4 (lane 9-10) shows that also the *fhbA* gene transcript levels are 5 times higher in the *pclA* disrupted strain compared to the wild-type. The wild-type and *pclA* disrupted strains were also grown on WK for 3 days. Northern analysis with total RNA isolated from the wild-type and *pclA* disrupted strain revealed that, on the WK, the expression of the *fhbA* gene was about 2 times higher compared to the wild-type (Fig. 4, lane 11-12).

DISCUSSION

In this study we describe the isolation of the *fhbA* gene encoding *A. niger* FHb and its comparison with other reported putative FHb-encoding genes of other filamentous fungi. The fungal genome databases revealed that the obligate aerobe filamentous fungi, *N. crassa*, *G. zeae* and *A. nidulans* all have two putative FHb-encoding genes. Other fungal genomes like that of the facultative anaerobe *S. cerevisiae* contains one FHb encoding gene in its genome while *C. albicans* contains 3 different FHb's. Interestingly, the different FHb's from the same species do not cluster together in preliminary phylogram analysis (not shown). Moreover, eukaryotic and bacterial FHb's do not clearly cluster in separate clades (not shown) suggesting that the FHb may have an unusual phylogeny. This unusual phylogeny is further corroborated by analysis of *Basidiomycetous* FHb sequences. *P. chrysosporium* and

C. neoformans have a single FHb-encoding gene, while other *Basidiomycetes* with available genome sequences, *Ustilago maydis* and *Coprinus cinerea* did not have any gene product that resembled a FHb. Also the fungi related *Oomycete Phytophthora sojae* and *Phytophthora ramorum* with completed genome sequences did not contain any gene product that resembled a FHb. Besides fungal homologues only few other lower eukaryotic species (*Dictostelium discodeum*, *Giardia lamblia)* have a FHb-encoding gene.

In contrast to the intron-less bacterial and *Ascomycetous* FHb-encoding genes, the *Basidiomycetous* genes are interrupted by respectively 1 (*P. chrysosporium*), 3 (*C. neoformans var. neoformans* (JEC21 and B-3501)) and 4 (*C. neoformans var. grubbii* H99) introns.

Besides the difference in the number of FHb-encoding genes per species, for a few of the encoded proteins an additional N-terminal domain was observed. The *A. niger, P. chrysosporium, M. grisea, S. pombe* and *C. neoformans* FHb's contain a N-terminal stretch of respectively 43, 15, 83, 24 and 79 amino acids. However, it is unknown what the functional relevance of these N-terminal residues is.

Attempts to elucidate the function of fungal FHb's have resulted in the conclusions that the protein might be involved in diverse functions such as oxygen storage (Kobayashi et al. 2002), protection from nitrosative stress (Lui et al. 2002, Jesus-Berrios et al. 2003) and oxidative stress (Zhao et al. 1996, Buisson and Labbe-Bios 1998). The unusual diversity in the number of FHb-encoding genes in the genomes of fungal species might therefore reflect the differences in requirement for FHb function in each individual species.

A difference in transcriptional regulation between the bacterial (reviewed by Frey and Kallio 2003) and fungal (Zhao et al. 1996, Buisson and Labbe-Bios 1998)

FHb gene regulation could reflect a difference in FHb function in the different species. In this study we show that gene expression of the *Aspergillus oryzae fhbA* gene is correlated to hyphal growth (Fig. 3, 4). These results are in agreement with what has been shown for *S. cerevisiae* YHB1 gene transcription (Gasch et al. 2000) but are in contrast with the induction of the *E.coli* FHb-encoding gene during the stationary growth phase that is mediated by the stationary phase-specific sigma subunit (sigma^S) of RNA polymerase (Membrillo-Hernandez et al. 1997). This suggests a need for FHb during stationary growth phase in bacteria while that of fungi is needed during hyphal growth.

The *pclA* disrupted mutant showed increased transcript levels of the *mpkA* gene compared to the wild-type, indicating elevated MpkA activity in a *pclA* strain compared to the wild-type, in parallel with increased *fhbA* transcript levels (Fig. 4, Mizutani et al. 2004). In *S. cerevisiae*, the corresponding mitogen-activated protein kinase is part of a kinase cascade that activates transcription factor Rlm1p (Watanabe et al. 1995, Jung and Levin 1999) and other transcription factors (Ste12, Crz1, Tec1 and SBF) (Garcia et al. 2004, Heinish et al. 1999). These transcription factors are involved in induction of genes encoding proteins involved in invasive growth and the cell integrity pathway of *S. cerevisiae* (Breitkreutz et al. 1999, Heinish et al. 1999). Besides Mpk1 (Watanabe et al. 1995, Jung and Levin 1999), also protein kinases Fus3, Kss1 are involved in invasive growth of *S. cerevisiae* (Elion 2001 and references therein). In *C. albicans* it has been shown that *YHB1* expression is modulated during hyphal development and that this is controlled by the Cph1 transcription factor (Nantel et al. 2002). The *C. albicans* Chp1, the homologue of *S. cerevisiae* Ste12, is involved in the pseudohyphal and invasive growth phenotype (Lui et al. 1993, Roberts and Fink 1994). The transcription analysis in Fig. 3 and 4

suggests that in *A. oryzae* a similar signal transduction pathway mediated by the mitogen protein A kinase (MpkA) regulate the *fhbA* gene transcription. This mechanism of regulation of *fhbA* gene expression could reflect the need to store oxygen and/or deliver protection against nitrosative and oxidative stress, during hyphal growth of filamentous fungi.

CHAPTER 8

Overproduction of *Aspergillus* hemoglobin domains in *Aspergillus oryzae* grown on solid substrates improves biomass yield, growth rate and enzyme production

R. te Biesebeke, A. Boussier, N. van Biezen, M. Braaksma, C.A.M.J.J. van den Hondel, W.M. de Vos, P.J. Punt (2004) Overproduction of *Aspergillus* hemoglobin domains in *Aspergillus oryzae* grown on solid substrates improves biomass yield, growth rate and enzyme production**.** *Submitted for publication.*

ABSTRACT

DNA-fragments coding for hemoglobin-domains (HBD) were isolated from *Aspergillus oryzae* and *Aspergillus niger*. The encoded amino acid sequences showed conserved amino acids compared to the well-studied *Vitreoscilla* hemoglobin. The HBD of *A. niger* and *A. oryzae* were overproduced in *A. oryzae* by introduction of the hemoglobin-domain gene fragments under the control of the constitutively expressed *gpdA* gene. In the transformants the oxygen uptake was significantly higher than that of the untransformed wild-type strain, demonstrating functional expression of the HBD. Moreover, during growth on potato dextrose agar and 5% wheat-based solid medium (5% WSM) the developed wet weight biomass was at least 1.3 times higher in the HBD-overproducing strains compared to the wild-type. Growth rate of the HBD-overproducing strains was also significantly higher during growth on 5% WSM compared to the wild-type. During growth on 5% WSM and ground wheat kernels (WK) the amylase and protease activities in the extracts of the HBD-overproducing strains were 30% to 150% higher and glucoamylase activities were at least 9 times higher compared to those measured in the wild-type strain. These results suggest that the *Aspergillus* HBD-encoding gene can be used in a self-cloning strategy to improve biomass yield and protein production of *Aspergillus* species.

