VIIp-1

Cloning, expression, purification, and characterization of alpha-glucosidase from *Fusarium venenatum*

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Alpha-glucosidase (EC 3.2.1.20) is an exo-hydrolase which cleaves glucose from non-reducing ends of a glucan chain. Since they can convert starch to glucose, α-glucosidases have potential in fermentation processes in several industrial applications. The *agl1* gene encoding a family GH31 alpha-glucosidase from *Fusarium venenatum* was cloned and successfully expressed in *F. venenatum*. Agl1 expression peaked on day 4 to day 6 when the *F. venenatum* transformants overexpressing *agl1* were grown in shake flasks. Although the predicted molecular weight of Agl1 is 106 kDa, the SDS-PAGE displayed a major band at 65 kDa as well as two lower bands. Further analysis showed that these protein bands were truncated products. The α-glucosidase from *F. venenatum* was purified to one band of 140 kDa on a native gel. This enzyme showed weak dependence of specific activity on pH in the range 4.0-7.7 with maximum around pH 5.0-5.3. For the enzymatic hydrolysis of maltose at pH 5.0 and 37°C, K_m and k_{cat} have been determined to be 0.13 mM and 23 s^{-1}, respectively. This enzyme demonstrates good thermostability as it retains 75% of its specific activity after 5 min at 65°C. Agl1 demonstrates strong "substrate inhibition" that can be attributed to a transglycosylation activity.

A novel ß-glucosidase gene from *Rhizomucor miehei*

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The genus *Rhizomucor* comprises two well-established thermophilic fungal species, *R. pusillus* and *R. miehei*. Both of these fungi are well known from biotechnological applications in consequence of its effective extracellular enzyme production (e.g. proteases, lipases). In the frame of a recent study ß-glucosidase activity of several *R. miehei* isolates were tested by carbon source assimilation methods.

ß-Glucosidases play important roles in biology, including the degradation of cellulose biomass by fungi and bacteria, degradation of glycolipids in mammalian lysosomes, and the cleavage of glycosylated flavonoids in plants. Filamentous fungi are known to be good producers of ß-glucosidases and several fungal glucosidases have been cloned and analyzed. Unfortunatelly, zygomycetes are poorly characterized from this aspect. The aim of our present study is to identify and characterize the *R. miehei* ß-glucosidase gene (*bgl*).

A 493 bps long fragment was amplified from the genomic DNA of the *R. miehei* strain NRRL 5901 using a degenerate primer pair designed on the basis of known fungal ß-glucosidase sequences. The amplicon was isolated, cloned into plasmid and its sequence was determined. The analysed fragment showed high homology with the C-terminal domains of the ß-glucosidases belonging to the fungal family 3 glycoside hydrolases. The whole sequence of the *Rhizomucor bgl* gene and its flanking regions were determined using the inverse PCR technique. Analysis of the cloned sequence revealed the highest homology with the ß-glucosidase of the *Piromyces* sp. strain E2. The tree inferred from the phylogenetic analysis of the alignment of 14 glycoside hydrolase family 3 proteins fits well to the phylogeny described by Harvey et al (1).

Furthermore, two vector systems were constructed for gene expression studies: (a) ß-gucosidase gene was placed between the *Mucor circinelloides gpd1* promoter and terminator regions and (b) the amplified ß-glucosidase promoter were fused with a green fluorescent protein gene.

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VIIp-3

Expression of a heterologous laccase by *Aspergillus niger* cultured by solid-state and submerged fermentations

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Laccase gene IV from *Trametes versicolor* was inserted by REMI in *Aspergillus niger* C28B25. This laccase gene insert from *T. versicolor* was under the control of the α-amylase promoter and terminator of *Aspergillus nidulans*. Additionally this vector pPLF26 carried genes conferring resistance for both ampicillin and phleomycin; the latter was used as a selection marker to isolate stable transformants. One selected transformant (C28eco3) was subjected to a second round of transformation and a new transformant was isolated (C28eco3-13). Laccase production was compared for these strains in submerged fermentation (SmF) and solid-state fermentation (SSF) using glucose or maltodextrin at different concentrations (10, 50 and 100 g/L). For SSF, polyurethane foam (PUF) was imbibed with liquid media, using 20 ml of medium per gram of PUF. Enzymes yields were higher with glucose as a carbon source. Repression by glucose on laccase expression in both transformats was found to a greater extent in SmF, since laccase activity dropped up to 80% when glucose concentration increased from 10 to 50 g/L. In addition, total repression was observed in SmF with 100 g/L as initial glucose concentration. In contrast, production profiles in SSF showed that strain C28eco3 increased laccase levels almost 6 times when glucose was modified from 10 to 50 g/L. In a similar behaviour, a 7-fold increased was observed for transformant C28eco3-13 with glucose at 50 g/L in SSF. Repression was only observed when glucose was increased up to 100 g/L in SSF for both transformants. Moreover, variations in laccase activity could not be explained by differences in biomass among wild type and transformant strains in SSF. It was rather the ability acquired after transformation with the heterologous gene what accounts for such differences as verified by comparing $Y_{E/X}$ for every strain. These results show that REMI as a means to integrate heterologous DNA, in combination with expression on SSF, can result in a convenient approach to increase heterologous expression of proteins by filamentous fungi.

Improved enzymes for production of cellulosic ethanol

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In 2001, Novozymes began work on a DOE-sponsored contract to reduce the cost of cellulases for ethanol production from biomass. Over the past four years we have utilized all the biotechnological tools at our disposal to discover new, more efficient fungal cellulases for the conversion of dilute acid pretreated corn stover to fermentable sugars. During this time a 30-fold cost reduction was obtained by increasing the efficiency of the enzymes and cutting the cost of enzyme production. This presentation will describe some aspects of this work and our role in making cellulosic ethanol a significant fuel in the years to come.

VIIp-5

Microarray and reaction array systems for functional determination of cytochrome P450 molecular species from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*

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As many as 154 cytochrome P450 genes were annotated from the genomic sequence of the lignin-degrading basidiomycete *Phanerochaete chrysosporium.* At least, 144 genes were so far confirmed to be expressed. A phylogenetic analysis revealed that about 120 genes were unique in basidiomycetes. Although a number of genes was less than that found in plant genomes, the P450 gene density of 5.1 genes/Mb genome and the P450 ratio over a total gene number of 1.4% were the highest among organisms whose genomic sequences have been reported. Gene structure of fungal P450s was complicated with 11.4 introns per gene and with relatively high appearance of phase 1 and 2 intron insertions. Most striking characteristics were found in the presence of microexons (coding 3-10 amino acid residues) in 112 Phanerochaete P450 genes. Furthermore, 82% of microexons were located at the substrate recognition site (SRS). Modeling study strongly suggested that the insertion of microexons into SRS region diversified the shape of the substrate binding space. Microexon insertion seems to be an evolutional strategy in lignin-degrading fungi to acquire a functional diversity. Thus, microexon was thought to be a key to design Phanerochaete P450 function. To clarify a catalytic function of each P450 gene of *P. chrysosporium*, the P450 reaction array system was constructed. Over 130 full-length cDNAs were obtained from 154 P450 genes. Heterologous expression systems using *Escherichia coli, Pichia pastoris*, and *Saccharomyces cervisiae*, which contain a fungal cytochrome P450 reductase gene, were successfully applied to recover many of Phanerochaete P450 activities as well as active P450 proteins. This fungal P450 reaction array system combined with a high-throughput product analysis system such as LC-MS and FT/ICR-MS enabled us to examine a large variety of exogenous substrates with a comprehensive manner. Furthermore, a DNA microarray system using 133 full-length cDNAs of fungal P450s as a probe was constructed to determine the gene expression against exogenous addition of a wide variety of compounds.

