CLONING AND HETEROLOGOUS EXPRESSION OF A GENE ENCODING PYRANOSE OXIDASE FROM THE WHITE-ROT FUNGUS *TRAMETES MULTICOLOR*

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Pyranose oxidase (pyranose 2-oxidase, P2O; EC 1.1.3.10) is an enzyme that widely occurs in basidiomycetous fungi. It catalyzes the *C*-2 oxidation of several aldopyranoses to form the respective 2-keto derivatives, transferring electrons to molecular oxygen to yield hydrogen peroxide. There is indication of an involvement of P2O in lignocellulose degradation; the exact physiological role of P2O, however, is not clearly understood to date.
P2O is a potentially interesting enzyme for biotechnological applications, its reaction products (2-keto sugars) can be attractive intermediates in the production of food additives, such as fructose, tagatose, or isomaltulose, and can easily be produced in high yields. *Trametes multicolor* is a potent producer of P2O activity and forms P2O constitutively during growth on a number of carbon sources. Although high levels of the enzyme can be produced by fermentation using lactose or whey powder as substrates, production by heterologous expression of the respective gene is an even more attractive alternative. We therefore isolated a clone from a genomic library encoding P2O, including 5´ regulatory regions, and used the sequence information to amplify a cDNA clone by RT-PCR. Delimitation of introns and exons was determined by comparison of the cDNA and genomic sequences. This is the first report of a genomic sequence of a pyranose oxidase.
The cDNA was re-amplified with primers containing suitable restriction sites, inserted into the bacterial expression vector pET 21d+ and successfully expressed in *E. coli*. Purification of the enzyme was done either by an established two-step purification method or by utilizing the poly-His-tag that was fused to the protein via the expression vector. Properties of the heterologous protein and its use in carbohydrate transformations will be discussed.

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372. FACTORS INFLUENCING GLYCOSYLATION OF TRICHODERMA REESEI CELLULASES

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Glycosylation of fungal proteins has not been studied thoroughly. Furthermore, as Cel7A and Cel7B from *Trichoderma reesei,* literature reports are very divergent both on the structural aspects as on the occupancy of the glycosylation sites.

The present study indicates that for *T. reesei* the number of strain-specific differences in glycosylation is quite low. Cellulases isolated from the wild type and QM derived strains carry predominantly $Man_5GlcNAc_2$. The occurrence of monoglucosylated N-glycans with strains Rut-C30 and RL-P37, could be due to an inefficient glucosidase II of the glycosylation pathway. The conflicting results in literature are mainly the result of post-secretorial hydrolysis. We show that the glycosylation of *T. reesei* cellulases varies considerably when the fungus is grown under different conditions. *T. reesei* produces an array of extracellular hydrolases. These enzymes (including an enzyme with Endo H-like activity) modify the glycan structures during cultivation. The effects are most prominent in corn steep liquor enriched media, where the pH is closer to the pH optimum (5-6) of these extracellular hydrolases. Fully *N*- and *O*-glycosylated Cel7A can only be isolated from minimal medium and this probably reflects the initial complexity of the protein upon leaving the glycosynthetic pathway (alltough at the low pH of the final cultivation medium proteases are particurlarly active).
As such, the *N*-glycans present on secreted cellulases can range from small mammalian-like high-mannose structures (decorated with phosphodiesters) to single GlcNAc residues. Also the extent of O-mannosylation and phosphorylation can vary considerably. Cellulases isolated from a minimal cultivation (pH 3) have, therefore, a higher molecular mass (ESI-MS) and a more acidic electrophoretic pattern (IEF-PAG) compared to glycoproteins from a corn steep liquor enriched cultivation (pH 5). The extracellular trigger to phosphorylation is still unknown but mannophosphorylation may be regulated intracellularly by α-((1→2)-mannosidases and phosphomannosyl transferases competing for the same intermediate in the glycosynthetic pathway.

Our study shows that not only the choice of the *T. reesei* strain but also the cultivation conditions are important for its use as a host for the production of heterologous proteins for industrial and pharmaceutical applications. Ongoing research aims to identify genes encoding for manno(phosphoryl) transferring enzymes as well as for the endoglycosidase responsible for the extensive trimming observed under certain culture conditions.

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PURIFICATION AND CHARACTERISATION OF TWO FORMS OF CELLOBIOHYDROLASE I (CEL 7A) FROM *CHRYSOSPORIUM LUCKNOWENSE*.

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Chrysosporium lucknowense,an ascomycetous cellulolytic fungus, is being developed as a (hemi)cellulases producer. Sequence analysis of peptides isolated from the major cellulase secreted by *C. lucknowense* showed similarity to published cellobiohydrolases. PCR amplification with primers based on these peptides resulted in the isolation of DNA fragment homologous to family 7 (Cel7A) of glycoside hydrolases. This PCR fragment was used for the isolation of the cbh1 gene. Analysis of the primary amino acid sequence of the CBH1 protein, deduced from the gene sequence, confirmed the isolation of a Cel7A glycoside hydrolases. Two forms of CBH1 were purified from the culture filtrate (52kD and 65 kD). Their content makes up about 20% of the total extracellular protein. Analysis of the enzymatic properties of the two proteins showed that the 52kD protein displayed a much lower avicel and cotton hydrolysis rate than the 65 kD protein, indicating the absence of a cellulose binding domain in the 52 kD protein. Moreover, the thermostability of the 65 kD enzyme was significantly better than that of the 52kD protein, and even better than that of a commercial *T.reesei* CBH1 protein. Development of a transformation system for *C.lucknowense* has opened means to selective overexpression of the *C.lucknowense cbh1* and other cellulase genes.

VIIIp-4

A SECOND REGULATORY SYSTEM IN ASPERGILLUS NIDULANS RESPONSIVE TO ALCOHOLS, KETONES AND ESTERS OPERATES INDEPENDENTLY FROM AlcR, THE ACTIVATOR OF ETHANOL CATABOLISM.

