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A new method for screening and isolation of hypersecretion mutants in *Aspergillus niger*

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Abstract Although filamentous fungi have a unique property of secreting a large amount of homologous extracellular proteins, the use of filamentous fungi as hosts for the production of heterologous proteins is limited because of the low production levels that are generally reached. Here, we report a general screening method for the isolation of mutants with increased protein production levels. The screening method makes use of an *Aspergillus niger* strain that lacks the two major amylolytic enzymes, glucoamylase (GlaA) and acid amylase (AamA). The double-mutant strain grows poorly on starch and its growth is restored after reintroducing the catalytic part of the glucoamylase gene ($GlaA_{512}$). We show that the fusion of a heterologous protein, a laccase from *Pleurotus ostreatus* (Pox2), to the catalytic part of glucoamylase ($GlaA_{512}$–Pox2) severely hampers efficient production of the glucoamylase protein, resulting in a slow-growth phenotype on starch. Laccase-hypersecreting mutants were obtained by isolating mutants that displayed improved growth on starch plates. The mutant with the highest growth rate on starch displayed the highest laccase activity, indicating that increased glucoamylase protein levels are correlated with higher laccase production levels. In principle, our method can be applied to any low-produced heterologous protein that is secreted as a fusion with the glucoamylase protein.

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Introduction

Filamentous fungi, such as *Aspergillus niger*, have the capability to secrete large amounts of proteins into their growth medium. Because several products produced with *A. niger* have the GRAS (Generally Recognized As Safe) status, this filamentous fungus is an attractive host for production of homologous and heterologous proteins (Gouka et al. 1997a; Radzio and Kück 1997; Archer 2000; Conesa et al. 2000; Punt et al. 2002). In general, the extracellular production of heterologous proteins is at least one order of magnitude lower than production of homologous proteins, thereby limiting the commercial potential of fungi as a production organism (Archer et al. 1994; Gouka et al. 1997a; Radzio and Kück 1997). The aim of this study was to improve *A. niger* as a host for protein production and, as an example, we have chosen to improve laccase production in *A. niger*. Laccases are interesting proteins for industrial applications because extensive studies have shown the potential of fungal phenol oxidases as a biological alternative for chemical oxidative processes, e.g. phenol removal from waste water, pulp bleaching (Karam and Nicell 1997; Breen and Singleton 1999) and ethanol production (reviewed by Mayer and Staples 2002). However, the application of laccases in industrial processes is limited due to the inability to efficiently produce large amounts of this class of enzymes in the original host fungus or by heterologous production in *Aspergillus* or in other filamentous fungi (Conesa et al. 2000). *pox2* encodes an extracellular laccase from the basidiomycete *Pleurotus ostreatus* (Giardina et al. 1996) and is used in this study as a model laccase to optimize production.

Over the last 10 years, several different approaches have been used to optimize production of extracellular heterologous proteins. These approaches include (1) the introduction of the heterologous gene under control of an efficient fungal promoter, preferably in high copy number to maximize transcription of the gene of interest (Verdoes et al. 1994), (2) the use of extracellular (Mattern et al. 1992) and intracellular (van den Hombergh et al. 1997) protease-deficient host stains and (3) the optimization of cultivation

conditions (Schrickx et al. 1993; MacKenzie et al. 1994; Xu et al. 2000; Conesa et al. 2001). In most cases, the most successful improvement has been the so-called gene fusion approach in which the heterologous gene of interest is fused to a naturally well secreted protein, a so-called carrier protein (reviewed by Gouka et al. 1997a). In *A. niger*, glucoamylase is mostly used as a carrier protein. Glucoamylase (GAM, EC 3.2.1.3) is a multiple-domain glycoprotein consisting of three domains. The first 470 amino acids (aa) encode a large catalytic domain that catalyze the hydrolysis of alpha-1,4- and 1,6-glycosidic linkages of polysaccharides, normally starch, into glucose residues. The C-terminal part of the protein (aa509–aa616) has been shown to contain a starch-binding domain (SBD). The catalytic domain and the SBD are connected by a linker domain (aa471–aa508) that contains many serine and threonine residues. The SBD is not essential for the activity of the catalytic domain (Sauer et al. 2000; Cornett et al. 2003). Fusion of the catalytic domain to other proteins has often been used in combination with the other approaches to improve production of heterologous proteins (Punt et al. 1991; Verdoes et al. 1994; Gouka et al. 1997b). However, even with the use of the gene fusion approach, the levels of production of heterologous proteins are usually lower than that obtained for homologous proteins (Gouka et al. 1997b).