Characterization of laccases from *Coprinopsis cinerea*

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Laccases (EC 1.10.3.2) from basidiomycetes are used for various industrial applications such as paper pulp bleaching, bioremediation, textile dye decolourization, wood composite production, wine and beer stabilization and biosensor manufacture. Overexpression of basidiomycete laccases in ascomycete species does not give satisfactory yields and often the recombinant enzymes show altered properties. Therefore, we developed a basidiomycete expression system for over production of laccases using the basidiomycete *Coprinopsis cinerea* as a host. In a laccase-free monokaryon, we homologously expressed all the 17 laccases genes (*lcc1* to *lcc17*) that occur in *C. cinerea* under the control of *Agaricus bisporus gpdII* promoter and laccase activities were obtained for transformants of 10 of the laccase genes. We also expressed 14 of the *C. cinerea* laccase cDNAs in *Saccharomyces cerevisiae* under the control of the galactose inducible *GAL1* promoter and laccase activities were obtained for six of the genes. Three of these (*lcc3*, *lcc9* and *lcc16*) failed before to produce detectable enzymatic activities in *C. cinerea* transformants. In conclusion, at least 13 of the *C. cinerea* laccase genes are principally functional. However, laccases distinguish in their substrate specificities. We have purified and characterized recombinant laccase Lcc1 from *C. cinerea* culture supernatants that exhibits identical properties to Lcc1 produced from the endogenous gene *lcc1* of another monokaryon. Characterization of other laccases is in progress.

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VIIp-7

Characterisation and heterologous expression of *Agaricus bisporus* lectins which inhibit proliferation of epithelial tumour cell lines

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Colorectal cancer is the second most common cause of cancer-related deaths in the Western World with 28,000 cases per year in the United Kingdom.

Agaricus bisporus is an economically important edible fungus. Despite its importance, the *A. bisporus* genome has remained under-exploited. Previous studies have identified lectins in *A. bisporus* which inhibit epithelial colon cancer cell growth. The *A. bisporus* lectins are part of a group of proteins that binds to the Thomsen- Friedenreich antigen (T-antigen) which is overexpressed in malignant and premalignant colonic epithelium. This T-antigen consists of the disaccharide galactose-β1-3-N-acetyl-galactosamine-α (Galβ1-3GalNAc). Binding of the *A. bisporus* lectin causes dose-dependent inhibition of the proliferation of these cells and is a reversible, non-cytotoxic effect. It has been shown that the lectin binds in the cytoplasm to a truncated form of the oxygen-regulated sialylated form of the lectin ligand and blocks nuclear localisation signal-dependent nuclear protein import. Four isoforms of the lectin have been detected previously in crude mushroom extract. It is yet unknown if these isoforms are formed by post-translational modification, if there are different genes for the four isoforms or if it is a combination of both possibilities. Recent work at Warwick HRI has identified two closely related lectin genes. The aims of this project are to clone these two lectin genes into vectors and transform them in *E. coli* for heterologous protein expression. The total cellular protein was purified via immobilised metal affinity chromatography. This was possible by the binding of the His-Tag (present on the heterologously expressed proteins) to Nickel on an iminodiacetic sepharose column. After purification of the protein, the His-Tag was removed by thrombin proteolysis. After high amounts of purified lectins were produced, the activity of the different lectins and the mechanism of action of the anti-poliferative effect were assayed.

Identification and characterisation of hydrophobins from *Fusarium graminearum*

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Fungal infection of barley and malt, particularly by strains of the genus *Fusarium*, is known to be a direct cause of undesirable beer gushing. Gushing is a phenomenon in which beer spontaneously, without agitation, vigorously over foams out from the package immediately on opening. We have shown previously that small fungal proteins, hydrophobins, act as gushing factors in beer. The aim of our work was to isolate and characterise hydrophobins from a gushing active fungus *Fusarium graminearum*. We generated profile hidden Markov models (profile HMMs) for the hydrophobin classes IA, IB and II from the multiple sequence alignments of their known members. We searched *Fusarium graminearum* genome database of predicted proteins (http://www.broad.mit.edu) with the models. The best matching sequences and the corresponding genes were isolated and characterised. One of the putative hydrophobin genes was expressed in *Trichoderma reesei* and the hydrophobin-like protein was isolated from the culture filtrate of the transformant. A concentration of 0.03 ppm of the RP-HPLC purified protein was observed to induce beer gushing.

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Isolation and characterization of the HFBIII hydrophobin of *Trichoderma reesei*

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Hydrophobins are surface active proteins produced by filamentous fungi. They have a role in fungal growth as structural components and in the interaction of fungi with their environment. Due to their physico-chemical characteristics they are very interesting proteins and suited also for various applications. We have been characterizing the hydrophobins of *T. reesei* on molecular and functional level. Previously we have reported characterization of the HFBI and HFBII hydrophobins. Recently, we have isolated a third hydrophobin, HFBIII, from *T. reesei*. The *hfb3* gene was cloned based on internal peptide sequences obtained for HFBIII found in the supernatant of lactose cultures. HFBIII is a classII hydrophobin and very similar to both HFBI and HFBII the most specific feature of it being an extra cysteine residue between the regular 4th and 5th cysteines. Expression of *hfb3* was studied and revealed that it is expressed similar to *hfb2* on complex plat materials and lactose but the expression levels are lower. To characterize HFBIII at a protein level, a *T. reesei* strain overexpressing HFBIII in a HFBII deletion background was constructed. A purification prodedure for HFBIII was developed. HFBIII is largely retained in the mycelium in vegetative cultures. Our findings show that different treatments are needed for removal of HFBIII from the mycelium than for HFBI, which can be found in the cell walls of glucose-grown mycelia. This can indicate different structural roles for the two proteins.