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The ethanol utilization pathway in A. nidulans is a regulatory model system the elucidation of which has contributed a great deal to our understanding of the regulation of transcription in filamentous fungi and whose characteristics are particularly useful for heterologous expression in both the fundamental and applied fields. Previously, five ketone-inducible transcription units were identified in the immediate vicinity of the alcohol dehydrogenase gene, alcA, and the regulatory gene specific for ethanol catabolism, alcR, on chromosome VII. One of them, designated alcP, encodes a putative extracellular protein with similarity to a lactonohydrolase from Fusarium oxysporum. We have studied the induction spectrum of the Aspergillus gene and found that it is similar but not identical to that of alcA. Notably, transcription is not induced by aldonate and aromatic lactones which are good substrates of the Fusarium enzyme. Ethanol turned out to be an extremely poor inducer but larger alcohols and aldehydes as well as methyl ketones provoke substantial induction of alcP as do small aliphatic esters like ethylacetate and propylacetate. However, alcP transcription is also provoked by 3-pentanone, a ketone that does not induce alcA. This observation suggests that alcP could be expressed independently of AlcR. This was confirmed in an alc500 deletion strain that lacks all seven ketone-inducible genes comprising the alc gene cluster and into which a single copy of the alcP gene was re-introduced. The induction characteristics in this alcR-deficient background were essentially identical to those exhibited by wild type strains, demonstrating the existence of a second regulatory system responsive to alcohols, ketones and esters (ake). We have renamed the gene akeP as it does not depend on AlcR for its expression and we are currently investigating the biochemical activity it codes for. It remains an open question whether the novel ake regulatory system is involved in alc gene transcription in response to common inducers like n-butanol, 2-methylbutyraldehyde and 2-butanone, all of which provoke substantially higher induction levels than the physiological inducer of ethanol utilization, acetaldehyde.

EVOLUTION OF RECOMBINANT (t-PA) TRICHODERMA REESEI IN CHEMOSTATS SELECTS MUTANTS DOWN-REGULATED IN CELLULASE PRODUCTION

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In order to study the stability of a recombinant strain of T.reesei expressing tissue plasminogen activator (t-PA), the recombinant strain 306/36 (with t-PA under the control of the native cbh1 promoter) and its parent strain (Rut-C30) were grown in chemostat cultures at a dilution rate of 0.05 h^{-1} in lactose limited chemostats and the secreted levels of recombinant t-PA and native cellulases determined.
It was found, that t-PA and native cellulase production by the recombinant strain 306/36 was not stable with morphological mutants displacing the parental strain. This displacement was correlated with a loss in native cellulase and t-PA secretion. Strain Rut-C30 yielded similar morphological mutants, but their appearance was not correlated with a loss of native cellulase production and some mutants at the end of the cultivation even showed enhanced cellulase secretion.
Isolated mutants were grown in batch cultivation and the cellulase and t-PA levels compared to parental strain 306/36 and wild type Rut-C30.
All mutant isolates derived from the recombinant strain 306/36 showed severe reduction in their ability to secrete native cellulases and t-PA production was virtually undetectable, although Southern blotting revealed no apparent loss in gene copy numbers of the t-PA::cbh1 construct.
The reduction may be well due to the fact, that recombinant protein production challenges the fungal secretion machinery to an extent that favours mutants in which recombinant protein production is suppressed. As both t-PA and native cellulase are down-regulated, we are investigating the potential role of the transcriptional regulators of the cellulase genes, ACEI and ACE II.

HETEROLOGOUS EXPRESSION OF T.REESEI SECRETORY PROTEIN IN S.CEREVISIAE PMT MUTANTS.

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The saprophytic fungus Trichoderma reesei secretes a wide range of enzymes which are of considerable biotechnological importance. It was postulated that O-mannosylation is necessary for protein secretion in T.reesei.
Due to the complicated genetics of the fungus it is very difficult to study this process in T. reesei. Thus to follow an effect of O-mannosylation on the secretion of T.reesei protein CBHII (cellobiohydrolase II) containing a heavily O-mannosylated linker between the catalytic and cellulose binding domains, we have decided to use S.cerevisiae. In S.cerevisiae seven genes, PMT 1-7 ,coding for protein-O-mannosyltransferases, the first enzyme starting synthesis of O- glycosidic linkage sugars, were identified . CBHII was expressed in yeast pmt mutants and in a parental strain. Enzyme activity was detected on the plates containing medium with beta-glucan. Hydrolysis of the substrate was observed only in pmt4 mutant and in parental strain. pmt1 and pmt2 mutants bearing cbh2 gene were not exhibiting CBHII activity on the plate. CBHII protein secreted by the pmt4 mutant and the parental strain was glycosylated to the same extend what means that CBHII is not glycosylated by Pmt4p. Our results indicate that most likely Pmt1,2 protein complex is needed for CBHII O-mannosylation. If the O- mannosylation is necessary for protein secretion the protein should be accumulated in the cells of pmt1 and pmt2 mutants. However, immunodetection of CBHII in the cells free extracts of the pmt1 and pmt2 strains indicates absence of the protein in the cells. In pmt2 mutant this effect was corrected by Trichoderma pmt gene homologous to the yeast PMT2.
The latter does not confirm however direct dependence between secretion on O-mannosylation. Thus the proteolytic stability of the non glycosylated CBHII protein is also considered.

VIIIp-7

AN EFFICIENT DEGRADATION OF POLYCHLORINATED AROMATIC HYDROCARBONS BY USING MOLECULAR-GENETICALLY BRED CORIOLUS HIRSUTUS STRAINS WITH HIGH LIGNIN PEROXIDASE ACTIVITY