In this study, we describe a new and generally applicable genetic selection system to select for strains with higher production levels of a heterologous protein that is fused to the catalytic domain of glucoamylase. To verify our new screening method, we have fused the catalytic domain of glucoamylase to a laccase from the basidiomycete *P. ostreatus* encoded by the *pox2* gene. Selection of mutant strains with improved glucoamylase production after UV mutagenesis resulted in the isolation of several laccase hypersecretion mutants.

Materials and methods

Strains, transformation procedures, media and growth conditions

A. niger strains N402 (a *cspA1* derivative of ATCC9029; Bos et al. 1988) and MGG029 (*prtT*, *glaA::fleo*^{r}*pyrG*) (Conesa et al. 2000) were used. *Escherichia coli* XL1-Blue was used for construction and proliferation of vector DNA. Fungal strains were transformed following the method described by Punt and van den Hondel (1992). Transformants were selected for on acetamide plates (Kelly and Hynes 1985). *Aspergillus* strains were cultivated at 30°C on minimal medium (MM) (Bennett and Lasure 1991) with 1% of glucose, 1% of soluble maize starch (BDH 30261 CM), or 2% of xylose as a carbon source.

Liquid cultivations were performed in 300-ml shake flasks containing 100 ml *Aspergillus* complete medium, which consists of minimal medium supplemented with 0.5% casamino acids and 1% (w/v) yeast extract. Cultures were inoculated with 1×10^{7} spores/ml and incubated for 17 h at 30°C at 250 rpm. Mycelium was separated from the medium by filtration through Myracloth and transferred to fresh medium.

The growth curves of the different transformants were determined by dry weight measurements. One hundred milliliters of 1% starch medium was inoculated with 1×10^{7} spores/ml of the different transformants and grown at 30°C at 250 rpm. Mycelium was harvested at different time points (t=52, 76, 120 and 132 h), filtered, washed with distilled water and dried.

Construction of *aamA* disruption vector

A 1.5-kb fragment of the *A. niger* acid amylase gene (*aamA*) was amplified from chromosomal DNA from N402 using primer AMY1 (5′-GGAATTCGGAGTTTCGGCCAATG AGAG-3′) and AMY5 (5′-GGAATTCGGRTTRTCRTG RTTYTC-3′) and cloned in pGEM-T resulting in vector pAAMA#10. Primer AMY1 was designed based on a partial cDNA encoding the acid amylase (D. Archer, unpublished data). Primer AMY5 was designed based on a conserved amino acid region around amino acid 315 present in various *A. niger* acid amylase sequences present in the database (database entries A35282, BAA22993, P56271). Acid amylase disruption vector pAO4.2*ΔaamA* was constructed by inserting an internal 0.9-kb BglII fragment from pAAMA#10 into the BglII site of pAO4.2 (de Ruiter-Jacobs et al. 1989).

Laccase expression cassettes and plasmids

Expression vectors were constructed using standard cloning procedures (Sambrook et al. 1989). pUR7894 (obtained from Dr. R. Gouka, Unilever, the Netherlands) contains the glaA2 coding sequence of *A. niger* fused to the *pox2* cDNA gene of *P. ostreatus* between *A. niger PexlA* promoter and *TexlA* terminator sequences. The fusion gene consists of the glucoamylase catalytic domain (512 aa) at the 5′ site and the laccase coding sequence (*pox2*) at the 3′ end (GlaA2::POX2). pXW4 was constructed to place the glucoamylase–laccase fusion gene under control of the inducible *A. niger* glucoamylase promoter and *A. nidulans trpC* terminator sequences. Plasmid pXW4 was constructed by ligating the 1.5-kb NotI/BglII fragment of expression vector pAN56-2 (van den Hondel et al. 1991) (Z32690), containing the *Pgla* promoter region and 5′ end of *glaA* coding region, the 2.6-kb BglII/AflII fragment of pUR7894 liberated by partial digestion, containing the 3′ end of glucoamylase gene fused to the *pox2* coding sequence and the 3.5-kb NotI/AflII fragment of pAN52-12 Aspergillus expression vector (P.J. Punt, unpublished data) together. Finally, the *A. nidulans amdS* selection marker was isolated as a 5.1-kb NotI fragment from pAmdS-Not (Kelly and Hynes 1985; P.J. Punt, unpublished data) and inserted in pXW4 at the unique NotI site to obtain the glucoamylase–laccase expression vector pXW5 (Fig. 1). Vector pXW6 was derived from pAN56-2, by inserting the 5.1-kb NotI