A proteomic approach for the basidiomycete *Sclerotium rolfsii*

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The plant pathogenic fungus *Sclerotium rolfsii* is the causative agent of the southern blight disease that annually results in severe crop losses. Interestingly, compounds excreted by *S. rolfsii* during the infection process represent biotechnologically valuable products such as industrially relevant enzymes and scleroglucan, a polysaccharide that is an excellent material for several technical and life-science applications. Both, being a plant pathogen and a biotechnologically important fungus make this organism an interesting candidate for obtaining a deeper insight into the plant-host interaction and for its exploitation for industrial purposes.
To gain a more comprehensive understanding of the proteins associated with the infection process, we have initiated a proteomics project. However, proteomic sample preparation represents a challenge as no universal extraction method exists. We have therefore applied and assessed three different protein extraction methods for *S. rolfsii*: i) extraction of membrane proteins, ii) extraction of soluble proteins and iii) extraction of soluble and unsoluble proteins using phenol. The protein extracts where subsequently analysed by 1D and 2D SDS-PAGE. Protein yield and abundance was highest using the phenol extraction method, however, additional protein spots could be identified with the other two methods in a 2D SDS-PAGE analysis. These results indicate that it is necessary to analyse the protein extracts from complementing extraction methods in order to isolate the majority of *S. rolfsii*'s proteom.

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Agrobacterium tumefaciens-mediated and protoplast-mediated transformation of the basidiomycete *Sclerotium rolfsii*

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The basidiomycete *Sclerotium rolfsii* is a plant pathogen with more than 500 different host species. Beside this, the fungus is known to excrete a series of enzymes and the polysaccharide scleroglucan. Mannanases, cellulases and acid-stable laccases, for example, are interesting for industrial applications. Scleroglucan is used as a natural thickener in cosmetics and oil production.

However, a transformation method that makes *S. rolfsii* accessible for genetic manipulations, a prerequisite for understanding its pathogenicity or for strain improvement, has not been developed so far. In an attempt to facilitate the genetic transformation of *S. rolfsii*, we have tested two different transformation approaches, the *Agrobacterium tumefaciens*-mediated transformation (ATMT) and the PEG-mediated transformation of protoplasts (PMT). For both methods, we used a hygromycin-resistance cassette as dominant selection marker, based on the *Escherichia coli hph* gene under control of either the *Agaricus bisporus gpd* promoter (*gpdAB*) or the *Aspergillus nidulans gpd* promoter (*gpdAN*). Both methods were successfully adapted to *S. rolfsii* and resulted in hygromycin resistant clones which were confirmed by PCR to contain the *hph* gene. However, the promoters used were critically important for conferring full hygromycin resistance. Whereas the *gpdAB::hph* construct resulted in a good transformation efficiency and stable hygromycin resistant clones, the *gpdAN::hph* construct was ineffective. Southern analyses are currently under way in order to examine the fate of the transforming DNA.

The EuroBloodSubstitutes Project: initial production of recombinant haemoglobin in yeast

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The *EuroBloodSubstitutes* Project is supported by the European Union 6th Framework Programme and is developing a technological baseline for producing novel haem proteins and blood substitute components using bacteria and fungi. Initial studies investigated the effect of carbon source and host mutations on the production of adult human haemoglobin (Hb) by *Saccharomyces cerevisiae*. The α and β globin genes were inserted into an episomal vector containing the bi-directional GAL7-10 promoter to allow simultaneous, balanced expression of the two genes. Equal expression of both globin monomers is known to be critical for efficient formation of functional Hb tetramers ($\alpha 2 \beta 2$). The GAL promoters are induced by galactose and repressed by glucose. The induction is controlled by Gal4p, a DNA-binding transcription factor, which activates expression of GAL genes, and by the Gal80p which inhibits transcriptional activation by Gal4p in the absence of galactose. Strains of *S. cerevisiae* possessing knockout mutations were transformed with the Hb expression plasmid and tested for their ability to produce cytosolic Hb. Such strains included a gal80Δ mutant, which would allow Gal4p to remain active in the absence of galactose, and a gal1Δ mutant, which lacks galactokinase activity and hence, cannot consume galactose. Both mutant strains were grown in selective minimal media containing a GAL-repressing carbon source (glucose), an inducing carbon source (galactose), a non-repressing carbon source (glycerol), or a mixture of inducing and non-repressing (glycerol and galactose). It was found that, in contrast to the wild-type and gal1Δ mutant, the gal80Δ mutant expressed Hb under non-repressing conditions (glycerol) and produced an even higher yield (1.7-fold greater) when grown on a mixture of glycerol and galactose. The gal1Δ mutant performed less well than either the gal80Δ mutant or the wild-type strain when grown under inducing conditions. This indicated that availability of galactose to induce the expression cassettes was not a limiting factor. The mixture of glycerol and galactose was the most favourable carbon source for Hb production for all three strains, allowing both mutant strains to accumulate 30-40% more Hb than the control (wild-type grown on galactose). However, the wild-type strain reached an even higher yield of Hb (180% increase) when grown on glycerol and galactose than on galactose alone. These results provide a baseline for the exploitation of fungi to produce blood substitute components.

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Agrobacterium tumefaciens-mediated transformation of *Monascus ruber*

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Agrobacterium tumefaciens-mediated transformation (ATMT) was successfully applied to the ascomycete fungus *Monascus ruber* using the binary vector pBHt2 containing the hygromycin B phosphotransferase gene (*hph* gene) as a selection marker. The pre-treatments of *Agrobacterium tumefaciens* AGL-1 cells during induction with acetosyringone (AS) produced more transformants than without AS, however, there was no significant difference. In contrast, the presence of AS in co-cultivation medium (CM) was imperative for transformation. The optimum co-cultivaiton time was 84 hr with the efficiency of 600 to 1,000 transformants when the same volume (200 μℓ) of 1×10 spores of *M. ruber* and bacterial cells were used. The stability of transformants was over 95% after several generations in the presence of hygromycin B (100 μg/Mℓ). The presence of the *hph* gene was confirmed by PCR and Southern blot analysis of randomly selected transformants. The development of transformation system by ATMT will enable us to study *M. ruber* genetically for understanding of biosynthesis of secondary metabolites.

High-yield production of the bacterial *Nonomuraea flexuosa* Xyn11A xylanase in *Trichoderma reesei* by expression of a truncated *Nf xyn11A* gene

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Xylanases that are active and stable at high temperature and alkaline pH are desirable in several industrial processes, e.g. in pre-bleaching of kraft pulp. We have previously successfully expressed the *Nf xyn11A* gene encoding a thermostable xylanase originating from an actinomycete *Nonomuraea flexuosa* (1) using *Trichoderma reesei* as a production host (2). The culture supernatants of the *T. reesei* transformants and those of the gene donor strain were found to contain, in addition to the full-length xylanase, Nf Xyn11A forms that were cleaved at the linker between the core and the carbohydrate binding module (CBM). The core polypeptides had better thermostability than the full-length xylanase and were effective in the bleaching application (1). For high-yield production of the Nf Xyn11A core protein for industrial applications, a truncated *Nf xyn11A* gene lacking the region encoding the CBM was expressed in *T. reesei* under the control of the strong *T. reesei* cellobiohydrolase 1 (*cbh1/cel7A*) promoter. The xylanase polypeptide was fused 3´ to *T. reesei* carrier polypeptides with an intact domain structure, either to the mannanase I (Man5A) core/hinge or to the cellulose binding domain (CBD) of cellobiohydrolase II (Cel6A). Also, as a control, a construct without a carrier polypeptide was prepared. Expression and production of the heterologous xylanase protein were studied from single-copy isogenic strains in which the expression cassette replaced the *cel7A* gene. Both the expression of xylanase mRNA and production of the recombinant xylanase into the culture medium were increased several fold when the truncated gene was used in the expression cassettes, compared to the corresponding constructs with the full-length gene. The best amount of xylanase was obtained when the Cel6A CBD was used as a carrier polypeptide. The truncated Nf Xyn11A constituted up to 25% of the total proteins secreted by the transformants. The expression of the bacterial gene, either as truncated or full-length form, did not induce the “unfolded protein response” (UPR) pathway, according to results from Northern blots probed with *pdi1* and *hac1*.