INTRODUCTION

Oxygen is essential for maximal energy yield and optimal utilisation of substrate in every aerobic organism (Frey and Kalio 2003). During growth of *A. oryzae* on solid substrates, the aerial hyphae account for 70% of the oxygen uptake (Rahardjo et al. 2001). It is shown that diffusion of oxygen is limited in the

filamentous fungal layer that covers the solid substrate and that the substrate penetrative hyphae are limited in oxygen consumption and growth (Oostra et al. 2001, Rahardjo et al. 2001). Therefore, oxygen supply to filamentous fungal cells that are in close contact with the substrate is considered as a bottleneck in solid state fermentation (Oostra et al. 2001, Rahardjo et al. 2001).

Hemoglobins bind oxygen reversibly and have been discovered in a wide range of organisms including vertebrates, invertebrates, higher plants, fungi and bacteria (Weber and Vinogradov, 2001). Despite the fact that all known hemoglobins have a highly variable primary amino acid sequence, they all show a 6 to 8 α-helical arrangement that facilitates binding of heme in the hydrophobic core of the protein (Frey and Kallio 2003). Hemoglobin bridges a wide variation in oxygen tensions at the sites of oxygen loading and unloading and plays therefor a major role in oxygen transport although specific hemoglobins may be specialised for other particular functions (Weber and Vinogradov 2001). Several hemoglobin domain (HBD) genes have been recently identified in various fungi, including *Aspergillus* species (te Biesebeke et al. 2004e). These HBD are a part of flavohemoglobins containing N-terminal hemoglobin and C-terminal reductase domains.

The expression of *Vitreoscilla* hemoglobin in *Eschericia coli* (Yu et al. 2002, Andersson et al. 2003) and *Enterobacter aerogenes* (Geckil et al. 2003) has been shown to correlate with improved protein synthesis, enhanced intracellular ribosome and tRNA contents and improved growth/survival properties. Moreover, *Vitreoscilla* hemoglobin production in *Yarrowia lipolitica* (Bhave and Chattoo 2003), *Pichia pastoris* (Wu et al. 2003), and *Acremonium chrysogenum* (DeModena et al. 1993) resulted in higher enzyme production, improved growth and higher cephalosporin C production, respectively. Production of *Vitreoscilla* hemoglobin in *Aspergillus terreus*

resulted in improved itaconic acid production (Lin et al. 2004). However, correlation with improved biomass production or enzyme production has not been made.

To evaluate whether besides *Vitreoscilla* hemoglobin, also the HBD of *A. oryzae* and *A. niger* can be used to improve fungal growth and enzyme production, *A. oryzae* strains were constructed that overproduce the *Aspergillus* HBD. The strains were analysed for growth, biomass yield and the production of secreted amylase and protease activities.

MATERIAL AND METHODS

Strains and media. *A. oryzae* ATCC16168 was used throughout this study. Growth on ground wheat kernels (WK) and 5% wheat-based solid medium (5% WSM) was performed as described (te Biesebeke et al. 2004a, 2004b). Potato dextrose agar (Oxoid) (PDA) was prepared as described by the manufacturer. Complete medium (CM) consisted of 1% glucose, 0.1% Yeast extract, 0.1% casamino-acids, 0.2% peptone, 2 mM $MgSO_4$, 10 mM $NaNO_3$, spore elements. Minimal medium is CM without peptone, yeast extract and casamino-acids. For membrane cultures nitrocellulose membranes (3 μm pore size, Millipore) that were placed on 25 ml of the agar-solidified substrates in petridishes inoculated with $2.5X10^7$ conidia as described (te Biesebeke et al. 2004b).

Isolation of the HBD encoding DNA fragments. To amplify the DNA fragment (444 nucleotides) of the HBD gene of *A. niger*, primers 57ANFHB1(5'CATGCCATGGCGCTCACACCAGAGCAGATC3') and 58ANHB2 (5'GGAAGATCTTTAGCCCTGGCTTTGCTTGTAGAGTGC3') were designed on the basis of the flavohemoglobin encoding gene (GenBank accession no AJ629189, te Biesebeke et al. 2004e). To amplify the DNA fragment (444 nucleotides) coding for the HBD of *A.*

oryzae, primers 50HbAONCO (5'CATGCCATGGCGCTCTCCCCTGAACAAATC3') and 53HbOBAM (5'CGCGGATCCTTATCCGTCGGCCTGCTT3') were designed on the basis of the flavohemoglobin gene from *A. nidulans* (Accession no AACD01000122, region: 103592 to 104824) and the AoEST04885 sequence (nrib.go.jp/ken/EST/db/blast.html). Primers were constructed in such a way that *Nco*I and *Bam*HI restriction sites were introduced in the DNA fragment at the 5' and 3' terminal sites respectively. In both 3' primers, an in frame stop codon was introduced 5' of the *Bam*HI restriction site. *Taq* DNA polymerase (Boehringer) was used with *Aspergillus* chromosomal DNA in 40 cycles PCR amplification (30 s at 94°C, 1 min at 45°C, 30 s at 72°C) according to the manufacturers protocol. DNA fragments were purified from 1% agarose gel electrophoresis with the Qiaquick DNAeasy columns (Qiagen, UK) and cloned in pGEM-T easy vectors (PROMEGA) and sequenced. Sequencing was performed with the Cycle Sequencing Kit from Pharmacia according to the manufacturer protocol. Sequence data were obtained with the ABI Prism 310 Genetic Analyser from Applied Biosystems (Perkin-Elmer division). The M13 Forward and Reverse sequencing primers (Table 1) were used for sequence analysis of the cloned HBD DNA fragment from *A. niger* and *A. oryzae*. Nucleotide sequences for the *A. oryzae* and *A. niger* HBD DNA fragments were assigned Genbank accession numbers AJ628839 and AJ62840. The sequence of the DNA fragment of *A. oryzae* contained a *Nco*I restriction site that restrained the chosen cloning strategy. Therefore, a silent point mutation was introduced at the *Nco*I restriction site by using the overlap PCR extension method (Yolon and Shabarova 1990, Yon and Fried 1989) and primers 51HbOmut1 (5'GGACCTCGCCCATTGCCTCCAAC3') and 52HbOmut2 (5'GTTGGAGGCAATGGGCGAGGTCC3'). Subsequently, the mutated *A. oryzae* DNA fragment was cloned and sequenced confirming the presence of the desired DNA sequence.