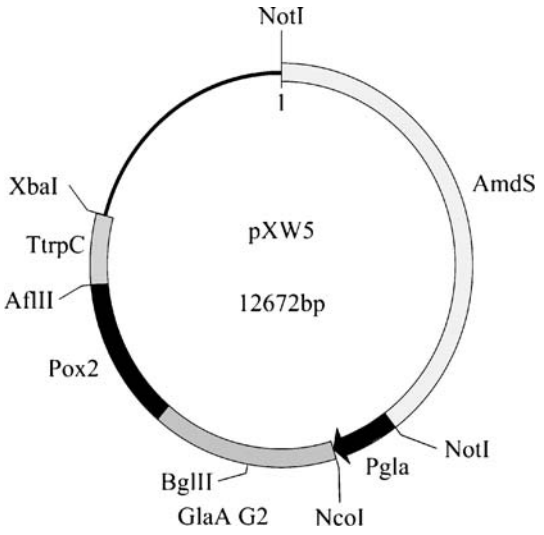

Fig. 1 Schematic representation of the pXW5 expression vector containing the fusion gene that consists of the sequence coding for the *A. niger* glucoamylase catalytic domain (GlaA G2, aa 1–512) and the *P. ostreatus* laccase gene (*pox2*), regulated by the *A. niger* glucoamylase promoter (Pgla). *AmdS A. nidulans* acetamidase, *TtrpC A. nidulans trpC* terminator sequence

fragment containing the *amdS* selection marker in the unique NotI restriction site of pAN56-2. The *pox2* gene in XW5.2 and XW5.2#64 was amplified from genomic DNA using MBL644 (5′-CACCACTACGACGGCTAC-3′) and MBL858 (5′-ATATCCAGATTCGTCAAGCTG-3′), which correspond to sequences flanking the *pox2* fusion in pXW5 using SuperTaq-Plus.

Mutagenesis and enrichment

UV mutagenesis was performed on freshly isolated spores diluted in 0.9% (w/v) NaCl to a final concentration of 1×10^7 spores/ml. Spores were irradiated with 250 mJ in 5-s time intervals up to 1 min (Bio-Rad GS gene linker). After determining the survival rate of the spores at the different time points, spores with a survival rate between 40 and 70% were plated on 1% starch medium and incubated at 30°C. Each Petri dish (∅ 9 cm) was inoculated with a concentration of approximately 10^4 viable spores, and mutant strains conidiating within 5 and 7 days were assigned as potential hypersecretion mutants since the non-mutagenized strain conidiates only after 7 days of incubation. Potential hypersecretion mutants were purified and re-screened for both increased laccase activity and improved growth on starch.

Laccase activity assays

Transformants and potential hypersecretion mutants were analyzed for laccase activity according to Palmieri et al. (1997), with some minor adjustments. Laccase plate assays were performed by spotting 5×10^5 spores on agar plates containing minimal glucose medium and incubated for 2 days at 30°C. Phenol oxidase activity was assayed using a top agarose assay. The ‘top agarose’ contained 0.5% 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and 1% p-hydroxybenzoic acid mixed with 1% agarose dissolved in 40°C McIIIvaine’s citrate–phosphate buffer (pH 4.5). Top agarose (12 ml) was pipetted onto the fungal colonies resulting in a covering layer in a Petri dish (∅ 9 cm). The plates were incubated in the dark for 17 h at room temperature and checked for development of green color.