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VIIp-15

Engineering novel beta-lactam producers: Cephalosporin production in *Penicillium chrysogenum* strains.

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Penicillium chrysogenum is industrially used as penicillin producer. The overproducer strains selected by the penicillin producing companies have the capability to produce and secrete more than 50 g/L of penicillin. Therefore, *P. chrysogenum* is a good host for the production of other -lactam antibiotics. Cephalosporins are produced by the filamentous fungus *Acremonium chrysogenum*, by a biosynthetic pathway that involves six genes of which the first two are common to the penicillin pathway. In this work, we have constructed *P. chrysogenum* strains that produce cephalosporins efficiently. We have introduced in *P. chrysogenum* npe6 (blocked in the last step of the penicillin pathway) the genetic information from *A. chrysogenum* necessary for the biosynthesis of cephalosporins. *P. chrysogenum* TA64, TA71 and TA98 have been transformed with the *cefD1*, *cefD2*, *cefEF* and *cefG* genes from *A. chrysogenum*. The recombinant strains TA64, TA71 and TA98 produce a similar amount of total -lactams than *P. chrysogenum* Wis 54-1255 (the npe6 parental strain) in complex production medium. The recombinant strains TA64, TA71 and TA98 produce a high extracellular amount of deacetylcephalosporin C but cephalosporin C is not detected in the culture broth. Low DAC-acetyltransferase activity (encoded by *cefG*) is present in TA71 and TA64 transformants, while TA98 has a higher enzymatic activity. HPLC analysis showed that TA64, TA71 and TA98 strains produce deacetylcephalosporin C and a small amount of intracellular cephalosporin C. HPLC analysis of TA98 revealed that it produces a higher amount of cephalosporin C. Mass spectra analysis confirmed that TA98 produces true deacetylcephalosporin C and cephalosporin C. The low level of cephalosporin C production may be the result of the low expression of *cefG* in the heterologous host or alternatively might be due to a cephalosporin secretion barrier.

Alternative cephalosporin secretion systems: Amplification of the cefT2 gene increases isopenicillin N and penicillin N secretion in *Acremonium chrysogenum* C10.

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In *Acremonium chrysogenum* the CefT protein is involved in cephalosporin secretion. Targeted inactivation of *cefT* gene revealed that it is not essential for cephalosporin secretion, suggesting the presence of redundant systems involved in cephalosporin exportation. Alternative secretion systems were searched in the regions adjacent to the cephalosporin gene clusters. A gene library of the *A. chrysogenum* C10 strain was screened with a probe internal to the 'early' gene cluster (330 bp *SalI-EcoRV* fragment of the *cefD1* gene). Six phage plaques gave positive hybridization with this probe. They corresponded to phages F2, F3, F4, F5, F6, F8 with six different inserts of *A. chrysogenum* DNA. Sequence analysis of the region downstream of the *cefD1* gene (from F8) revealed the presence of a new open reading frame named cefT2, encoding a multidrug efflux pump belonging to the Major Facilitator Superfamily (MFS) of membrane proteins. The CefT2 protein has 12 transmembrane spanning (TSM) domains and contains motifs A, B, C, D2 and G characteristic of the Drug: H^+ antiporter 12-TMS group of the major facilitator superfamily. The amplification of the *cefT2* gene in *A. chrysogenum* C10 results in increments in penicillin (isopenicillin N and penicillin N) production and in reductions in the cephalosporin production. It seems that the CefT2 protein is involved in isopenicillin N and penicillin N secretion in *A. chrysogenum*, although we can not exclude other possibilities at this time.

VIIp-17

Construction of large-scale genomic DNA deletions in the koji mold *Aspergillus sojae*

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Aspergillus sojae is mainly used in the industrial production of enzymes and oriental fermented food products such as soy sauce and miso. However, the modification of this fungus by genetic engineering is a difficult and time-consuming process because of low gene targeting frequency (1). To overcome this difficulty, we established an efficient gene targeting system using an *A. sojae ku70* disruption mutant. The *ku70* gene may play a key role in the nonhomologous end-joining pathway and the gene targeting frequency is greatly increased in the *ku70* disruption mutants of *A. sojae* (2, 3).

We have recently developed a method to efficiently construct large-scale deletion mutants by using a combination of a *ku70* disruption mutant of the koji mold *A. sojae* and a *pyrG*-mediated transformation system following 5FOA selection.

A. sojae does not produce aflatoxins but has a homolog of an aflatoxin biosynthetic gene cluster that is approximately 60-kb long. The removal of this cluster is desirable for food safety. However, due to the low frequency of the homologous recombination, it is difficult to construct a deletion mutant of the cluster in *A. sojae*. In addition, phenotypic selection was not available because the genes involved in the cluster were not transcribed. Therefore, we attempted to construct deletion mutants of the aflatoxin gene cluster in *A. sojae* by using this system.

To construct deletion mutants, the *pksA*-targeting vector pVb3-PG2 consisting of a *pyrG* marker and a 1.7-kb DNA fragment containing *moxY*, a gene located at one end of the aflatoxin gene cluster, was integrated into the *pksA* locus-located at the other end of the cluster-of the *A. sojae ku70* disruption mutant. Next, 5FOA-resistant and *pyrG*-negative transformants were selected from the strain. PCR and southern analysis confirmed the deletion of the entire aflatoxin gene cluster in the *A. sojae* 5FOA-resistant strains.

In conclusion, the method described here is very useful for making large-scale deletion mutants and enables multiple gene targeting in *A. sojae*.

(1) Takahashi *et al.* (2004) Mol Gen Genet 272: 344-352.
(2) Takahashi *et al.* (2006) Biosci Biotechnol Biochem 70: 135-143.
(3) Takahashi *et al.* (2006) Mol Gen Genet (in press).

PCR-based gene targeting in *Aspergillus niger*

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Bipartite gene-targeting is a flexible PCR-based method for modifying, inserting and deleting genetic material into the genome of an organism without bacterial sub-cloning. With the recent increase in genome sequences for filamentous fungi, and especially the Aspergilli, customized versions of the technique for filamentous fungi have been made possible. The bipartite method additionally reduces false positives from non-specific insertions in the genome.