Construction of the expression vectors and fungal transformation. Plasmid pAN52-1 Not (GenBank accession number Z32524) containing the promoter region of the *gpdA* gene of *Aspergillus nidulans* was used for all constructs. Plasmid pHBN and pHBO were constructed by introducing the 0.4 kb *Nco*I/*Bam*HI digested PCR amplified *A. niger* and *A. oryzae* hemoglobin encoding DNA fragment in plasmid pAN52-1 Not. Sequencing of the constructed plasmids confirmed the presence of the correct insert sequence.

Plasmids pHBN and pHBO were used in a co-transformation experiment with plasmid pAB4-1 (vanHartingsveldt et al. 1987) containing the *A. niger pyrG* auxotrophic selection marker gene as described by van den Hondel (1992) to transform the *A. oryzae* ATCC16868*pyrG* (te Biesebeke et al. 2004d). Co-transformants were selected for growth in the absence of uridine (Verdoes et al. 1993). From each transformation a dozen of transformants were analysed by colony hybridisation (Sambrook et al. 1989) using a ^{32}P-labelled *trpC* terminator probe, a DNA fragment which is part of the expression vector pAN52-1Not (Z32524). In all cases, transformants that showed significant hybridisation to the trpC-probe were used for further analysis.

Analysis of HBD production. A method to detect the presence of an active HBD was based upon consumption of oxygen of exponentially grown wild-type and transformed cells in complete medium (CM) similar as was described previously (Yu et al. 2002). The quantitative determination of dissolved oxygen (DO) was determined in a shake flask using an oxygen electrode connected with the control system of a New Brunswick fermentor as was described previously (Yu et al. 2002). Oxygen calibration was carried out with cell free CM medium saturated with oxygen after 15 min of bubbling of pure oxygen through the medium set at 100% saturation. Equal

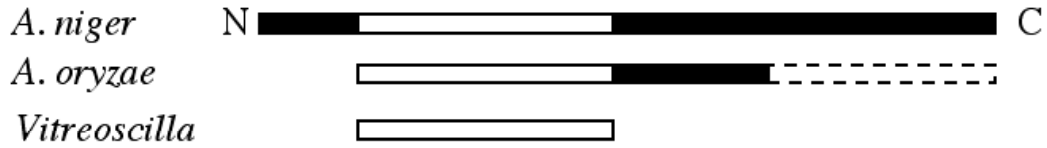
amounts (3 g) of exponentially grown wild-type or transformed cells were transferred to 100 ml 100% oxygen-saturated CM medium and DO changes were measured. As control experiments DO changes were measured in 100 ml CM with 100% oxygen saturation and in 100 ml CM with 100% oxygen-saturation with 15 g of wet weight wild-type biomass.

Analysis of secreted enzyme production. Extracts from the *A. oryzae* wild-type and the transformants harboring pHBN and pHBO were grown for 5 and 6 days on ground WK or for 3 days on 5% WSM were prepared and analysed for α-amylase, glucoamylase and protease activities as described by te Biesebeke et al (2004a, 2004b).

RESULTS

Cloning of the *Aspergillus* HBD genes. The DNA fragments of the HBD-encoding gene of *A. oryzae* and *A. niger* were PCR amplified and sequenced. The deduced amino acid sequences were aligned to that of the *Vitreoscilla* hemoglobin and the secondary structure elements were assigned (Fig. 1) (Ermler et al. 1995, Ilari et al. 2002). The heme molecule is embedded in a hydrophobic crevice formed by the 6 α-helices (Weber and Vinogradov 2001, Ilari et al. 2002, Frey and Kallio 2003), showing a number of invariant residues. Overall the amino acid sequences are 44% identical and the residues that are involved in stabilisation of the heme bound dioxygen (Tyr-B10, Gln-E7) are conserved. Moreover, the His-F8, Tyr-G5 and Glu-H23 residues that are involved in formation of the catalytic triad at the proximal site (Frey and Kallio 2003) are also conserved between the fungal and *Vitreoscilla* hemoglobin.

A



B

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                                    B10           CD1         E7 E11
                                     *             *           *  *
AN  MPLTPEQIKIIKATVPVLQEYGTKITTAXYMNMSTVHPELNAVFNTANQVKGHQARALA 60
AO  MPLSPEQIQLIKATVPVLQQHGTTITTVFYNNMLTAHPELNAVFNNANKVNGHQPRALA 59
VT  -MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQESLEQPKALA 58
    >__________A__________<>_____B_____<             >______

                               F7H8        G5
                                **          *
AN GALFAYASHIDDLGALGPAVELICNKHASLYIQADEYKIVGKYLLEAMKEVLGDACTDDI 120
AO GALFAYASHIDDLGALGPAVELICNKHASLYIQPEQYQIVGKFLLEAMGEVLGDALTPEI 119
VT MTVLAAAQNIENLPAILPAVKKIAVKHCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDI 118
    ___E_______<>_________F___________< >_____G_______< >______

                      H23
                       *
AN LDAWGAAYWALADIMINREAALYKQSQG 147
AO LDAWATAYWQLADLMIGREAELYKQADG 147
VT LDAWGKAYGVIADVFIQVEADLYAQAVE 146
   _____________H_____________<
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Fig. 1 A. *Schematic representation of the A. niger and (partial) A. oryzae flavohemoglobin proteins, and the Vitreoscilla hemoglobin. The hemoglobin domains (HBD) are shown as white boxes. The black box at the N-terminus (N) represents the N-terminal extension of the A. niger protein. The black box at the C-terminal side (C) of the A. niger protein represents the reductase domain. The unidentified part of the A. oryzae reductase domain is shown as a dotted line.* ***B****. Alignment of the predicted amino acid sequence of the A. niger (AN; CAF32308.1), A. oryzae (AO; CAF32307.1), and Vitreoscilla (VT; P04252) HBD sequences using clustalW 1.82. In the A. niger expression vector pHBN a methionine codon is introduced at the position corresponding to the methionine codon predicted from the A. oryzae sequence. > < mark the limits of the domains. Identical amino acids are shown in bold. The 6 α-helixes (A, B, E, F, G, H) and the residues that might be involved in hemoglobin function (B10: Y, CD1: F, E7: Q, E11 : L, F7: K, H8: H, G5: Y, H23: E) are marked with an asterisk. The amino acids were identified according to Frey and Kalio (2003); see text for further details.*