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_Polychlorinated aromatic pollutants have been one of the most important public concerns. Lignin-degrading enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) produced in white-rot basidiomycetous fungi are known to be involved in the degradation of polychlorinated aromatic hydrocarbons (PCAHs). So we attempted to produce Coriolus hirsutus strains with high LiP activity and examine the degradation of the PCAHs by them. The chromosome-integrating vector MIp30 carrying the C. hirsutus gpd gene promoter—Lentinula edodes priA gene terminator and the selectable marker of C. hirsutus ARG1 gene was constructed. The C. hirsutus LiP gene (lip) was fused between the promoter and terminator of MIp30 and the resulting recombinant plasmid MIp30-lip was introduced into protoplasts of monokaryotic C. hirsutus arg1 strain, followed by selection of Arg^+Lip^+ colonies. Southern-bolt analysis revealed that two of the Arg^+Lip^+ transformants, named ChTF3-1(Ch.LiP) and ChTF3-2(Ch.LiP), possess the plural number of copies (applox. 5) of the promoter—lip—terminator cassette on their chromosomes. Northern-blot analysis showed that both Arg^+Lip^+ transformants contained large amounts of lip transcripts. The mycelial cells of the transformants were cultivated in BK medium containing 25 g brewer's grains and 100 ml Kirk Basal III medium per liter and grown at 25°C. The LiP activities of the ChTF3-1(Ch.LiP) and ChTF3-2(Ch.LiP) in their culture supernatants were found to be about 5-times as high as that of the Arg^+ control. The degrading activities of PCAHs, i.e., pentachlorophenol (PCP) and 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD) were analyzed as follows. The reaction mixtures containing PCP or 2,7-DCDD and the culture supernatants were incubated for 20 hr at 25°C and the remaining PCP or 2,7-DCDD was extracted by hexane, followed by gas chromatographic analysis. The supernatants of ChTF3-1(Ch.LiP) and ChTF3-2(Ch.LiP) showed remarkably high PCP degradation activities: at the time when only 22.0 % of PCP was degraded by the control Arg^+ transformant, 80.5 % and 78.2 % of PCP were degraded by ChTF3-1(Ch.LiP) and ChTF3-2(Ch.LiP), respectively. The 2,7-DCDD degrading activities of the supernatants of ChTF3-1(Ch.LiP) and ChTF3-2(Ch.LiP) were also higher than that of the control Arg+ transformant, 80.5 % and 78.2 % of PCP were degraded by ChTF3-1(Ch.LiP) and ChTF3-2(Ch.LiP), respectively. The 2,7-DCDD degrading activities of the supernatants of ChTF3-1(Ch.LiP) and ChTF3-2(Ch.LiP) were also higher than that of the control Arg+ transformant: at the time when only 33.5 % of 2,7-DCDD was degraded by the control Arg+ transformant, 73.7 % and 63.5 % of 2,7-DCDD were degraded by ChTF3-1(Ch.LiP) and ChTF3-2(Ch.LiP), respectively.

VIIIp-8

TRANSCRIPTIONAL RESPONSES TO SECRETION STRESS IN THE FUNGI TRICHODERMA REESEI AND S. CEREVISIAE REVEAL ESSENTIAL DIFFERENCES AND COMMON FEATURES

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Trichoderma reesei is an industrial protein production host known for its exceptional protein secretion capability. This study aims at uncovering the transcriptional responses occurring in T. reesei cells exposed to secretion stress and comparing these responses to similar experiments carried out in S. cerevisiae. Secretion stress is caused by compromised protein folding or transport in the secretory pathway. It induces a number of genes involved in different aspects of secretion through the unfolded protein response (UPR) pathway. In T. reesei it has also been shown that secretion stress down-regulates genes encoding secreted proteins.

We constructed cDNA subtraction libraries and made cDNA-AFLP (amplified fragment length polymorphism) experiments from cells under secretion stress. A transformant expressing human tissue plasminogen activator (tPA), treatment with the chemical DTT (dithiothreitol) that prevents correct protein folding and a transformant over-expressing IREI protein (sensor protein of the UPR_pathway) were analysed. Around two hundred unique ESTs were retrieved by these methods and the expression pattern of about 50 was confirmed by Northern experiments. A rank sum test for the Northern data was used to define those genes that show upregulation in all the three conditions. Data from DTT and tunicamycin treatment, foreign protein production and IRE1 and HAC1 (UPR transcription factor) deletion experiments in S. cerevisiae were combined from litterature.

The transcriptional responses of T. reesei and S. cerevisiae show clear overlap, especially with respect to genes involved in protein translocation, folding and glycosylation in the ER. However, there seems to be major differences in regulation of amino acid biosynthesis and nucleosome genes. The GCN4/CPC1 transcription factor and a limited set of its putative target genes are induced only in T. reesei. This response points to the upregulation of glutathione synthesis to relieve oxidative stress caused by compromised protein folding. Interestingly also a set of nucleosome genes is upregulated in T. reesei without a clear connection to cell cycle.

PLECTASIN: INDENTIFICATION AND RECOMINANT PRODUCTION OF THE FIRST DEFENSIN FOUND IN FUNGI.

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Defensins are antimicrobial peptides widely found in vertebrates, invertebrates, plants and mammals. These peptide antibiotics play an important role in the innate immune defence including that of humans. They contain a distinct pattern of disulphide bridges and generally have a basic pI. We describe the identification and heterologous expression of the first microbial defensin. Transposon assisted signal trapping (TAST) was employed to quickly identify the secreted protein diversity from a cDNA library made from the ascomycete Pseudoplectania nigrella. Among the hits identified was a protein with structural similarity to definsins which we named "Plectasin". The mature peptide was used to search for homology in all public sequence databases. All 6 structurally important cysteines are conserved and the overall sequence homology ranges from 40% to 60% to previously identified animal defensins.

Purified Plectasin was tested against a range of bacteria. Generally, Plectasin was active against Gram-positive species, including Bacillus subtilis, Enterococcus faecalis, Micrococcus luteus, Streptococcus pneumoniae, Staphylococcus epidermidis and Staphylococcus aureus.

The cost of chemical peptide synthesis and controlled oxidative refolding of defensin molecules has been a major obstacle for the exploitation of these molecules as potential drugs. Therefore we cloned the Plectasin encoding gene in a microbial production host. By one step hydrophobic charge induced chromatography, we were able to recover grams of purified Plectasin. This result is promising for the use of these complex molecules in research and pharma industry.

CLONING AND CHARACTERIZATION OF A NEW LACCASE FROM THE WHITE-ROT FUNGUS *Pleurotus eryngii*

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White-rot fungi from the genus *Pleurotus* are being studied for both the development of new environmental friendly processes for paper manufacture and the design of bioremediation strategies for the removal of aromatic xenobiotics from waste-waters and contaminated soils. These studies are based respectively on the particular ability of *Pleurotus* species to degrade lignin from wheat straw, showing limited attack to cellulose fibers, and the wide substrate specificity of the ligninolytic enzymes, including laccases, versatile peroxidases (VP) and aryl-alcohol oxidase (AAO). The molecular characterization of VP and AAO from *P. eryngii* have been carried out and structure-function studies with these enzymes are in course. In this work we performed a molecular characterization of laccases secreted by this fungus since it has been recently reported that they are able to degrade *in vitro* 2,4-dichorophenol and benzo(a)pyrene as models of phenolic and non-phenolic aromatic recalcitrant compounds.