Aspergillus niger is a platform for cell factories and has found wide applications. Especially organic acids and enzymes are produced readily. With the release of the genomic sequence for *A. niger*, bipartite gene-targeting has become possible, and with it, metabolic engineering on a genome scale.

In the present work, we present bipartite techniques developed for *Aspergillus nidulans* adaptated to *A. niger*.

VIIp-19

Genomics approaches to study the regulation of the proteolytic system of *Aspergillus niger*

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The proteolytic system of *Aspergilli* is quite complex. A broad range of proteolytic activities has been identified from *A. niger* over the past years. Several of the genes involved have been characterized and studied in more detail. Random mutagenesis and targeted disruption of genes encoding proteases have resulted in mutants with significantly lower protease activities. However, even with the use of protease mutants as host strains for heterologous protein production, proteolytic degradation is still a major problem. Although different regulatory systems have been described, until now only a very small part of the (regulatory) components of the proteolytic system have been characterized in detail. It is the objective of our project to follow a rational approach for analysis of the regulation of the proteolytic system, by using a combination of various genomics tools in combination with extracellular protease profiling. For this purpose a protease assay has been developed.

For these genomics experiments a number of different growth conditions have been selected. These include variation in carbon sources, nitrogen sources and pH and limitation of phosphate and sulfate. These conditions are tested in batch fermentations for growth, production of protein and protease activity. For the final genomics experiments a selection of conditions will be made and samples from fermentations run under these conditions will be analyzed with metabolomics and transcriptomics tools. Correlations between the different data sets will be used to identify and understand the biological processes involved in the regulation of the fungal proteolytic system.

The final goal is to identify the strain and cultivation conditions that reduce proteolytic activities in order to further improve heterologous protein production.

Production of two types of pectinolytic enzymes by some *Aspergillus* section *Nigri* members on submerged and solid state fermentation systems

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Production of the two types of pectinolytic enzymes named polygalacturonase and polymethygalacturonase active towards to homogalacturonan regions of pectin backbone, by the strains of ***Aspergillus niger***, ***A. tubingensis***, ***A. aculeatus***, *A.* ***foetidus*** **var.** ***pallidus*** and ***A. carbonarius*** which are taxonomically included in *Aspergillus* section *Nigri* were evaluated using two fermentation systems one of which is well-known submerged and the other solid state.

After pectinolytic capabilities and activities of the indicated strains were determined and compared on two different systems their enzyme activities on solid state were found higher than submerged system. Highest producer strain belong to *Aspergillus foetidus* var. *pallidus* was chosen in order to improve enzyme activities on solid state fermentation system. Through this aim different agroindustrial residues, some chemical and physical parameters that are substrate composition (as sole wheat bran and pre-treated sugar beet pulp and different combinations two of them), C:N ratio (10:1, 16:1, 28:1, 31:1, 37:1) moisture level (70%, 75%) and amount of inoculum (10^7, $2x10^7$, $5x10^7$, 10^8 per 2,5 gr of substrate) that affect the enzyme production were tried.

VIIp-21

Molecular analysis and improvement of protein production by *Aspergillus oryzae* grown on solid substrates

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Aspergillus oryzae grown in submerged and solid state fermentations revealed a number of differences in molecular, physiological and morphological aspects that are correlated with the different growth conditions. First, the aerial hyphae that occur only in solid state fermentation are very important for oxygen uptake and thus fungal growth. Second, solid state fermentation is characterised by gradients in temperature, water activity, oxygen and nutrient concentrations. Third, pellet growth in submerged and mycelial growth in solid state fermentations show different gene expression and protein secretion patterns. Using both classical and X-omics based approaches we aim to expand our basic knowledge of mechanisms of growth of filamentous fungi on solid substrates and to exploit the biotechnological applications. An example of a molecular genetic strain improvement approach based on our results will be presented.

Genetic stability of multiple gene insertions in *Aspergillus nidulans*

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In many filamentous fungi, including *Aspergillus nidulans*, transformation of recombinant DNA often leads to the integration of multiple copies of this DNA either as inverted or direct repeats. For many industrial applications transformants with multiple gene copies are obtained from screening because such strains often have high gene expression levels. However, multiple gene insertions often result in genetic instabilities due to homologous recombination between the repeated sequences.

We have developed a conidiospore color assay in *A. nidulans* that allows the detection of spontaneous or induced recombination events within a fungal colony and also determines whether the event occurred as a loop-out or a gene conversion event. The assay provides a practical way to measure the recombinogenic effects of different configurations of repetitive sequences and also a means to compare the effect of various gene mutations on recombination. The assay system includes a unique restriction site that can be cleaved *in vivo* by induction of an endonuclease. We have expanded the assay to include a hetero-allelic diploid that allows the study of inter-chromosomal recombination. The assays can be used to screen for genes involved in genomic stability as well as favorable insertion points and repeat configurations of heterologously expressed genes.

VIIp-23

α-amylase from a filamentous fungus – *Ophiostoma floccosum*

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Ophiostoma floccosum, an ascomycete, is being developed as a new expression system for the production of foreign proteins. Enzymes for starch degradation and several proteases are amongst the most efficiently secreted proteins of *Ophiostoma*. The organism secretes only a few proteins into the culture medium, which provides a considerable advantage for the purification of any recombinant gene product.

Several mutants of *O. floccosum* derived by UV mutagenesis have been isolated and the total the amount of secreted protein was increased by 4 to 6 times. The amylase activity of the best mutant was improved 240-fold compared to the parental strain. The proteinase profiles in the culture supernatants of several key mutants have been characterised for the selection of a suitable expression host for a particular gene product. The regulatory sequences and the protein-encoding region of α-amylase, one of the dominant secreted proteins, have been isolated. A series of expression vectors containing the α-amylase regulatory sequences and sequences encoding the mature α-amylase enzyme gene have been constructed. The expression system is being tested using *dsRed* as a reporter gene.

Highly efficient generation of knockout mutants using a *Sordaria macrospora* strain deficient in the mammalian *ku70* ortholog

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Targeted gene replacement via homologous recombination is a routinely used approach to elucidate the function of unknown genes. Integration of exogenous DNA in the genomic DNA requires the action of double-strand repair mechanisms. The filamentous ascomycete *Sordaria macrospora* is a model system for studying fruiting body development in fungi. In contrast to the budding yeast *Saccharomyces cerevisiae*, but similar to many filamentous fungi, plants and animals, transformed DNA is ectopically integrated into the genome of *S. macrospora*. Most probably this occurs by non-homologous end joining (NHEJ). A mechanism which involves the binding of the Ku heterodimer (Ku70/Ku80) at the ends of a DNA double-strand break (DSB). Phylogenetic analysis of Ku70 orthologs of fungal, plant, and animal origin reveals that the Ku70 protein is well conserved among eukaryotes. To improve gene targeting efficiency in *S. macrospora*, we identified and deleted the *S. macrospora ku70* gene. No impairment of the Δku70 mutant in vegetative or fruiting body nor ascospore development was observed making this strain an ideal recipient for gene targeting of developmental genes. As a case study, the *S. macrospora* Δku70 strain was used for targeted deletion of the pheromone gene *ppg2* and the pheromone receptor gene *pre2* [1, 2]. PCR generated deletion constructs containing 1000 bp of homologous flanking sequence resulted in a drastically increased gene targeting efficiency. As a consequence, almost all transformants generated carried a disrupted target gene.