A. oryzae Strain	*O_2 consumption (%*min^{-1})*	*Growth Rate (mg/h)*	*Amylase (U/mg)*	*Glucoamylase (U/mg)*	*Protease pH 5.5 (U/mg)*	*Protease pH 7 (U/mg)*	*Protease pH 8.5 (U/mg)*
WT	3.4	37	952	0	95	148	667
PHBN	5.1	51	1524	11	214	233	1095
PHBO	5.0	58	1773	9	190	281	1238

Table 1. *Oxygen consumption, growth rate and enzyme activities of the hemoglobin-producing and wild-type strains. Oxygen consumption rates were determined from results shown in Fig. 2 presuming that the oxygen consumption was constant during the first 2 min and expressed in decreased O_2 percentage per min (%*min^{-1}). Growth rates were determined from the results in Fig. 3B presuming that they were constant during the first 30 h of growth on 5% WSM and were expressed in amount of wet weight biomass formed per h (mg/h). Enzyme activities were measured in extracts after 3 days of growth of the wildtype, and the HBD-producing strains (harboring plasmid pHBN and pHBO) grown on 5% WSM. Extracts were prepared as described (te Biesebeke et al. 2004b). Enzyme activities were expressed as Units per mg of wet weight solid state fermentation sample (U/mg).*

HBD overexpression in *Aspergillus oryzae* transformants. The *A. oryzae* and *A. niger* HBD-encoding genes were overproduced in *A. oryzae.* One transformant of each overproduction plasmid (pHBO and pHBN, respectively) was selected for further analysis. To determine whether the transformants produced HBD, cell-free extracts were analysed by SDS-PAGE. As production of the 16 kDa HBD could not clearly be detected in the protein extracts of the transformants, an alternative method to demonstrate HBD production was chosen. Cells were harvested after 20 h of growth in complete medium and transferred to oxygen-saturated complete medium. The change of dissolved oxygen (DO) in the growth medium was determined after addition of the same amounts of either wild-type or transformed cells (Fig. 2). This analysis shows that the cells producing the *Aspergillus* HBD show a marked faster decrease in the amount of dissolved oxygen compared to the wild-type cells (Tabel 1). These results indicate that both the *A. niger* and *A. oryzae* HBD genes are expressed

and produce proteins with an active conformation. Moreover, this implies that HBD overproduction increases the respiratory capacity of *A. oryzae*.

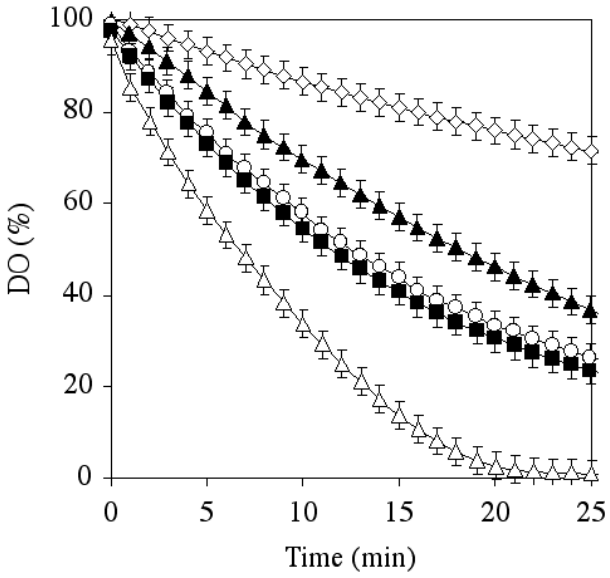

Fig. 2. *Oxygen consumption in A. oryzae wild-type and transformants. Dissolved oxygen (DO(%)) was determined in oxygen saturated complete medium (CM) after addition of 3 g wet weight wild-type (▲), or HBD-producing strains (pHBN: ▪,* pHBO: O). *The open triangles (Δ) represent the time course with 15 g wild-type cells, and the open diamonds (◊) represent that of CM without addition of biomass. Results are the average of 2 independent experiments.*

Growth, Growth rate and Enzyme production. To determine the impact of overproduction of the Aspergillus HBD on growth of A. oryzae, transformants were grown on filters that were placed on top of minimal medium (MM), 5% WSM and potato dextrose agar (PDA). Fig. 3 shows that with the HBD-overproducing strains, the biomass yield is significantly higher (at least 1.3 times) when grown on the different media compared to the wild-type strain. Dry weight measurements confirmed the observed difference between the HBD-overproducing strains and the

wild-type (not shown). These results suggest that the substrates are faster converted by the HBD-overproducing strains than the wild-type strain.

Besides biomass weight, also different enzyme activities were measured in the extracts of the HBD-overproducing and wild-type strains grown for 3 days on 5% WSM. Table 2 shows that the α-amylase, glucoamylase and protease activities are all higher in the HBD-overproducing strains compared to the wild-type. In another approach the HBD-overproducing and wild-type strains were grown for 5 and 6 days on ground WK (te Biesebeke et al. 2004a and 2004d) and thereafter enzyme activities were determined in the extracts of these cultivations. Table 2 shows that the α-amylase activity in the extract of the HBD-overproducing strains is at least 30% and 60% higher compared to that of the wild-type after respectively 5 and 6 days of growth. The glucoamylase activity is at least 9 times higher in the extracts of the HBD-overproducing strains compared to that of the wild-type strain. The protease activities measured in the extracts of the HBD-overproducing strains are at least 3.8 and 4.5 times higher, respectively, compared to that of the wild-type strain after 5 and 6 days of growth on the ground WK.

A. oryzae Strain	*Time (d)*	*Amylase (U/g)*	*Glucoamylase (U/g)*	*Protease pH5.5 (U/g)*	*Protease pH7 (U/g)*	*Protease pH8.5 (U/g)*
WT	5	129	0.8	12	10	13
pHBN	5	168	7.4	53	57	47
pHBO	5	200	12.8	59	64	48
WT	6	134	1.0	14	16	14
pHBN	6	214	12.9	88	96	61
pHBO	6	229	11.7	91	109	83

***Table 2**. Hydrolytic enzyme production in A.oryzae wild-type and transformants. Enzyme activities were determined in extracts after 5 and 6 days (D) of growth of the wild-type, the A. niger (pHBN) and A. oryzae (pHBO) HBD-overproducing strains grown on ground wheat kernels (WK). Extracts were prepared as described (te Biesebeke et al. 2004a). The results are the average of 2 experiments. Standard errors did not exceed 15% of the shown values.*