One laccase DNA probe was obtained by PCR using as primers degenerated oligonucleotides corresponding to conserved sequences in other fungal laccases. This probe, around 550 bp, was used for screening the *P. eryngii* genomic library, previously built in λEMBL3. The DNA of positive clones was analyzed and transferred to *pBluescript*. The cDNA sequence for this protein encodes a new *P. eryngii* laccase (N-terminal different to those reported for other laccases isolated in this fungus). A molecular model for this protein has been obtained using as template the structure of two fungal laccases recently crystallized, and the SWISS-MODEL program. The isolation and biochemical characterization of this new enzyme is in progress.

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HETEROLOGOUS EXPRESSION OF A LACCASE GENE FROM THE ASCOMYCETE MELANOCARPUS ALBOMYCES IN TWO FUNGAL HOSTS

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Laccases belong to the family of multicopper oxidases and they oxidise phenolic compounds with broad substrate specificity. A large variety of filamentous fungi have been shown to produce laccases, and these enzymes have been implicated in e.g. degradation of lignin, pigment formation, sporulation and plant pathogenesis. The best characterised laccases are those of the bacidiomycetous white-rot fungi. We have characterised a laccase enzyme from the ascomycete Melanocarpus albomyces and shown that it has very interesting technical properties, good thermostability and a neutral pH optimum. The three-dimensional structure of this laccase was solved and the gene encoding it was sequenced. These studies revealed some interesting points including N- and C-terminal processing of the protein and protrusion of the four C-terminal amino acids inside the protein near the active site.

The M. albomyces laccase was expressed in *Saccharomyces cerevisiae* and *Trichoderma reesei*. Production in yeast yielded up to 3 mg/l of the enzyme, an expression level well adequate for protein engineering purposes. Both the use of a yeast prepro-sequence for expression and the truncation of the expression construct at the natural C-terminal cleavage site markedly improved the production level. When the laccase cDNA was expressed in Trichoderma reesei under the cbh1 (cellobiohydrolase I) promoter, laccase production of more than 200 mg/l was obtained in shake flask cultivations. Fermentor cultivations have resulted in still higher laccase levels. The recombinant enzyme was purified and shown to have very similar biochemical and technical properties as the one produced by the native host. Northern analysis of the T. reesei transformants producing laccase suggested that expression of this laccase does not cause secretion stress to the host cells.

VIIIp-12

PROTEIN PRODUCTION AND UNFOLDED PROTEIN RESPONSE IN FERMENTATIONS OF TRICHODERMA REESEI AND ITS TRANSFORMANT EXPRESSING ENDOGLUCANASE I WITH A HYDROPHOBIC TAG

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The effect of induction of protein production was studied in bioreactor cultures of the T. reesei strain Rut-C30 and its transformant expressing endoglucanase I (EGI, Cel7B) fused with a hydrophobic tag. The peptide tag was previously designed for efficient purification of the fusion protein in aqueous two-phase separation. The first phase of the bioreactor cultivations was carried out on glucose containing minimal medium. At the stage when glucose was nearly depleted, the medium was supplemented with rich medium containing lactose as a carbon source to induce production of cellulases. The transformant produced somewhat less secreted protein and cellobiohydrolase I (CBHI, Cel7A) activity than the parental strain. Western analysis of intracellular proteins showed that the fusion protein $EGI_{core\text{-}P5}(WP)_4$ accumulated inside the cell, indicating impaired secretion of the protein. Two-dimensional gel analysis suggested that the fusion protein was possibly trapped early in the secretory pathway. The mRNA levels of the UPR (unfolded protein response) target genes, bip1 and pdi1, and the level of the activated hac1 transcript encoding the UPR transcription factor, increased at the same time with an increase in the transcript levels of cellulase genes, suggesting UPR activation in response to cellulase induction. However, only a minor increase in *pdi1* and bip1 transcript level was observed in the transformant expressing the fusion protein compared to its parental strain. In addition, slightly lower CBHI production and cbh1 mRNA levels were measured in the transformant as compared to the parental strain, indicating activation of the novel repression mechanism of genes encoding secreted proteins in response to secretion stress, RESS (repression under secretion stress).

CODON OPTIMIZATION IMPROVES EXPRESSION OF RECOMBINANT MITE ALLERGEN, DER F7, IN ASPERGILLUS ORYZAE

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Aspergillus fungi have attracted considerable attention as promising hosts for large-scale production of proteins; however, in most cases the secreted yields of heterologous proteins were much lower than those of homologous proteins. In order to overcome these disappointed results, we first investigated the effect of codon optimization on the expression and production of heterologous proteins in Aspergillus oryzae using a major mite allergen, Der f7, as a model protein.

We synthesized a codon-optimized der f7 gene according to the preferential codon usage of A. oryzae, and then inserted it downstream of the high-level expression promoter, Pgla142. Whereas no detectable signal was observed in the transformant containing a native der f7 construct by western analysis, two discrete signals corresponding to glycosylated and non-glycosylated Der f7 were detected in that with a codon-optimized construct. Northern analysis revealed the significant difference in the amount of the der f7 transcript between the both transformants, suggesting that codon optimization of the heterologous der f7 improves the transcription efficiency and/or stability of the mRNA and results in a significant increase in the yield of Der f7. In addition, improvement of the Der f7 yield by codon optimization was also demonstrated in a fusion protein, in which Der f7 was fused to a homologous secreted protein through a KEX2 cleavage site. Finally, we constructed the transformant strains that had multiple copies of thus codon-optimized fusion gene and achieved the secreted Der f7 yield more than 100-fold compared with the transformant containing a native der f7 fusion gene.

Investigation of network topology and quantification of fluxes in central carbon metabolism of *Aspergillus nidulans* under different conditions of glucose repression

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Aspergilli are producers of industrially relevant products, namely organic acids (e.g. citric acid, *A. niger*) and enzymes (e.g. a-amylase, *A. oryzae*), as well as polyketides (e.g. statins, *A. terreus*). In large-scale production, it is common to use complex sugar mixtures as substrate and a major problem encountered is connected to the occurrence of carbon repression. The effect of this regulatory mechanism is that readily metabolizable carbohydrates (such as glucose) repress the synthesis of enzymes related to catabolism of alternative carbon sources ensuring preferential utilization of the most favored carbon source present in the medium. In *A. nidulans* and *A. niger*, carbon repression is mediated by the protein CREA [1]. Our work was focused on the phenotypic investigation of *A. nidulans* cells grown under different conditions of glucose repression, through the quantification of fluxes in their central carbon metabolism. The approach adopted in this study was metabolic flux analysis based on stationary carbon isotope labelling experiments, using fractional enrichment data [2]. Carbon labelling experiments were performed using a reference strain and a derepressed mutant (creAΔ4), grown on glucose. In addition, the mutant cells were grown on a mixture of glucose and xylose, which is also a strongly repressing carbon source [1], although to a smaller extent than glucose. A metabolic model comprising all relevant biochemical conversions and transport processes as well as the fate of every carbon atom throughout the metabolic network of *A. nidulans* was developed, based on a metabolic reconstruction for *A. niger* [3]. Fractional enrichment data and measurements of extracellular rates, as well as information on the biomass composition of the fungus, were then combined with the metabolic model for the identification of network topology and estimation of *in vivo* fluxes in the central carbon metabolism of *A. nidulans*.