[1] Pöggeler S (2000) Two pheromone precursor genes are transcriptionally expressed in the homothallic ascomycete*Sordaria macrospora*. Curr Genet 37: 403-411
[2] Pöggeler S, Kück U (2001) Identification of transcriptionally expressed pheromone receptor genes in filamentous ascomycetes. Gene 280: 9-17

VIIp-25

Identification of novel peptidase-encoding sequences and expression profiles in the biocontrol fungus *Trichoderma harzianum*

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Current molecular and genomic methods are being applied to *Trichoderma* spp. because of its importance in agriculture for the biological control of plant pathogens. Despite the potential relevance of the proteolytic activity for the *Trichoderma* biocontrol properties, the number of genes cloned to date is relatively low compared to those of other traditionally biocontrol-associated enzymatic systems (i.e. chitinases). We have inspected a set of 2672 unique ESTs (1629 singlets and 1043 contigs) generated from *T. harzianum* CECT 2413 mycelium growing under simulated antagonistic conditions, with the aim to identify extracellular peptidases that may participate in the *Trichoderma*-pathogen interaction. BlastX analyses and further manual screening based on the functional identity and cellular location of the best matches, allowed us to select a total of ten unisequences encoding novel putative peptidases in *T. harzianum* CECT 2413. The encoded proteins included six serine endopeptidases (EC 3.4.21) from S1, S8 and S53 families, two aspartic endopeptidases (EC 3.4.23) belonging to family A1, a metalloendopeptidase (EC 3.4.24) from family M35, and an aminopeptidase (EC 3.4.11) as a member of family M28. Full-length amino acid sequences were obtained and compared at phylogenetic level with peptidases from other organisms. Expression analyses by Northern blot demonstrated that the corresponding genes are differentially regulated in response to different culture conditions, suggesting that they have distinctive functions.

Identification of *Trichoderma virens* genes encoding for chitinolytic enzymes using the expressed sequence tag approach

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Locally isolated *Trichoderma virens* spp. UKM1 was able to utilize chitin and its derivatives, glucosamine, *N*-acetylglucosamine and chitosan for growth. The ability to grow on chitin and its aminosugar compounds as sole carbon source indicates that this fungus can break down chitin or its derivative to its simplest form and use it as an energy source for cell growth and development. To execute this function, the fungus has to secrete all enzymes required for chitin and chitin derivatives degradation or modification. To identify genes involved in the aminosugars degradation or modification, we constructed a number of cDNA libraries and carried out expressed sequence tag analysis (EST) on some of the clones. The fungus was grown in chitin or its derivatives (colloidal chitin, chitosan, glucosamine or N-acetylglucosamine) as sole carbon source or in rich medium, the potato dextrose broth. Fungal total RNA was extracted from the pooled mycelia harvested from different days of growth and the mRNA was isolated using PolyA-Tract mRNA Isolation Systems kit (Promega, USA). The cDNA libraries were constructed using the cDNA construction kit from Stratagene (LA Jolla, USA). A total of 2880 clones have been selected, subjected to single pass sequencing from the 5'end of the vector and analysed using a STACKPACK v2.1.1.software. Out of 2880 sequences, 77% produced good quality sequence with phred quality of >20 and the sequence length of more than 150 bp. Clustering and assembly analysis resulted in the identification of 1660 unique transcripts. Sequence similarity searches against gene sequences in public database were carried out using the BLASTX program. Based on the BLAST report we identified a number of genes that encode for chitinolytic enzymes such as endochitinase (EC 3.2.1.14), β-N-acetylhexosaminidase (EC 3.2.1.52), chitobiase (EC 3.2.1.52), exo-β-D-glucosaminidase (EC 3.2.1.-) and chitin deacetylase (EC 3.5.1.41). Work is currently ongoing to screen for more genes encoding for chitinolytic enzymes from this fungus using a cDNA microarray approach. Identification of these genes will facilitate the production and functional analysis of recombinant chitinolytic enzymes of *T. virens*, which could have different properties from the currently available commercial chitinases.

VIIp-27

Heterologous production of laccase enzymes from *Thielavia arenaria*

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Laccases are multicopper oxidases catalyzing oxidation of various phenolic compounds, aromatic amines, and even inorganic compounds by a one-electron transfer mechanism. Laccases are common enzymes in nature, especially in plants and fungi. Fungal laccases are involved in plant pathogenesis, pigment production, and lignin biodegradation. Recent interest of using laccase in various industrial enzyme applications is based mainly on broad substrate-specificity of the enzyme. We describe molecular cloning of four laccase genes from the thermophilic ascomycete *Thielavia arenaria* as well as their heterologous expression in *Trichoderma reesei*. Characterization of the purified recombinant enzymes indicated that the *Thielavia arenaria* laccases are clearly distinct proteins from each other having unique catalytic properties. The predominant *Thielavia arenaria* laccase, here referred as TaLcc1, was produced as 72 kDa protein in *Trichoderma* with pH optimum of 6.0 on guaiacol and a half-life of 5 h at 60 °C. Several ascomycete laccases were tested in denim treatment. TaLcc1 enzyme was found to be superior in decolorization of Indigo dye and denim bleaching being, thus, a promising candidate for textile applications.

Premature polyadenylation and consequent nonstop mRNA degradation reduce the mRNA level of heterologous gene in *Aspergillus oryzae*

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Aspergillus oryzae has been used for the Japanese fermentation products owing to its ability to secrete a wide range of enzymes. It has been, therefore, paid an attention as a host organism for heterologous protein production. However, the secretion level of heterologous proteins is generally lower than that of the homologous proteins.

In the previous study, using a mite allergen protein, Der f 7 as a model, we compared the expression levels of codon optimized Der f 7 (Der/opt) and of native codon Der f 7 (Der/ntv), and showed that codon optimization improved the secreted yield of Der f 7 by increasing the mRNA level in *A. oryzae*. In this study, we analyze the effect of codon optimization on the Der f 7 mRNA level. At first, we constructed chimeric Der f 7 genes, Der/opt-ntv and Der/ntv-opt, in which codons of 5' half or 3' half of the gene were optimized respectively. Interestingly, codon optimization of 3' half of the Der f 7 gene was sufficient for increasing the mRNA level. On the other hand, 3'-RACE analysis revealed that poly(A) tail was added at several positions within the 3' half of the transcripts of Der/ntv and Der/opt-ntv, whereas poly(A) tail was added only at the termination region of Der/ntv-opt and Der/opt. Taken together, these observations suggest that the 3' half of the native Der f 7 gene contains several potential poly(A) addition signals which could be eliminated by codon optimization. Moreover, insertion of termination codons upstream of Der f 7 resulted in the increased level of the Der f 7 mRNA, suggesting that premature polyadenylation and consequent mRNA degradation by the nonstop mRNA decay pathway reduce the Der f 7 mRNA level. In conclusion, our results suggest that premature polyadenylation and consequent nonstop mRNA degradation is one of the critical factors that reduce the mRNA level of heterologous gene.