Laccase stability in *A. niger* growth medium was measured using purified laccase from *P. ostreatus* (gift from R. Gouka, Unilever, the Netherlands). Growth media were taken from cultures of 0, 8, 11, 22 and 26 h after mycelium transfer and to 1 ml of the growth medium 100 ng of purified laccase was added. Laccase activity was determined using the spectrophotometric measurement at 420 nm as described by Palmieri et al. (1997) after incubation for 1.5 h at 37°C. Laccase activity in cell pellets was also measured by the spectrophotometric measurement. Pellets from 22-h shake-flask cultivations were isolated by filtration through a Myracloth filter and squeezed in a sandwich of three paper towels for 1 min with a weight of 1 kg on top. Mycelium with a wet weight of 2.5 g was resuspended in 15 ml McIIIvaine buffer and incubated in a shaker with an agitation of 250 rpm at 37°C. After 2 h of incubation samples were centrifuged for 10 min at 2,000 rpm. A volume of 990 µl from the upper face was mixed with 10 µl of 10 mM ABTS and incubated for 20 h at 30°C. The amount of laccase was determined spectophotometrically using purified laccase from *P. ostreatus* as a standard.

Results and discussion

Construction of starch non-utilizing *A. niger* strain

As a starting strain for the mutant screen, an *A. niger* mutant was constructed that lacks the two major starch-hydrolyzing enzymes, glucoamylase (GlaA) and acid amylase (AamA). The acid amylase gene was amplified from chromosomal DNA using the specific primers AMY1 and AMY5. The PCR product was cloned into vector pAO4.2 resulting in vector pAO4.2*ΔaamA*. This vector contains a 1.5-kb amylase coding sequence of *A. niger* flanked by the *A. oryzae pyrG* upstream and downstream sequences.

To obtain the *A. niger* mutant strain that lacks both the glucoamylase and the acid amylase encoding genes, strain MGG029 (*prtT glaA::fleo*^{r}*pyrG*, Conesa et al. 2000) was used. *A. niger* MGG029 is deficient in the expression of several protease genes due to a regulatory mutation (Mattern et al. 1992) and contains a deleted glucoamylase gene. Strain MGG029 was transformed with vector pAO4.2*ΔaamA* to disrupt the acid amylase gene. Integration of the disruption cassette at the amylase gene locus by a single crossover will result in the disruption of the amylase gene. *A. niger* MGG029-*ΔaamA* transformants were obtained after selection for uridine prototrophy and

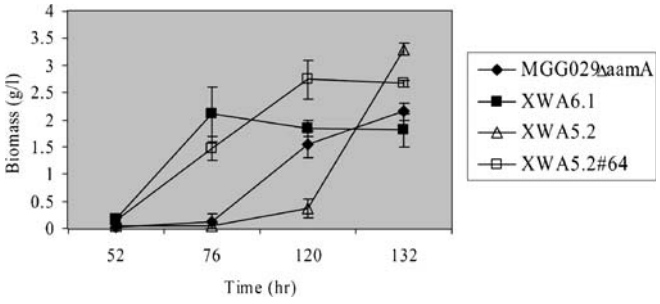

Fig. 2 Growth curve of various *A. niger* strains in batch culture using 1% (w/v) starch as sole carbon source. Biomass production of MGG029-Δ*aamA*, XWA6.1, XWA5.2, and XWA5.2#64 was determined at several time points during growth (52, 76, 120 and 132 h after inoculation of equal amounts of spores). Dry cell weight measurements were performed in duplicate from two independent cultivations. The *error bars* indicate the standard deviation of the mean dry weights

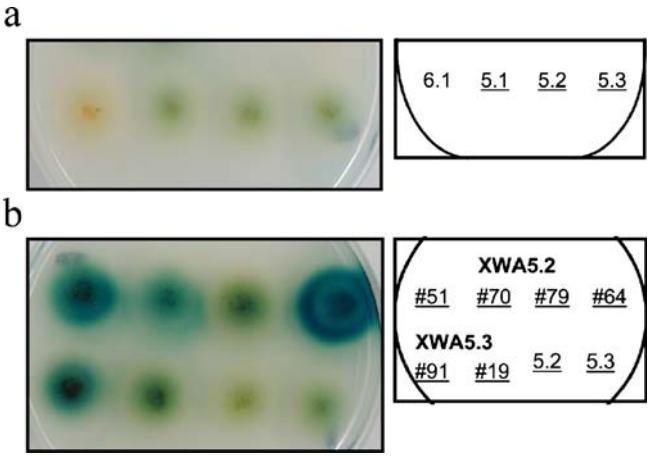

Fig. 3 Plate assay for analysis of laccase (Pox2) production. **a** ABTS overlay assay on MGG029-Δ*aamA* transformants, containing the GlaA2::Pox2 expression cassette (XWA5.1, 5.2, and 5.3) and the expression cassette with the catalytic part of glucoamylase only (XWA6.1). **b** Detection of laccase production using ABTS overlay assay for a selection of mutants that displayed a fast-growing phenotype on starch medium after UV mutagenesis. All colonies displayed in **a** and **b** are covered with ABTS overlay agarose and incubated at room temperature for 17 h. Strains showing the *green color* are positive for laccase activity. Strain positions are indicated in the drawn template and laccase-producing strains are *underlined*