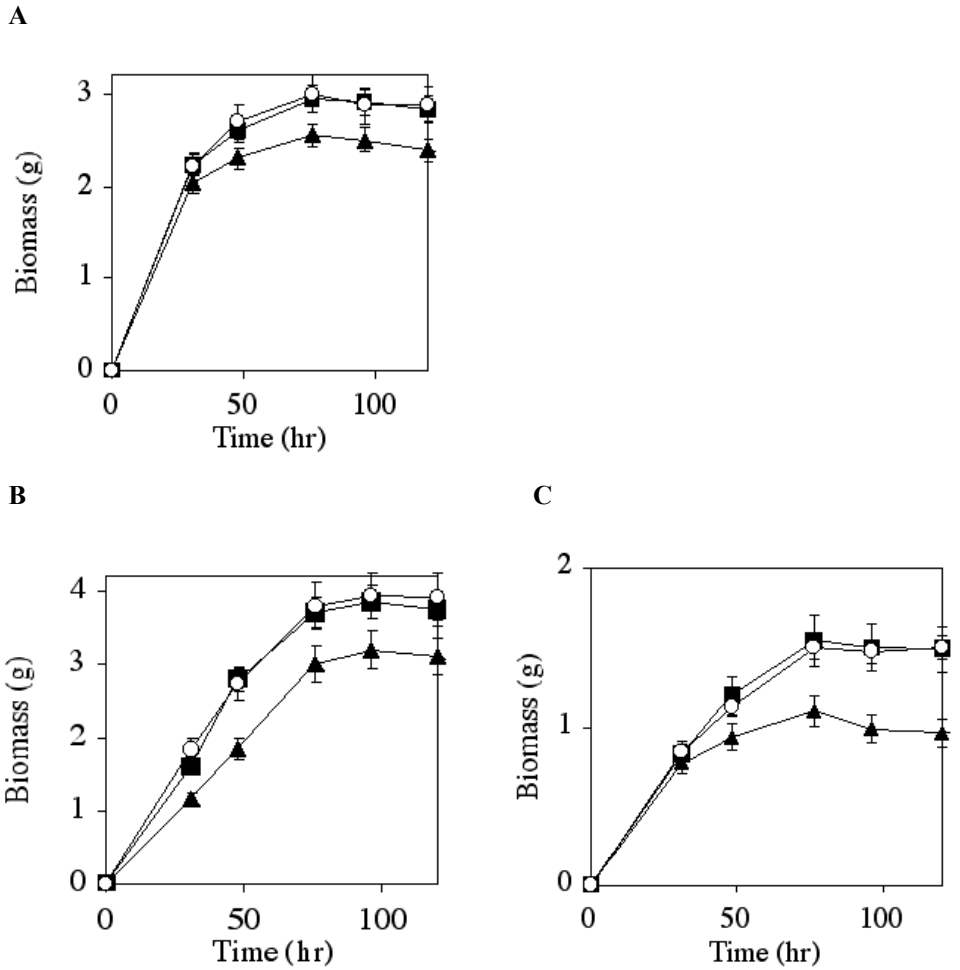

Fig. 3 A B C. *Biomass in A. oryzae wild-type and transformants*. *Biomass development was determined as gram wet weight of the wild-type (▲) and HBD-overproducing A. oryzae strains (pHBN: ▪, pHBO:* O*) during cultivation for 120 h in minimal medium (**A**), 5%WSM (**B**) and PDA (**C**).*

DISCUSSION

In bacterial (*E. coli*, *E. aerogenes*) and fungal (*P. pastoris*, *A. chrysogenum*, *Y. lipolytica*) hosts, the expression of the gene encoding *Vitreoscilla* hemoglobin has been shown to correlate with improved growth as well as improved protein and product synthesis (DeModena et al. 1993, Kang et al. 2002, Yu et al. 2002, Wu et al. 2003, Bhave and Chattoo 2003, Geckil et al. 2003, Andersson et al. 2003, Lin et al. 2004). In this paper, we describe the expression of DNA fragments encoding the

Aspergillus HBD in *A. oryzae* analysis of the resulting transformants grown on different solid substrates. As was observed for the *Vitreoscilla* hemoglobin production in different fungal species, the overproduction of the *Aspergillus* HBD in *Aspergillus oryzae* have beneficial effects on growth rate, biomass and enzyme production (Table 1 and 2, Fig. 2, 3). This was further sustained by the analysis of one other transformant of each overexpression plasmid (pHBO and pHBN, respectively) that showed similar results for oxygen consumption and protein production (not shown).

A possible mechanism has been suggested for the observed increase in growth rate of *Vitreoscilla* hemoglobin-overproducing *E.coli* cells. Although it is impossible to produce crystalline forms of many docked electron transfer complexes, indirect evidence suggests interaction between hemoglobin and components of the electron transport chain (Liu et al. 2004, Kidd et al. 2002, Naito et al. 2001). The cytochrome bo subunit I of *E. coli*, *Vitreoscilla* and *Pseudomonas aeruginosa* were found to interact significantly with *Vitreoscilla* hemoglobin (Park et al. 2002). This subunit I is the terminal oxidase that is responsible for the reduction of oxygen and, therefore, it has been suggested that *Vitreoscilla* hemoglobin transports oxygen to cytochrome bo and thereby improving the efficiency of respiration and growth in these bacteria (Tsai et al. 2002, Park et al. 2002). Since a large sequence homology occurs between *Vitreoscilla* and *Aspergillus* HBD (Fig. 1), it is tempting to assume that similar interactions of the terminal oxidases of *Aspergillus oryzae* with the *Aspergillus* HBD occur.

The higher growth rate and biomass yield that is observed in the *Aspergillus* HBD-domain producing *A. oryzae* strains grown on the different substrates suggest that these strains convert more substrate compared to the wild-type. Microscopic observations showed that the spore germination and branch frequency of the HBD-

expressing strains are not differing from that of the wild-type (not shown). Therefore, it may be conceived that the hyphae of the HBD-overproducing strains penetrate the substrate better than the wild-type due to improved oxygen supply to these penetrative hyphae. During filamentous fungal growth on solid substrates, it is generally assumed that there is a limitation in the oxygen supply to the cells that are in close contact with the substrate (Oostra et al. 2001, Rahardjo et al. 2002). Therefore, it is likely that the *Aspergillus* HBD function as an oxygen transport- or storage-protein in the overproducing *A. oryzae* strains.

Our results show that besides overproduction of the bacterial *Vitreoscilla* hemoglobin in bacterial and fungal species, also *Aspergillus* HBD-production in *Aspergillus oryzae* improves growth and enzyme production during growth on a solid substrate. Therefore, it is now possible to follow self-cloning strategies, aiming at strain improvements, with the *Aspergillus* HBD-coding DNA fragments in industrial used *Aspergillus* strains.