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A gene cluster for ergovaline biosynthesis from a fungal endophyte of perennial ryegrass

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Clavicipitaceous fungal endophytes of the *Epichloë/Neotyphodium* group form mutualistic symbioses with grasses of the family Pooideae. Some strains produce the ergopeptine alkaloid ergovaline, which is implicated in livestock toxicoses caused by ingestion of endophyte-infected grasses. Cloning of a non-ribosomal peptide synthetase (NRPS) gene and characterisation of flanking sequences from *N. lolii* identified a gene cluster containing a single-module NRPS, *lpsB*, and other genes orthologous to those in the ergopeptine gene cluster of *Claviceps purpurea*. This cluster was shown by Southern analysis to be linked to *lpsA* and *dmaW*, two previously-characterised ergovaline biosynthetic genes, but separated by highly AT-rich regions, containing remnants of putative transposable elements. Targeted disruption of *lpsB* showed that this gene encodes the single-module NRPS that activates D-lysergic acid, for incorporation by the tri-modular NRPS, LpsA, into ergovaline. Real-time RT-PCR analysis shows that all genes in the ergovaline biosynthetic gene cluster are preferentially expressed *in planta*. Growth in various minimal media, including limiting phosphate, carbon and nitrogen levels, showed that expression was not nutritionally regulated, suggesting a specific plant factor may be required. However, exposure to a ryegrass extract was insufficient to induce ergovaline gene expression, suggesting more complex signalling may be required between endophyte and host plant.

Characterization of two gene clusters for glycolipid biosynthesis in *Ustilago maydis*

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Under conditions of nitrogen starvation, *Ustilago maydis* produces two classes of extracellular glycolipids that act as biosurfactants. Ustilagic acid (UA) consists of a cellobiose moiety glycosidically linked to the terminal hydroxyl group of 15,16-dihydroxypalmitic acid. In addition, *U. maydis* secretes mannosylerythritol lipids (MELs), which consist of the disaccharide mannosylerythritol esterified with acyl and acetyl groups at the mannosyl group. We had previously identified a glycosyltransferase involved in MEL production and a cytochrome P450 monooxygenase essential for UA biosynthesis. Since both genes were highly expressed under conditions of nitrogen starvation we used DNA microarray analysis to isolate further genes involved in glycolipid biosynthesis. We could identify two large gene clusters, which are responsible for glycolipid production. Database comparisons and mutational analysis allowed us to propose biosynthetic pathways for both of these unusual secondary metabolites. Since *U. maydis* is very amenable to genetic analysis this organism can be used for metabolic engineering of these extracellular glycolipids. Deletion of a MEL-specific acetyl transferase resulted in the production of deacetylated versions of this glycolipid. Disruption of another UA-specific P450 monooxygenase lead to secretion of a modified version of ustilagic acid, whose molecular structure is currently under investigation. Nitrogen dependent expression of the UA cluster genes is regulated by a novel Zn-finger transcription factor, which is part of the gene cluster. Constitutive expression of this regulator results in glycolipid production even in the absence of nitrogen limitation.

VIIp-31

Pyranose dehydrogenase encoding genes from *Agaricus spp.*

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PDH is an enzyme that was described only several years ago in a number of ecologically related litter-decomposing fungi (*Agaricales*, *Gasteromycetales*). It catalyzes the C-3 and/or C-2 oxidation of several aldopyranoses to the respective keto sugar derivates. Considering the broad substrate tolerance (which also varies with the source of the enzyme), PDH provides a new convenient tool for high yield production of 3-keto-oligosaccharides and 3-keto-glycosides. PDH oxidizes most major sugar components of wood polysaccharides, and is implicated to play a role in lignocellulose degradation. *Agaricus bisporus*, the white button mushroom, is an economically significant agricultural crop. Deeper insight in the physiological role of PDH may provide help for mushroom growers to increase yield, improve quality or make new sources of raw materials utilizable. We isolated the PDH-encoding gene from *A. bisporus* as well as from the related mushroom *A. meleagris*. Further investigations revealed that *A. meleagris* harbors two additional, closely related genes, encoding as yet uncharacterized proteins. We also investigated the transcriptional regulation of these genes by different carbon sources on a defined minimal medium by real-time PCR.

A self-replicating vector for efficient transformation and screening of gene libraries in *Chrysosporium lucknowense*

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An integrated technology platform is currently under development utilizing *Chrysosporium lucknowense* gene expression technology coupled with high-throughput screening. In order to satisfy the needs for the various stages of protein discovery and development, an ideal fungal expression system will have the following characteristics. It will have: (i) a high transformation frequency to allow efficient library construction and screening (ii) the ability to express a wide variety of eukaryotic proteins in a biologically active form; (iii) compatibility with laboratory automation; (iv) the ability to produce sufficient quantities of protein to support the confirmation of hits and the many other activities involved in transforming a hit into a drug; and (v) capabilities for further scale up to commercial protein production in flexible and robust fermentation processes.

Within a strain improvement program the wild-type strain of *C. lucknowense* was improved with respect to fungal morphology and protein production levels. One of the advantages of the low viscosity mutants obtained is that they can be cultivated in microtiter plates and can be used in a robotic liquid-handling system (1).

In this paper we describe the construction of a self-replicating vector that facilitates efficient transformation, high level expression and retention of DNA sequences in a fungal host. The integration of this approach with the associated high-throughput robotic technology (2) for library screening is presented.

(1) Burlingame RP and Chandra R (2005) Gene discovery and protein production technology. An integrated system to discover, develop and manufacture enzymes and other proteins. Industrial Biotechnol. 1, 35-37.
(2) Patent application WO 01/79558 (2001). High-throughput screening of expressed DNA libraries in filamentous fungi.

VIIp-33

Risk assessment: usage of genetically modified yeasts during wine making

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In contrast to USA, public in Europe, primarily in Germany, has still a critical position towards the application of genetic engineering, particularly in agriculture and food production. Therefore studies that demonstrates the safety of GMO for human consumption and the environment are required to convince the public to accept the use of GMO. Up to now, little is known about the environmental fate of GMO and their potential effect on naturally occurring organisms, especially during the process of wine making.

The aim of this study was to compare the behaviour of an industrial wine yeast (*Saccharomyces cerevisiae* VIN 13) and genetically modified yeasts derived from it in a model systems. The GM yeasts are expressing an α-amylase originally cloned from *Lipomyces kononenkoae* (designated GMY1)(Steyn *et al.*, 1995), and an endo-β-1,4-glucanase and endo-β-xylanase originally cloned from *Butyrivibrio fibrisolvens* H17c and *Aspergillus niger* (designated GMY2) (Strauss, 2003). The main focus was on the amylolytic strain GMY1, where a null hypothesis was investigated that states that no difference exists between the ecological behaviour of the wild-type strain VIN 13 and GMY1. This hypothesis was specifically tested on grapevines in a secluded greenhouse and in spontaneous and controlled fermentations.