reduced halo formation on starch medium. Strains with a reduced halo were purified and Southern blot analysis confirmed the disruption of the acid amylase gene (data not shown). As expected, strain MGG029-*ΔaamA* has severely reduced growth rate on starch compared to the strain (MGG029) in which only the glucoamylase is deleted. Residual growth of the MGG029-*ΔaamA* strain is probably sustained by the expression of enzymes that are capable of degrading starch, e.g. alpha glucosidase or additional amylases. The slow-growth phenotype of the MGG029-*ΔaamA* strain was reversed by transforming the strain with the catalytic part of the glucoamylase ($GlaA_{512}$) under the control of the endogenous glucoamylase promoter. This transformant (XWA6.1), carrying vector pXW6 (Pgla-GlaA2-TtrpC), showed faster growth on starch medium, indicating that growth on starch of the MGG029-*ΔaamA* strain could be restored by the expression of the catalytic domain of the glucoamylase gene (Fig. 2).

Isolation of *A. niger* transformants expressing *pox2*

To produce the *P. ostreatus* laccase protein in *A. niger*, the laccase-coding gene (*pox2*) was fused with the catalytic domain of glucoamylase. The fusion gene (*glaA2::pox2*) was placed under the control of the inducible glucoamylase promoter (*Pgla*) from *A. niger* resulting in the expression vector pXW5 (*Pgla-GlaA2::POX2-TtrpC*) (Fig. 1). pXW5 was transformed to the *A. niger* MGG029-*ΔaamA* strain using AmdS as a selection marker. Transformants that were able to grow on acetamide were purified and analyzed for laccase production. Southern blot analysis of pXW5 transformants confirmed the presence of the GlaA2::POX2 expression cassette (data not shown). Transformants carrying pXW5 were analyzed for their laccase production using a laccase activity plate assay. Three transformants (XWA5.1, XWA5.2 and XWA5.3) were cultivated on inducing (glucose) medium for 48 h followed by an ABTS-agarose overlay assay. These transformants showed a development of a weak green color after 17–20 h at room temperature (Fig. 3a), indicating the production of very low amounts of laccase activity. No laccase activity was observed when the strains were grown on non-inducing xylose medium (data not shown), nor in the strain containing only the glucoamylase part (XW6.1) (Fig. 3a).

Production of *P. ostreatus* Pox2 laccase in *A. niger*

Laccase producing transformant XWA5.2 and the control strain XWA6.1 were used to study laccase protein production levels in shake-flask cultures. Both strain XWA5.2 and XWA6.1 were cultured for 17 h in shake-flasks under inducing conditions using glucose as a carbon source. Mycelium was harvested and transferred in fresh growth medium and cultivation was continued. Medium samples were taken 8, 11, 22 and 26 h after transfer. These medium samples were assayed for laccase activity as described in Materials and methods. In contrast to the results on agar plates, in none of the medium samples could laccase activity be measured. This suggests that the laccase protein is inactive or rapidly degraded in the liquid cultivation medium or the laccase protein is not secreted in the medium and is retained in the cell wall. To address the first possibility, we determined the stability of purified laccase protein after incubation in growth medium. Purified laccase protein from *P. ostreatus* (100 ng/ml) was incubated in growth medium derived from cultures grown for 4, 8, 11, 22 and 26 h after the mycelium transfer. Incubation of the laccase protein in these growth media showed more than a 50% decrease in activity when incubated for 1.5 h at 37°C (data not shown). This suggests that the laccase protein itself is not very stable in the growth medium. To check whether the laccase fusion protein produced under sub-

merged cultivation conditions retains in the cell wall, fungal cell pellets obtained from shake-flask cultures were analyzed for laccase activity. Pellets from different time points taken from submerged cultivations (4, 8, 11, 22 and 26 h) were put in solid ABTS–top agarose and were incubated at room temperature. A green color was observed around all pellets, indicating that the laccase fusion gene is actually produced by these pellets and retained within the cell wall. A more intensive green color was found for mycelium that was cultivated longer, indicating an increase in laccase protein production with time. Laccase activity in cell pellets was quantified by incubating equal amounts of fresh weight cell pellets in McIIIvaine buffer for 2 h. This incubation resulted in the release of the laccase from the cell wall. The amount of laccase was determined spectophotometrically using *P. ostreatus* Pox2 as a standard. Under inducing conditions, strain XWA5.2 showed a laccase activity of 0.75 ng/g wet weight mycelium, whereas strains that do not contain the glucoamylase–laccase fusion protein do not produce any laccase activity (Fig. 4).