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VIIIp-15

IDENTIFICATION OF A THERMOTOLERANT FUNGAL STRAIN AND INDUCING PATTERN OF ITS LACCASES

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Laccases are copper-containing enzymes that catalyze the oxidation of phenolic substrates and couple this to the reduction of oxygen to water. These enzymes have found novel biotechnological applications that have led the search for new laccase sources in nature. Fungal strain EUM-1, previously isolated from the tropic in Southern Mexico has shown a thermotolerant phenotype. The present work describes the identification of this strain by means of the analysis of Internal Transcribed Spacer (ITS) sequences amplified by PCR and their comparison with other ITS fungal sequences. These studies assigned EUM-1 to the Polyporaceae family belonging to group II within Trametes. Thus our strain is now identified as Trametes sp. EUM-1.
On the other hand, using defined Kirk medium, a group of 10 compounds were tested as possible inducers of laccases by Trametes sp. EUM-1. The effect of two incubation temperatures (28 and 39ºC) was also studied. In a primary selection on Petri dishes containing 2,2-azinobis-[3-ethylbenzthiazoline-6-sulfonate] (ABTS), only 2 out of 10 compounds proved to be real inducers: Ferulic Acid (FA) and Veratric Acid (VA), since they triggered bigger oxidation halos than control medium. After 7 days in liquid cultures at 28°C, laccase activity was 4 times higher in medium containing FA than in control medium, 1.2 and 0.3 U/L, respectively. There was not a substantial change in laccase production throughout the time after 30 days. Interestingly, at 39°C a continuous increase in laccase activity was determined for both FA containing and control media, reaching 18 and 16 U/L after 30 days, respectively, which represented enzymatic levels greater in one order of magnitude than those values observed at 28°C. In addition, crude extracts obtained at 39°C retained at least 85% of total laccase activity after incubation for 1 hour at 60°C. However, VA did not show any inducer character for laccases under the assayed conditions, which could be related to a toxic effect since this compound decreased fungal growth measured as radial extension rate on Petri dishes.
These results are in agreement with some reports describing that inducers can significantly change their effects according to the fungal strains. Additionally, it would be remarkable to elucidate the inducing mechanism of laccases by temperature linked to the production of thermostable enzymes in this strain.

VIIIp-16

A NOVEL EXPRESSION SYSTEM FOR RECOMBINANT PROTEIN PRODUCTION IN MUCOR CIRCINELLOIDES

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With the identification and characterization of strong and regulated promoters, the development of dominant selective markers, reporters and transformation procedures to work in Mucor circinelloides we have added significantly to the genetic tools available to work in this fungus. In collaboration with others, we have shown the involvement of the cAMP-dependent protein kinase A in the control of morphology and branching in M. circinelloides. The expression tools developed have also been used in other zygomycetes for the expression of genes.

We are using these genetic tools to evaluate the potential use of M. circinelloides for recombinant protein production. Current production levels for a model protein (the Aspergillus niger glucose oxidase (GOX)) are over 100 mg/L of secreted active protein. These levels have been obtained using strains with an integrated single copy of the expression cassette using the native GOX signal peptide.

Among other proteins produced in M. circinelloides to date are secreted fungal enzymes but also a rat GPCR receptor that is made as a glucoamylase fusion protein and is correctly processed.

An overview of the expression system, the challenges ahead (genetic control of morphology, fermentation technology, homologous integration for knock-out construction, novel markers, etc) and the strategies currently being used will be presented.

A method for creating transgenic fungal strains that lacks antibiotic genes for potential future commercial use.

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We have created a system for transformation of filamentous fungi lacking antibiotic genes in the final transgenic strains. Antibiotic genes are very convenient and often used for selection of transgenic strains and those are not needed in the final transgenic product. One of the concerns for future approval of transgenic organisms is the possibility of genomic exchange of genes from one microorganism to another. Potential and unwanted spread of antibiotic genes must be avoided by not having these genes in the final transgenic organisms. This transformation system may have potential for future use of genetically modified fungal products.

TRANSFORMATION OF THE CLAVARIC ACID PRODUCER BASIDIOMYCETE HYPHOLOMA SUBLATERITIUM BASED IN THE T-DNA TRANSFER AND INTEGRATION

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Hypholoma sublateritium is a basidiomycete that produces clavaric acid, an antitumour isoprenoid molecule inhibiting the farnesylation of the RAS protein, an event essential for the development of tumoral cells.
An effective transformation technique is an useful tool in order to characterize H. sublateritium genes and to elucidate the biosynthetic pathway of clavaric acid. Several methods such as PEG-mediated protoplast transformation and electroporation are used in different filamentous fungi; however, none of these methods showed to be effective due to the low yield and pour viability of Hypholoma sublateritium protoplasts.
Arthrospores of this fungus were successfully transformed to hygromycin B resistance by Agrobacterium tumefaciens-mediated transformation. Five different promoter sequences that control the expression of the E. coli hph (hygromycin phosphotransferase) gene were tested. Among the heterologous promoting sequences only one, carrying the Agaricus bisporus gpd promoter, proved to be useful. Three promoters from ascomycete fungi were ineffective to express the hygromycin resistance marker following A. tumefaciens-mediated transformation, revealing the critical importance of the chosen promoter, in order to express heterologous genes in the basidiomycete H. sublateritium. The actin (act) gene promoter of H. sublateritium has been cloned and is being developped as an homologous promoter for constitutive gene expression. Hygromycin B resistant clones showed a ramdom integration pattern and both, single- and multiple-copy integrations, were observed. All the analyzed transformants were mitotically stable and maintained the integrated T-DNA in absence of antibiotic.