No substantial changes in the variety of wild yeasts were detected due to the introduction of VIN 13, GMY1 and GMY2 on the secluded vineyard. Particularly there is no difference between the behaviour of VIN13 and GMY1 on grapevines. Since no significant differences were observed between the fermentations of berries that were inoculated with the modified and unmodified strains of VIN13 the natural fermenting ability of the unmodified strain was conserved in the recombinant strains. In addition the recombinant yeast strains did not have an advantage during spontaneous fermentations. A significant decline in the concentration of GM yeasts on the grapevines was observable each year. A low number of the recombinant strain GMY2 were detectable two years after the last release of these strains, whereas strain GMY1 was not detectable at all. These findings indicate that case by case studies are an important tool to display the actual behaviour of modified yeasts.

Transformation of *Aspergillus nidulans* using the orotidine monophosphate decarboxylase gene of *Myrothecium gramineum*

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Myrothecium gramineum is a filamentous *Ascomycete*, which is used as a new expression host in ongoing research. In view of using *Myrothecium* as a host for the production of industrial enzymes, an efficient expression system is required, as well as an easy selection method for clones carrying the introduced expression cassette.

In the literature, several methods are described to select transformants of fungi. One can use dominant selection markers, such as antibiotic resistance markers, or auxotrophic markers, e.g. based on nitrate reductase. Since antibiotic resistance markers cannot be used in view of specific industrial applications and since homologous systems are more efficient, the 5'-orotidine monophosphate decarboxylase gene (OMPD-gene) was here chosen as selection marker. The OMPD selection system is based on the fluoro-orotic acid resistance of mutants that are defective in the conversion of orotate to uridine-5'-monophosphate (UMP), which is catalysed by OMPD and orotic acid phosphoribosyltransferase. The mutants are uracil or uridine auxotrophic. An advantage of the system is that both positive and negative selection is possible for OMPD-wild type as well as for OMPD-deficient strains.

The OMPD-gene of *Myrothecium gramineum* was isolated and its nucleotide sequence and amino acid sequence were deposited at the GenBank database (No. **DQ359751**). To utilize the gene as a selection marker, a vector pOV, containing 2050 bp of this gene was constructed. To prove that pOV contains an OMPD-gene coding for a functional enzyme, pOV was used to complement an OMPD negative strain of *Aspergillus nidulans*: this strain, A722, can only grow when uracil is added and is FOA-resistant. $1.2.10^8$ protoplasts were transformed with 61.8 µg pOV and these protoplasts were plated on AMM medium. 597 colonies were obtained and 97% were stable transformants. A selection of 18 complemented colonies were tested as to their 5-FOA sensitivity by plating them on AMM containing 1g/l FOA. None of the transformants grew, which again proves that a functional OMPD enzyme was present in these transformants. The colonies were further tested by PCR with primers specific for the OMPD-gene of *M. gramineum*. All PCR's resulted in the expected 762 bp fragment, which confirms the presence of at least one copy of the OMPD gene of *M. gramineum* in the genome of the *Aspergillus* colonies.

It can be concluded that a successful transformation system has been developped for *A. nidulans* based on the OMPD-gene from *M. gramineum*. This gene will also be used to work out an efficient transformation method for *M. gramineum*.

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Proteome and transcriptome analysis of the cellular responses to production of the heterologous protein, *Melanocarpus albomyces* laccase, in the filamentous fungus *Trichoderma reesei*

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Trichoderma reesei Rut-C30 and its transformant producing *Melanocarpus albomyces* laccase were cultivated in lactose-containing minimal medium in chemostats and subjected to proteome and transcriptome analysis to identify differentially expressed genes under these conditions. The cultures were carried out using a dilution rate (0.03/h) previously shown to be optimal for production of endogenous extracellular proteins under these conditions. The TRAC method for multiplexed transcriptional analysis was first applied to analyse the expression levels of a selected set of genes and to monitor the steady state in the chemostat cultures. The results indicated that UPR (unfolded protein response) pathway was not activated as a response to laccase production. On the contrary, the expression levels of e.g. the foldase and chaperone genes pdi1 and bip1 were expressed at a slightly higher level in the host. Also the expression levels of the genes encoding the endogenous secreted cellulases, egl1 and cbh1, were slightly higher in the host strain. Both proteome and genome wide transcriptional analysis using oligonucleotide microarrays revealed only a modest number of cellular responses to laccase production. Altogether 32 genes were differentially expressed between the strains, only five of the genes being upregulated in the laccase producing strain, and the rest having a higher expression level in the host strain and including many genes encoding extracellular enzymes. Proteome analysis using 2D gel electrophoresis with DIGE showed increased expression of a group of proteins including e.g. heat shock proteins as well ubiquitin associated proteins and proteins involved in ER-associated protein degradation. The result indicates that although laccase expression does not induce apparent UPR, other type stress responses are induced e.g. activated protein degradation. The results were also compared to those obtained in previous studies on the production human tissue plasminogen activator (tPA) in *T. reesei*.

Citrinin-free mutants of the hyperpigmenting *Monascus purpureus* IB1 strain: Cloning of the citrinin synthase gene

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Monascus purpureus and *Monascus rubber* azaphilone pigments are widely used as food colorants in far East countries including "anka" (red rice). *Monascus* pigments are added to a variety of foods. Concern about the utilization of *Monascus* pigments in Europe has been motivated by the presence of small amounts of citrinin (a nefrotoxic compound) in the culture broth of *M. purpureus*. A hyperpigmenting *M. purpureus* IB1 strain was selected in our Institute (Campoy *et al.*, 2003; 2006). HPLC studies showed that this strain produces small amounts of citrinin. In a screening program mutants lacking completely the ability to synthesize citrinin were obtained. Those mutants still overproduce azaphilone pigments. Viceversa, non-pigmented (white) mutants were obtained by REMI transformation that are still able to synthesize citrinin.

A fragment of the citrinin synthase gene was cloned by PCR and used as probe to isolate a phage containing the entire citrinin synthase cluster region from a *M.purpureus* l phage library. The citrinin synthase is an iterative type I polyketide synthase. The citrinin synthase gene (*cis*) encodes a 2593 amino acid protein containing in a single module domains for a ketosynthase (KS), an acyltransferase (AT), an acyl-carrier (ACP) and a methyltransferase (MT), suggesting that the nascent polyketide is methylated by the same enzyme. This pattern of precursor activation and polymerization corresponds exactly to the expected domain organization for the biosynthesis of the citrinin molecule but it is different from the expected module organization for the biosynthesis of the azaphylone synthase. In conclusion the dissociation of the ability to synthesize citrinin and azaphylone pigments and the information obtained on the domain structure of the citrinin synthase indicate that each of these secondary metabolites is synthesized by a different polyketide synthase.

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