Besides low laccase production levels, the fusion of the laccase to the glucoamylase also affected the efficiency by which the glucoamylase carrier was secreted. Growth assays on starch indicated that the growth of strain XWA5.2 was less compared to the control strain XWA6.1, which contains only the catalytic part of the glucoamylase ($GlaA_{512}$) under the control of the endogenous glucoamylase promoter (Fig. 2). The low production levels and the slow-growth phenotype of the transformant expressing the glucoamylase–laccase fusion protein on starch plates made it possible to set up a general genetic mutant screen to isolate potential hypersecretion mutants.

Isolation of hypersecretion mutants

The screening method aims for the isolation of hypersecretion mutants using growth on starch medium as a positive selection method. The rationale behind the screen is that *A. niger* hypersecretion mutants producing larger amounts of the glucoamylase–laccase fusion protein will grow better on starch medium than the parental strain. Transformants containing the glucoamylase–laccase fusion protein (XWA5.1, XWA5.2 and XWA5.3) were UV-irradiated and mutants were selected that grew better on starch plates. After purification, the mutant strains were rescreened for improved glucoamylase secretion and for laccase production to confirm the overproduction of the heterologous fusion protein.

A total amount of 500,000 viable spores (10^4 viable spores per plate) were plated on solid medium with 1% starch as a carbon source. In total, 252 mutants were identified as strains with an improved growth rate and conidiation on starch. After purification, these strains were screened for increased laccase production and improved growth on starch medium. Six mutants were selected in which the increased laccase production (Fig. 3b) correlated with an increased growth on starch (data not shown). One mutant, XWA5.2#64, showed the highest growth rate, together with the highest laccase activity determined by ABTS-agarose overlay assay, and was studied in further detail.

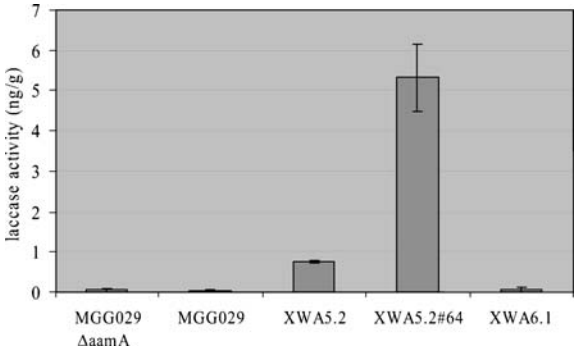

Fig. 4 Cultures were inoculated with equal amounts of spores and grown under induced cultivation conditions for 22 h. A defined wet weight, similar for each strain, was resuspended in McIIIvaine buffer and incubated for 2 h at 37°C at 250 rpm. Laccase activities were determined using a spectrophotometer in duplicate from two independent cultivations. The amount of laccase released from the cell pellet was determined using purified Pox2 from *P. ostreatus* as a standard. The *error bars* indicate the standard deviation of the mean of two independent experiments

Characterization of hypersecretion mutant XWA5.2#64

Analysis of the growth characteristic of strain XWA5.2#64 on starch plates indicated that this mutant showed an improved growth phenotype on starch that was comparable to strain XWA6.1, expressing the catalytic part of glucoamylase (data not shown). This observation was confirmed by comparing growth curves of the various strains (Fig. 2). This finding suggests that the secretion efficiency of the glucoamylase–laccase fusion protein is up-regulated in this mutant strain. To determine whether the improved production of the glucoamylase–laccase fusion protein was caused by an increased mRNA expression level, Northern blot analysis was performed. RNA from mycelium of XWA5.2 and XWA5.2#64 grown under identical submerged cultivation conditions was isolated and the expression of the glucoamylase gene was compared and identical expression levels were observed (data not shown). This suggests that the mutant strain XWA5.2#64 has been altered in a post-transcriptional step that results in a higher yield of the glucoamylase–laccase fusion protein.