VIIIp-19

ENDOPLASMIC RETICULUM STRESS LEADS TO THE SELECTIVE TRANSCRIPTIONAL DOWN-REGULATION OF THE GLUCOAMYLASE GENE IN ASPERGILLUS NIGER.

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We describe a new endoplasmic reticulum-associated stress response in the filamentous fungus Aspergillus niger. The inhibition of protein folding within the endoplasmic reticulum (ER) leads to cellular responses known collectively as the unfolded protein response (UPR) and we show that the selective transcriptional down-regulation of the gene encoding glucoamylase, a secreted protein, but not non-secreted proteins, is an additional consequence of ER stress. The inhibition of protein folding in the ER can be induced in a variety of ways. We have examined the effects of dithiothreitol (DTT), a reducing agent that causes the formation of unfolded proteins and have used antisense technology to lower the level of protein disulfide isomerase (PDI) in the ER of A. niger. We show that both approaches cause the down-regulation of transcription in genes encoding secreted glucoamylase and also aspergillopepsin but not genes encoding the non-secreted proteins γ-actin and glyceraldehyde 3' phosphate dehydrogenase. The DTT-treated fungal cells also show evidence for the induction of the UPR because expression of bipA and pdiA, encoding an ER-resident chaperone and foldase respectively, are up-regulated and splicing of hacA, the gene encoding the transcription factor responsible for induction of the UPR, occurs, allowing the production of an active protein. This response is not evident in the pdiA antisense strains, suggesting that the transcriptional down-regulation mechanism is controlled differently to the UPR. An analysis of the promoter of the glucoamylase gene using truncated glaA promoters to drive the _-glucuronidase reporter gene has shown that the down regulation effect is attenuated with a promoter length of 1kb but that the glaA promoter of 2kb exhibited the effect, suggesting that the motif(s) which mediate the response are situated within the region 1-2kb from the ATG.

VIIIp-20

Investigating the function of Six1, a small cysteine-rich protein secreted by

Fusarium oxysporum f.sp. lycopersici during infection of tomato

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Fusarium oxysporum f.sp. lycopersici (Fol), is a soil inhabiting fungus that can infect tomato plants via the roots and colonise the xylem. In xylem vessels Fol secretes a 12 kD, cysteine rich protein (Six1) which appears to be derived from a 30 kD precursor through proteolytic processing by either plant or fungal proteases. The SIX1 gene is required for resistance of tomato plants carrying the I-3 resistance gene against Fol. Apart from its apparent role in I-3 mediated resistance, nothing is known about the function of Six1. To find clues for a possible function, we are investigating how expression of SIX1 is regulated. SIX1 is expressed in planta, but not during growth in media that were designed to mimic the composition of xylem sap or in xylem sap itself. To monitor the induction of expression in planta we are currently constructing SIX1 promoter-GFP fusion genes. To investigate a possible elicitor activity of Six1, analyse its proteolytic processing, and find interacting proteins, we are producing the Six1 protein in Pichia pastoris.

Glycosylation as a tool for improved protein production in *Aspergillus niger*

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Production of heterologous proteins in *Aspergilli* results often in disappointing low yields. It is clear that secretion of the protein often is the bottleneck. To overcome this bottleneck in production of chymosin by an industrial *Aspergillus* strain we have increased the level of glycosylation of the chymosin molecule by a single amino acid change. This resulted in a significant increase in chymosin production.
In order to avoid changing the chymosin molecule we also developed an alternative strategy in which a small linker sequence, encoding an N-glycosylation site, was inserted between the secretion carrier *glaA* and prochymosin. In this case increased chymosin production levels were achieved, without any change to the final product.
Summarizing, we have shown that increasing glycosylation is an efficient way to overcome some of the bottlenecks in the secretion of heterologous proteins.

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INFLUENCE OF ACE2 ON THE EXPRESSION OF THE XYLANOLYTIC ACTIVITY OF *TRICHODERMA REESEI ON DIFFERENT CARBON SOUCES.*

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The xylanase system of the filamentous fungus *Trichoderma reesei* consists of two specific xylanases, Xyn1 and Xyn2, which are simultaneously expressed during growth on xylan but respond differentially to low molecular weight inducers. We have recently demonstrated that a deletion within the xylanase-activating element in the xyn2 promoter (XAE) completely eliminated binding of the cellulase and xylanase regulator Ace2 and thereby fully abolished transcription of xyn2 under both conditions.

In the presented study we made use of an Ace2 deletion strain to elucidate the influence of this factor on the transcriptional regulation of xyn1 and xyn2 gene expression. The parental strain T. reesei QM 9414 and the deletion strain were cultivated under non inducing (glucose) and inducing (xylan, cellulose) conditions. Thereafter the corresponding enzyme activities were detected and respective mRNA levels were quantified via Real Time PCR.

In contrast to the results obtained from the deletions in XAE of xyn2 the affects of the Ace2 deletion are less pronounced. These findings give strong indications for an additional factor contacting XAE thereby mediating expression of xyn2. The pattern of mRNA formation of xyn1 does not show significant changes comparing these two strains.

VIIIp-23

EXPRESSION OF RECOMBINANT PAF (PENICILLIUM ANTIFUNGAL PROTEIN) IN PENICILLIUM CHRYSOGENUM

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The filamentous fungus Penicillium chrysogenum secretes the protein PAF which is small, highly basic and cysteine rich. This protein exhibits antimicrobial activity towards a variety of filamentous fungi, e.g. opportunistic human pathogenic (Aspergillus niger, A. fumigatus) and plant pathogenic molds (Botrytis cinerea)[1].
In order to produce a recombinant histidine-tagged protein we use Penicillium chrysogenum itself as the expression system, where paf mRNA synthesis is xylose-inducible and the secretion of the protein occurs into the supernatant because of an intact prepro-sequence. The recombinant protein ist tagged with 6 histidine residues which facilitates the protein purification and which also allows to distinguish between the recombinant and the endogenous protein.
In consequence we want to perform a mutational analysis of the recombinant PAF in order to analyse the relation of the amino acid sequence and the structure with the antifungal activity of the protein. The results will help to improve our understanding of the growth inhibitory proterties of PAF and of antifungal proteins in common.
This work was supported by the Austrian science Foundation, grant FWF P15261.

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VIIIp-24

INCREASED ANTIFUNGAL AND CHITINASE SPECIFIC ACTIVITIES OF Trichoderma harzianum CECT 2413 BY ADDITION OF A CELLULOSE BINDING DOMAIN.