CHAPTER 9

General discussion and concluding remarks

Introduction

Aspergillus oryzae has been used for over 1000 of years in solid state food fermentations and *Aspergilli* have been used for decades for the production of proteins. Although the first industrial use of partially purified enzyme preparations of filamentous fungi involved *A. oryzae* in a solid state fermentation process (Takamine 1894,Takamine 1914) fundamental knowledge about the microbial biology of this process is limited compared to growth of filamentous fungi in submerged fermentation. However, recently, increasing research attention is given to growth of filamentous fungi on a solid substrate because of its high protein production capacity (Pandey et al. 1999, te Biesebeke et al. 2002, Holger et al. 2004). The general aim of the research described in this thesis was to expand our knowledge about gene expression and protein production by filamentous fungi grown on solid substrates and to use this knowledge to improve protein production of filamentous fungi and hence to improve the solid state fermentation process. The most remarkable feature of filamentous fungi during growth on a solid substrate is the very different hyphal growth phenotype compared to growth in submerged fermentation (Chapter 2). The morphological differentiation during growth on a solid substrate is suggested to be of functional relevance for protein secretion (Chapter 1). Moreover, the aerial hyphea are mainly responsible for oxygen uptake that becomes limited in the penetrative hyphae (Rahardjo et al. 2001, Oostra et al. 2001). Therefore, the focus in our research was on the relation between cellular morphogenesis and protein secretion, and, on the improvement of oxygen supply of *A. oryzae* during growth on a solid substrate.

Protein secretion

Growth of *A. oryzae* on solid substrates involves a substrate-specific polarised hyphal growth phenotype and secretion of carbohydrate-converting enzymes. Research on fungal protein secretion during growth on a solid substrate has mainly been focussed on the hydrolytic activities of proteins without determining the identity of the corresponding enzymes. In our research different secretory enzymes were identified as differentially expressed during growth on WK and in wheat-based liquid medium (chapter 3). This analysis showed that besides α-amylase also a neutral protease, a chitinase and an unidentified 22 kDa protein (Fig. 1) were secreted by *A. oryzae* during growth on wheat kernels (WK). The solid state fermentation was further characterised by the abundant secretion of two unique fungal proteins of 16 and 27 kDa (Fig. 1) with a presently unknown functionality.

Control mechanisms that regulate gene expression

Aspergillus species produce several secretory proteins more efficiently during growth in solid state fermentations compared to growth in submerged fermentation (Chapter 1). Transcription studies comparing gene expression under either of the cultivation methods however have been limited to the *glaB* gene encoding glucoamylase B, an enzyme that is mostly expressed during growth of *A. oryzae* on a solid substrate. It was suggested that the promoter region of *glaB* gene mediates the induction of transcription by starch, low-Aw, high temperature and physical barriers for hyphal extension (Ishida et al. 2000). Our preliminary northern analysis with probes for the *A. oryzae alpA* and *nptB* genes encoding alkaline and neutral protease, suggested also higher transcription of these protease genes during growth on a solid substrate than during submerged growth. Therefore, we analysed the gene regulatory

mechanisms of *glaB*, *alpA* and *nptB* of *A. oryzae* during growth in a liquid medium and on a solid substrate. In support of previous studies (Solis-Pereyra et al. 1993 and 1996, Ashokkumar and Gunasekaran 2002, Bakri et al. 2003, Kamra and Satyanarayana 2004, Holker et al. 2004), our results (Chapter 4) suggest that during growth on solid substrates catabolite repression is less pronounced compared to submerged fermentation due to direct consumption of glucose after liberation from the substrate. The transcription induction of the *alpA* and *nptB* genes appeared to be regulated by pH while maltose induced the transcription of the *glaB* gene (Chapter 4).

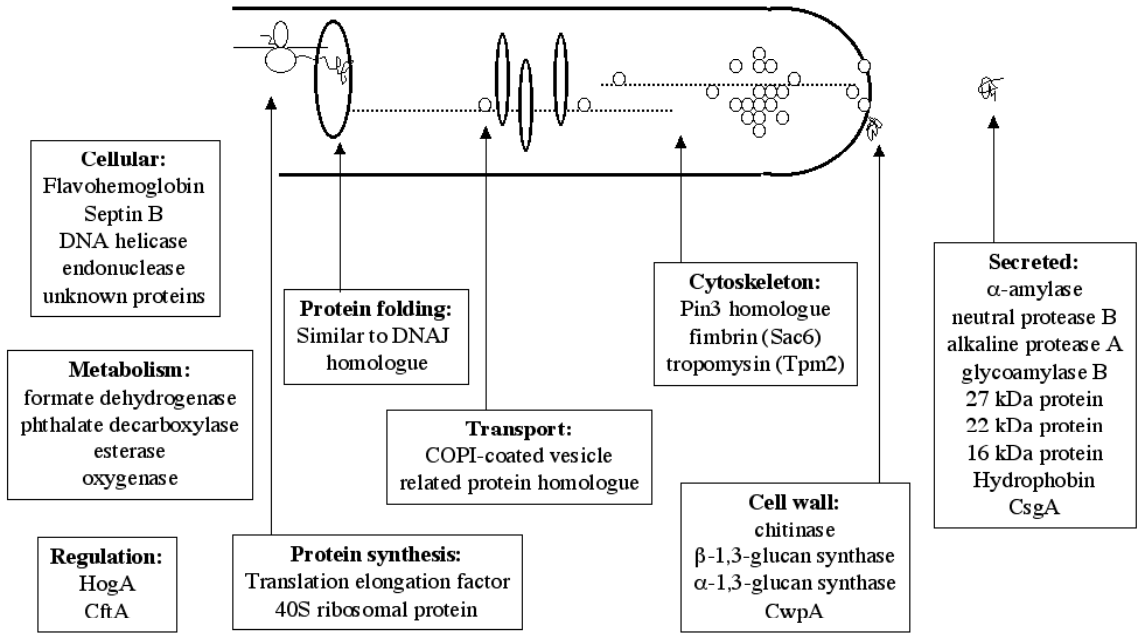

Fig. 1. *The various proteins and/or genes related to several aspects of growth on a solid substrate, which were identified in the research described in this thesis. The proteins and/or genes are indicated in relation to the hyphal growth phenotype.*

Polarised hyphal growth

Until now, genome wide expression analysis is rare for filamentous fungi in spite of the completion of various genomes (Archer and Dyer 2004, Maeda et al.

2004). In our research a heterologous macroarray analysis and a suppression subtractive hybridisation method were developed (Chapter 5) to identify genes that are differentially expressed during growth on a solid substrate and in a liquid medium. Akoa et al. (2002) identified with a subtraction library some genes encoding proteins involved in substrate degradation, metabolism, transport and cell wall synthesis that were associated to surface growth. We have extended this analysis and identified genes encoding proteins involved in cellular morphogenesis and polarised growth (Fig. 1) and associated them to the filamentous fungal growth phenotype on a solid substrate.