Peberdy (1994) proposed that the secretory capacity of filamentous fungi is intimately associated with the process of growth at the hyphal tip and might be enhanced by the increase of the number of hyphal tips. Morphological mutants with increased apical surface, called hyperbranching mutants, display a three- to fivefold increase of extracellular protein (Lee et al. 1998) and highly branched mutants of *Aspergillus oryzae* showed increased glucoamylase production in stirred batch cultures (Bocking et al. 1999). Therefore, we compared the morphology of stain XWA5.2#64 with the parental strain, XWA5.2, under submerged cultivation conditions. The morphology of XWA5.2#64 was

indistinguishable from XWA5.2, indicating that higher production was not caused by a morphological alteration.

Although the laccase activity in strain XWA5.2#64 on plates was higher compared to its parental strain (Fig. 3), still no laccase activity could be measured in the extracellular medium of XWA5.2#64 cultures directly. Therefore, laccase activity was determined on cell pellets as described above. The laccase activity of strain XWA5.2#64 showed a sevenfold increase compared with laccase activity found for the parental strain XWA5.2 (Fig. 4). The stability of purified laccase from *P. ostreatus* in the growth medium of XWA5.2#64 and XWA5.2 was compared and no differences in stability of laccase was found between the medium obtained from XWA5.2#64 and XWA5.2 (data not shown). To exclude the possibility that the improved production is caused by a mutation within the fusion gene, the *pox2* gene was amplified from both the parental strain (XW5.2) and the mutant (XW5.2#64) and sequenced. No differences in the sequences between the strains were found, indicating that the improved laccase production in this mutant is due to a posttranscriptional alteration.

Using this new screening method, we showed that it is possible to isolate potential hypersecretion mutants. The prerequisite for the screening is that the protein of interest is produced as a fusion protein with the glucoamylase gene. The initial screening on starch is simple, robust and fast. In our hands, we found that six of the eight glucoamylase hypersecretion mutants also secreted more of the protein of interest, in our case a laccase. Furthermore, more detailed molecular analysis and controlled batch fermentation of the hypersecretion mutant can contribute to the identification of factors that improve heterologous protein secretion in filamentous fungi.

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References

Archer DB (2000) Filamentous fungi as microbial cell factories for food use. Curr Opin Biotechnol 11:478–483

Archer DB, Jeenes DJ, MacKenzie DA (1994) Strategies for improving heterologous protein production from filamentous fungi. Antonie van Leeuwenhoek 65:245–250

Bennett JW, Lasure LL (1991) Growth media. In: Bennett JW, Lasure LL (eds) More gene manipulation in fungi. Academic, New York, pp 441–457

Bocking SP, Wiebe MG, Robson GD, Hansen K, Christiansen LH, Trinci AP (1999) Effect of branch frequency in *Aspergillus oryzae* on protein secretion and culture viscosity. Biotechnol Bioeng 65:638–648

Bos CJ, Debets AJ, Swart K, Huybers A, Kobus G, Slakhorst SM (1988) Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. Curr Genet 14:437–443

Breen A, Singleton FL (1999) Fungi in lignocellulose breakdown and biopulping. Curr Opin Biotechnol 10:252–258

Conesa A, van den Hondel CAMJJ, Punt PJ (2000) Studies on the production of fungal peroxidases in *Aspergillus niger*. Appl Environ Microbiol 66:3016–3023

Conesa A, van de Velde F, van Rantwijk F, Sheldon RA, van den Hondel CAMJJ, Punt PJ (2001) Expression of the *Caldariomyces fumago* chloroperoxidase in *Aspergillus niger* and characterization of the recombinant enzyme. J Biol Chem 276:17635–17640

Cornett CA, Fang TY, Reilly PJ, Ford C (2003) Starch-binding domain shuffling in *Aspergillus niger* glucoamylase. Protein Eng 16:521–529

de Ruiter-Jacobs YM, Broekhuijsen M, Unkles SE, Campbell EI, Kinghorn JR, Contreras R, Pouwels PH, van den Hondel CAMJJ (1989) A gene transfer system based on the homologous *pyrG* gene and efficient expression of bacterial genes in *Aspergillus oryzae*. Curr Genet 16:159–163