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Trichoderma harzianum is a widely distributed soil fungus that antagonizes numerous fungal phytopathogens. The antagonism of *T. harzianum* usually correlates with the production of antifungal activities including the secretion of fungal cell walls degrading enzymes such as chitinases. Chitinases Chit42 and Chit33 from *T. harzianum* CECT 2413, which lack a chitin-binding domain, are considered to play an important role in the biocontrol activity of this strain against plant pathogens. Hybrid chitinases Chit33-CBD and Chit42-CBD with stronger chitin-binding capacity than the native chitinases have been engineered by adding to these enzymes a cellulose-binding domain (CBD) from cellobiohydrolase II of *Trichoderma reesei*. Transformants that overexpressed the native chitinases displayed higher levels of chitinase specific activity and were more effective at inhibiting the growth of *Rhizoctonia solani*, *Botrytis cinerea* and *Phytophthora citrophthora* than the wild type. Transformants that overexpressed the chimeric chitinases possessed the highest specific chitinase and antifungal activities. Results confirmed the importance of these endochitinases in the antagonistic activity of *T. harzianum* strains, and demonstrate the efectiveness of adding a celllulose-binding domain (CBD) to increase hydrolytic activity towards insoluble substrates such as chitin-rich fungal cell walls.

VIIIp-25

THE CONSTRUCTION AND EXPRESSION OF THE EGIII EXPRESSION CASSETTE IN FILAMENTOUS FUNGUS TRICHODERMA REESEI

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The filamentous fungi *Trichoderma reesei* is known as an efficient producer of cellulase. According to the reports by literature, the amounts of heterogonous proteins can increase in protease-deficient strains. To promote the heterogonous proteins production by *Trichoderma reesei*, EMS mutagenesis of *Trichoderma reesei* Rut C30 was carried out and one protease-deficient strain *Rut* C30M3 was obtained. The protease activity of *Rut* C30M3 decreased about 70% than that of Rut C30, but the cellulase activities were not affected. So it is reasonable to take *Rut* C30M3 as the host strain to increase heterologous proteins production.

The strong promoter and terminator of cellobiohydrolase I (CBHI) gene from *Trichoderma reesei* 9414 were isolated by PCR technology and the expression vector pTRIL was constructed by inserting multiple cloning sites between the promoter and terminator with pUC19 as backbone. To confirm the usefulness of pTRIL, the DNA fragment encoding hygromycin phosphotransferase (*hph*) gene conferring resistance to the antibiotic hygromycin B was inserted into XhoI and SalI sites of pTRIL resulted the recombinant pRIL-hph. The pTRIL-hph containing the Pcbh1-*hph*-Tcbh1 expression cassette was introduced into the strain Rut C30 by protoplast transformation. 15 transformants were isolated in minimal medium plates contained 100μg /ml of hygromycin B. The hygromycin-resistant transformant H1 was analyzed by PCR and Southern analysis. The results clearly show that hph gene has been integrated into the chromosome DNA of H1 and expressed under the control of Pcbh1. The hygromycin B resistance of *T. reesei* H1 was 150μg/ml, which was two times higher than that of Rut C30. The construction of pTRIL is beneficial to molecular biological research on filamentous fungi and genetic modification of *T. reesei*.

To construct the expression cassette Pcbh1-eg3-Tcbh1, the eg3 gene was amplified by PCR and inserted into pTRIL between the Pcbh1 and Tcbh1 fragments, creating recombinant plasmid pTRIL-eg3. The *Trichoderma reesei* protease-deficient strain *Rut*C30M3 was co-transformed with the P*cbh*1-*eg*3-T*cbh*1 expression cassette and the plasmid pAN7-1. And 35 transformants were obtained. The special PCR amplifications with cbh1 gene primers were carried out to identify the homologous integration transformants. 2 transformants named L13 and L15 were selected as the potential cbh1 site-directed integration strains. Dot blot analysis with cbh1 fragment as probe and Western blot immunity analysis with cbh1 antibody were carried out. The results indicated that the expression Pcbh1-eg3-Tcbh1 cassette was integrated into the cbh1 site of T.reesei chromosomal DNA.

Meanwhile, the filter paper activity and CMC activity assay of L13 and L29 transformants were carried out. The results indicated that the filter paper activity of transformant L13 was reduced about 40% and the CMC activity was increased 20% than that of *Rut*C30M3.

VIIIp-26

Analysis of the Fusarium venenatum Glucoamylase Promoter by Deletion, Insertion, and Substitution Analyses.

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Filamentous fungi have the capacity to produce and secrete high levels (grams per liter) of extracellular enzymes into their environment which makes them suitable hosts for the production of industrial enzymes. Understanding fungal gene expression is essential in order to devise effective methods to increase the production of homologous and heterologous proteins. One level of control of the expression of fungal genes is transcription. This study focused on the analysis of the F. venenatum glucoamylase (gla) promoter and promoter variants. A site specific integration system at the niaD locus was developed to allow analysis of lipase expression when the expression cassette was integrated in single copy. Using this site-specific integration system, the study evaluated the importance of different promoter elements within the F. venenatum gla promoter by analyzing deletions, insertions, and substitutions generated by site-directed mutagenesis. The Thermocyces lanuginosus lipase gene was used as a reporter to determine the promoter strength of the variants. A comparison of the nucleotide sequence of the F. venenatum gla promoter with the promoters of Aspergillus amylase genes indicated that there were two putative conserved sequences designated Regions IIIa and IIIb located at -158 and -134 relative to the start codon, respectively. The deletion of the CGG triplet at -158 to -156 resulted in a significant decrease in lipase activity. Likewise, the deletion of the sequence of CCAATGAGGGC (-134 to -124) designated Region IIIb resulted in a significant decrease of lipase activity. This suggests that the CGG triplet and the putative Region IIIb are important for high-level expression from the gla promoter. Interestingly, the addition of the consensus sequence AAATTTAA commonly found in the Region IIIa of Aspergillus amylase genes led to a significant reduction of lipase activity, suggesting the Aspergillus consensus is not optimal for expression from the F. venenatum gla promoter. The introduction of the CGG triplet at position –147 to -145 which produced a CGG direct repeat Region IIIa of CGGCGTAATTTCGGCC significantly enhanced lipase activity by about three-fold. The introduction of a second putative Region IIIa with a CGG direct repeat at position –108 to –93 yielded a six-fold increase of lipase activity. Northern analysis showed that the differences in lipase activity between the promoter variants correlated very well with the relative amount of lipase mRNA.