In solid state fermentation, contact of the penetrative hyphae with the solid substrate is elementary for substrate utilization. To study the relation between the number of hyphal tips and protein secretion during growth on a solid substrate we have constructed two mutant strains with increased hyphal branching (Chapter 6). Both mutant strains were analysed during growth on a solid substrate and it was shown that the α-amylase, glucoamylase and protease activities were at least 50% higher compared to the wild-type strains. These results show that hyperbranching strains can improve the solid state fermentation process.

Flavohemoglobin, and, hemoglobin overproduction

The heterologous macro-array identified one differentially expressed gene that was of particular interest, since it encoded a putative oxygen-binding hemoglobin-domain. The encoded protein, a putative flavohemoglobin protein is only present, as far as currently known, in a selection of lower eukaryotic organisms, including several, but not all fungal species, and some bacterial species (Chapter 7). Some fungal species contain even up to 3 gene copies in their genomes. The

flavohemoglobin is characterised by a N-terminal hemoglobin domain and a C-terminal redox-active domain (Fig. 2) and its function has been associated with protection against nitrosative/oxidative stress or oxygen storage. Transcription regulation of the *A. oryzae* flavohemoglobin gene was growth-phase dependent, suggesting a possible requirement for flavohemoglobin during hyphal growth.

Fig. 2. *Schematic drawing of the three domains of flavohemoglobin (FHb) (Ermler et al. 1995, Ilari et al. 2002). The N-terminal hemoglobin domain has a high affinity for oxygen, the middle domain for FAD, and the C-terminal domain for NAD(P)H. The dotted line illustrates the variable length at the N-terminus of some species.*

Oxygen is mainly (for 70%) taken up by the aerial hyphae of *A. oryzae* (Rahardjo 2001). Moreover, there is a lack of oxygen supply to the penetrative hyphae during growth on a solid substrate. Hemoglobin binds oxygen reversible and plays a major role in oxygen transport in a wide variation of organisms (Weber and Vinogradov 2001, Frey and Kallio 2003). With the aim to improve the oxygen supply to the penetrative hyphae, *Aspergillus* hemoglobin domain (HBD) proteins were produced in *A. oryzae*. The HBD-overproducing strains showed faster oxygen uptake compared to the wild-type. Moreover, biomass yield, growth rate and enzyme production were higher in the HBD-overproducing strains compared to the wild-type. A likely explanation would be that the HBD-producing strains facilitate improved oxygen supply to the electron transport chain of the cell.

Conclusion

Production of proteins by filamentous fungi on solid substrates offers advantages over growth in submerged fermentation (Chapters 1 and 5). Our results suggest that the protein producing capacity during growth on a solid-substrate is different compared to submerged fermentation due to the difference in hyphal growth phenotype (Chapter 5). The importance of hyphal growth for the production of proteins during growth on a solid substrate, was corroborated by the results obtained with the hyperbranching strains (Chapter 6). In solid state and submerged fermentation, different control mechanisms regulate the glucoamylase and protease gene transcription (Chapter 4). However, the difference in spectra of secreted proteins (Chapter 3) in submerged and solid state fermentation suggests that other, not-yet identified, gene regulatory mechanisms that activate gene transcription in solid state fermentation await elucidation (Chapter 7). Based on the results obtained with the research described in this thesis, several approaches to improve the solid state fermentation process or to increase protein production by filamentous fungi have been realised by constructing hyperbranching mutants (Chapter 6) and *Aspergillus* hemoglobin domain-producing strains (Chapter 8).

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Samenvatting

Filamenteuze schimmels worden al eeuwen gebruikt voor het maken van gefermenteerde voedingsproducten zoals kaas (zoals bijv. Roquefort, camembert), worst (salami), soja saus en alcoholische drank (saké, wijn). In de afgelopen decennia werden de filamenteuze schimmels tevens gebruikt als miniatuurfabrieken voor de productie van eiwitten. Deze eiwitten hebben, na opzuivering, een industriële toepassing bij de productie van vruchtensappen, beer, wijn, brood, yoghurt, diervoeding maar ook in de papier, textiel, wasmiddelen en farmaceutische industrie (Bennet 1998, Pandey et al. 1999). De eiwitten worden geproduceerd door schimmels te laten groeien in grote fermentoren, gevuld met vloeibaar groeimedium. Het begrip over het groei- en eiwitproductie proces van en door schimmels in vloeibaar medium, heeft de afgelopen decennia een enorme ontwikkeling doorgemaakt.

Aspergillus oryzae is de eerste filamenteuze schimmel die gebruikt is voor de toepassing van (gedeeltelijk gezuiverde) enzym preparaten (Takamine 1894). Voor deze toepassing werd *Aspergillus oryzae* gegroeid op een vast substraat, zoals rijst- of graankorrels, in een proces dat vaste-stof fermentatie wordt genoemd. In de jaren 40-50 van de vorige eeuw bleek echter dat de industriële productie van penicilline en eiwitten veel gemakkelijker is wanneer de filamenteuze schimmel groeit in een vloeibaar medium. Hierdoor werd de aandacht voor vaste-stof fermentatie in de Westerse wereld minder. Vaste-stof fermentatie heeft recent echter weer hernieuwde aandacht gekregen omdat blijkt dat filamenteuze schimmels onder deze groeiconditie relatief meer eiwitten maken dan gedurende groei in een vloeibaar medium (Pandey et al. 1999, te Biesebeke et al. 2002, Holker et al. 2004). De doelstelling van de studie die in dit proefschrift beschreven staat, was te begrijpen wat er met *A. oryzae* gebeurt

wanneer deze op een vast substraat groeit. Omdat de morfologie van de schimmel bijzonder is tijdens vaste-stof fermentatie en groei beperkt wordt door zuurstofgebrek in het deel van de schimmel dat zich in het vaste substraat bevindt (Hoofdstuk 1 en 2), wordt in dit proefschrift onderzoek beschreven dat gericht is op het verkrijgen van meer inzicht in deze aspecten van het vaste-stof fermentatie proces. De verkregen kennis is gebruikt om het vaste-stof fermentatie proces te verbeteren en om de eiwitproductie van filamenteuze schimmels te optimaliseren.