Giardina P, Aurilia V, Cannio R, Marzullo L, Amoresano A, Siciliano R, Pucci P, Sannia G (1996) The gene, protein and glycan structures of laccase from Pleurotus ostreatus. Eur J Biochem 235:508–515

Gouka RJ, Punt PJ, van den Hondel CAMJJ (1997a) Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects. Appl Microbiol Biotechnol 47:1–11

Gouka RJ, Punt PJ, van den Hondel CAMJJ (1997b) Glucoamylase gene fusions alleviate limitations for protein production in *Aspergillus awamori* at the transcriptional and (post) translational levels. Appl Environ Microbiol 63:488–497

Karam J, Nicell JA (1997) Potential applications of enzymes in waste treatment. J Chem Technol Biotechnol 69:141–153

Kelly JM, Hynes MJ (1985) Transformation of *Aspergillus niger* by the amdS gene of *Aspergillus nidulans*. EMBO J 4:475–479

Lee IH, Walline RG, Plamann M (1998) Apolar growth of *Neurospora crassa* leads to increased secretion of extracellular proteins. Mol Microbiol 29:209–218

MacKenzie DA, Gendron LC, Jeenes DJ, Archer DB (1994) Physiological optimization of secreted protein production by *Aspergillus niger*. Enzyme Microb Technol 16:276–280

Mattern IE, van Noort JM, van den Berg P, Archer DB, Roberts IN, van den Hondel CAMJJ (1992) Isolation and characterization of mutants of *Aspergillus niger* deficient in extracellular proteases. Mol Gen Genet 234:332–336

Mayer AM, Staples RC (2002) Laccase: new functions for an old enzyme. Phytochemistry 60:551–565

Palmieri G, Giardina P, Bianco C, Scaloni A, Capasso A, Sannia G (1997) A novel white laccase from Pleurotus ostreatus. J Biol Chem 272:31301–31307

Peberdy JF (1994) Protein secretion in filamentous fungi—trying to understand a highly productive black box. Trends Biotechnol 12:50–57

Punt PJ, van den Hondel CAMJJ (1992) Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. Methods Enzymol 216:447–457

Punt PJ, Zegers ND, Busscher M, Pouwels PH, van den Hondel CAMJJ (1991) Intracellular and extracellular production of proteins in *Aspergillus* under the control of expression signals of the highly expressed *Aspergillus nidulans gpdA* gene. J Biotechnol 17:19–33

Punt PJ, van Biezen N, Conesa A, Albers A, Mangnus J, van den Hondel CAMJJ (2002) Filamentous fungi as cell factories for heterologous protein production. Trends Biotechnol 20:200–206

Radzio R, Kück U (1997) Synthesis of biotechnologically relevant heterologous proteins in filamentous fungi. Process Biochem 32:529–539

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Sauer J, Sigurskjold BW, Christensen U, Frandsen TP, Mirgorodskaya E, Harrison M, Roepstorff P, Svensson B (2000) Glucoamylase: structure/function relationships, and protein engineering. Biochim Biophys Acta 1543:275–293

Schrickx JM, Krave AS, Verdoes JC, van den Hondel CAMJJ, Stouthamer AH, van Verseveld HW (1993) Growth and product formation in chemostat and recycling cultures by *Aspergillus niger* N402 and a glucoamylase overproducing transformant, provided with multiple copies of the glaA gene. J Gen Microbiol 139:2801–2810

van den Hombergh JPTW, van de Vondervoort PJI, Fraissinet-Tachet L, Visser J (1997) *Aspergillus* as a host for heterologous protein production: the problem of proteases. Trends Biotechnol 15:256–263

van den Hondel CAMJJ, Punt PJ, van Gorcom RFM (1991) Heterologous gene expression in filamentous fungi. In: Bennett JW, Lasure L (eds) More gene manipulation in fungi. Academic, New York, pp 396–428

Verdoes JC, Punt PJ, Stouthamer AH, van den Hondel CAMJJ (1994) The effect of multiple copies of the upstream region on expression of the *Aspergillus niger* glucoamylase-encoding gene. Gene 145:179–187

Xu J, Wang L, Ridgway D, Gu T, Young Moo (2000) Increased heterologous protein production in *Aspergillus niger* fermentation through extracellular proteases inhibition by pelleted growth. Biotechnol Prog 16:222–227