Tijdens groei van *A. oryzae* op rijst- en graankorrels laten de hyphen van de schimmel een duidelijk gepolariseerde groei zien en worden enzymen uitgescheiden die de koolhydratenpolymeren in het substraat kunnen omzetten. In Hoofdstuk 3 staat de identificatie beschreven van α-amylase en een neutraal protease die hoofdzakelijk verantwoordelijk zijn voor de amylase- en protease-activiteiten tijdens groei op graankorrels. Daarnaast werden andere schimmelspecifieke eiwitten geïdentificeerd waarvan de functie tot op heden onbekend is. Om te begrijpen waarom eiwitten in hogere mate worden geproduceerd door schimmels tijdens groei op een vast substraat in vergelijking tot groei in een vloeibaar substraat, is de transcriptie bestudeerd van een 3-tal genen die coderen voor hydrolytische enzymen (Hoofdstuk 4). Het *glaB* transcript dat de informatie bevat voor de biosynthese van glucoamylase werd gesynthetiseerd in de aanwezigheid van hoge concentraties maltose. De synthese van de *alpA* en *nptB* transcripten die de informatie bevatten voor de biosynthese van, respectievelijk, een alkalisch en neutraal protease, werden beïnvloed door de zuurgraad. Het verschil in transcriptie van deze genen tijdens groei van *A. oryzae* op een vast en vloeibaar substraat kon worden verklaard door een verschil in aanwezigheid van controle mechanismen voor genexpressie.

De productie van eiwitten in filamenteuze schimmels verloopt via cellulaire processen die ook een invloed hebben op de morfologie van filamenteuze schimmels. Om andere genen te identificeren die differentieel tot expressie komen tijdens groei van *A. oryzae* op een vast en in een vloeibaar substraat is o.a een heterologe macroarray-analyse methode ontwikkeld (Hoofdstuk 5). De meeste genen die hoger tot expressie komen tijdens groei op een vast substraat coderen voor eiwitten die een rol spelen bij de morfologische ontwikkeling van *A. oryzae*. Voornamelijk eiwitten die betrokken zijn bij het aanmaken van de schimmel celwand en eiwitten die de richting (polarisatie) van groei bepalen werden geïdentificeerd. Hoofdstuk 6 laat zien dat morfologische mutanten die gemaakt zijn door genen uit te schakelen met behulp van moleculair genetische technieken en die meer gepolariseerde groei hebben, ook meer eiwitten produceren. De resultaten in Hoofdstuk 5 en 6 onderbouwen de hypothese dat de productie van eiwitten tijdens vaste-stof fermentatie hoger is in vergelijking met productie in vloeistof fermentatie doordat er meer gepolariseerde groei is tijdens vaste-stof fermentatie.

Met de heterologe macroarray is ook een gen geïdentificeerd dat codeert voor een flavohemoglobine, een eiwit dat een hemoglobine en een reductase domein bezit. Hemoglobine bindt zuurstof reversibel en heeft in zoogdieren een transport functie, terwijl reductases deel uit maken van de electronen transport keten die betrokken is bij het genereren van energie in de cel. Het flavohemoglobine eiwit blijkt deel uit te maken van een familie van eiwitten die alleen in sommige bacteriën, gisten, schimmels en enkele lagere eukaryote organismen voorkomen (Hoofdstuk 7). Flavohemoglobin beschermt bacteriën, gisten en schimmels tegen stikstof en oxidatieve stress en is mogelijk betrokken bij zuurstof transport/opslag. In Hoofdstuk 7 staan transcriptie studies beschreven die laten zien dat het flavohemoglobine gen

van *A. oryzae* tot expressie komt tijdens gepolariseerde groei. Deze resultaten suggereren dat, in tegenstelling tot wat we van bacteriën weten, tijdens groei van schimmels behoefte is aan functioneel flavohemoglobin.

Het proces van groei van *A. oryzae* op een vast substraat en het verbeteren van de eiwitproductie capaciteit van *A. oryzae* is bewerkstelligd door de constructie van mutanten m.b.v. de moleculaire genetica. Naast stammen die meer gepolariseerde groei vertonen en meer eiwit produceren (Hoofdstuk 6) zijn ook *A. oryzae* stammen gemaakt waarin het *Aspergillus* hemoglobine domein tot overexpressie gebracht is. In vergelijking met het wild-type, blijken deze over-producenten meer zuurstof op te nemen, sneller te groeien, meer biomassa per tijdseenheid te produceren en meer amylase, protease en glucoamylase te maken (Hoofdstuk 8). Deze resultaten suggereren dat overproductie van het *Aspergillus* hemoglobine domein in *A. oryzae* niet alleen een beter zuurstoftransport, maar ook een snellere ademhaling en daardoor een snellere energie vorming tot gevolg heeft.

In algemene termen draagt dit proefschrift bij aan de fundamentele kennis over groei en eiwitproductie van filamenteuze schimmels die industrieel gebruikt worden. Met behulp van genetische modificatie is bovendien de groei en eiwit productie verbeterd door de morfologie van de filamenteuze schimmels te veranderen en door hemoglobine intracellulair tot overproductie te brengen.

Curriculum vitae

Rob te Biesebeke was born 4 December 1968 in Berkeland (Eibergen, Gelderland, NL). In 1986, he graduated successfully from High school (HAVO, Haaksbergen, Overijssel, NL). After 2 years of additional education he obtained an undergraduate certificate from the technical school (1988, Hofstede MTS, Hengelo, NL). After 5 years of study, he was graduated from Saxion polytechnics (Saxion Hogeschool, Enschede, NL) with specializations in Biochemistry/Biotechnology (1992) and Microbial Environmental Technology (1993). He fulfilled internships with Prof. Dr. Andrea Parmeggiani and Dr. Piet Anborgh in the Biochemical laboratory of the Ecole Polytechnique (1991, Paris, France), Dr. Gerard Rouwendaal and Ing. Jan Springer at Wageningen University and Research Centre (1992, ATO-DLO Wageningen, NL) and, Dr. Sietze Keuning and Drs. Jaap van der Waarde at Bioclear (1993, Groningen, NL). After Saxion polytechnics, he subscribed to study Biology at Utrecht University (NL). During this study, he volunteered for a project at the Dutch Cancer Institute (1994, N.K.I., Amsterdam, NL). In 1996, he obtained his Master in Science (MSc.) in Applied Molecular Biology from Utrecht University after a stage with Dr. Marcel van der Vaart, Dr. John Chapman and Prof. Dr. Theo Verrips at the Unilever Research Laboratory (1996, Vlaardingen, NL). After his graduation, he worked on an Applied Molecular Biological project in collaboration with Prof. Dr. Theo Verrips. This work was performed at the University of Utrecht within the group of Prof. Dr. Arie Verkleij and Prof. Dr. Johannes Boonstra (1996-1997, Utrecht, NL). Subsequently, he joined the Biochemical laboratory of Prof. Dr. Andrea Parmeggiani at the Ecole Polytechnique (1997-2000, Paris, France). In 2000, he started a PhD fellowship with Prof. Dr. Willem de Vos, Prof. Dr. Cees van den Hondel, and Dr. Peter Punt. This thesis describes the results obtained during this fellowship. This work was performed within the framework of Wageningen Centre for Food Sciences (WCFS) at the TNO Life Sciences Research Institute (2000-2005, Zeist, Utrecht, NL).

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