VIIIp-27

EXPRESSION AND CHARACTERISATION OF THE EXOPOLYGALACTURONASE PGX1 CODING GENE OF Fusarium oxysporum f. sp. radicis lycopersici IN Pichia pastoris

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Polygalacturonases of pathogenic fungi are considered to be actively involved in the process of infection and symptom production in host plants. The relative importance of their role in pathogenesis is probably determined by their structural and functional features of the PG enzymes as well as the coordination of their individual expression pattern during the infection process.

Four polygalacturonase genes, encoding two ENDOPGs and two EXOPGs (pgx1 and pgx2) are present in a pathogenic strain of Fusarium oxysporum f. sp. radicis lycopersici (FORL), which produces root rot disease in tomato plants. Due to the difficulty to obtain individual PGs from in vitro fungal cultures, the use of heterologous systems to express PG coding genes represent a feasible approach to produce and facilitate the purification of PGs. In these work, we have cloned the pgx1 gene in the P. pastoris expression vector pPIC?a, in order to obtain PGX1. We have obtained several clones, which showed the extracellular production of the enzyme with PG activity. The physico-chemical features of this PGX1 have been analysed, including optimal pH (5.5) and temperature (45ºC) and have been compared and discussed in relation to the other EXOPG (PGX2), which were obtained from in vitro cultures of F. oxysporum f.sp. radicis lycopersici.

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VIIIp-28

***ASPERGILLUS SOJAE*, A NEW SYSTEM FOR EFFICIENT PROTEIN PRODUCTION**

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Aspergillus species are known as high-level expression hosts for the production of enzymes or metabolites. In the last two decades for the industrially used species for *Aspergillus niger, A. foetidus, A. tubigensis,* and *A. oryzae* expression systems have been developed. We describe the development of an expression system based on the koji mold *Aspergillus sojae* for the production of homologous and heterologous proteins. Transformation based on auxotrophic (*pyrG, niaD*) and/or dominant markers (*amdS*), was used to introduce the genes of interest. To improve the yield of produced protein, *Aspergillus sojae* mutants with lower protease activity, either by gene disruption or by UV mutagenesis, were isolated. To improve the fermentation yields of *Aspergillus sojae* also morphological and so-called fermentor adapted mutants with lower viscosity, were isolated. As one of the examples for heterologous protein production the production of human interleukin 6 was used.

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FLUORESCENT MARKER TRANSFORMATION OF TRICHODERMA VIRENS I10 DOES NOT ALTER MYCOPARASITIC ACTIVITY AGAINST SCLEROTIUM ROLFSII, SCLEROTINIA SCLEROTIORUM AND SCLEROTINIA MINOR.

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Mycoparasitic ability of an antagonistic isolate (I10) of Trichoderma virens previously co-transformed by the plasmid vectors pPgpd-DsRed and gGFP was evaluated. The first vector contains the DsRed-Express gene (Clontech) and the second the Green Fluorescent Protein (GFP) gene together with a gene for hygromycin resistance. Expression of all three genes was controlled by the constitutive Aspergillus nidulans glyceraldehyde-3-phosphate (gpd) promoter. Co-expression of both fluorescent markers was clearly evident in the same mycelium under the fluorescent microscope. The effects of the dual transformation on mycoparasitic ability of T. virens I10 was evaluated by incubating sclerotia of Sclerotium rolfsii, Sclerotinia sclerotiorum and S. minor in 24 wells microplates inoculated with transformed or wild type isolates. Four sclerotia were sown in each well and a row of six wells was considered as a replicate. 4 x 24 sclerotia were tested for each isolate. After 7 (S. minor), 11 (S. rolfsii) and 13 (S. sclerotiorum) days of incubation, firmness of sclerotia was evaluated. On the average, T. virens I10 demonstrated a good decaying ability of sclerotia of S rolfsii (48.3% decayed sclerotia) and S. minor (48.9%) while S. sclerotiorum (16.6%) was more resistant, probably because of the larger size of its sclerotia. Within each pathogen the dual marked transformant gave percentages of decayed sclerotia not significantly different compared to the wt. Results suggest that transformation using GFP and/or DsRed marker genes could be useful to monitor the behaviour of mycoparasitic T. virens isolates.

TRANSCRIPTIONAL AND PROTEOMIC CHANGES IN TRICHODERMA REESEI EXPRESSING A HETEROLOGOUS BACTERIAL XYNB GENE FROM THE THERMOPHILE DICTYOGLOMUS THERMOPHILUM

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We have taken a holistic approach into analysis of heterologous gene expression in Trichoderma involving enzyme activity measurements, gene transcription assays, zymogram gels, Western blotting and proteomics. The T. reesei transformant expressing the bacterial xynB gene encoding a thermophile xylanase was grown in a laboratory fermenter and assays were performed on culture supernatants collected from different time points. Transcription of the xynB under the T. reesei cbh1 (cellobiohydrolase 1) promoter was comparable to that of the native cbh1, however, the yield of the heterologous xylanase produced in this particular transformant was no more than about 100 mg/l. Transcription analysis of selected genes involved in UPR (unfolded protein response) indicated a slight increase in the expression of pdi (protein disulfide isomerase) but otherwise the UPR pathway seemed not have been induced. Comparative proteomic analysis of the culture supernatant from the non-transformed host and the transformant expressing thermophilic xylanase showed differential expression of 23 proteins. Analysis of these spots is underway. This multifaceted approach may reveal crucial proteins involved in heterologous gene expression in filamentous fungi in general and Trichoderma in particular.

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VIIIp-31

EFFECT OF bipA GENE ATTENUATION ON HOMOLOGOUS AND HETEROLOGOUS PROTEIN SECRETION IN Aspergillus awamori.

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The BiPA protein is a molecular chaperone that contains an HDEL targeting signal and is present in the endoplasmic reticulum (ER). BiPA plays an important role in the protein traffic process by binding to the newly synthesized polypeptides and promoting their proper folding. It also binds to aberrant proteins preventing them from leaving the ER to continue through the secretory pathway; i.e, it exerts a “quality control” discriminating between properly folded proteins to be exported (secretion competent) and inadequately folded ones.

The effect of different chaperone levels on heterologous protein production has been studied in different organisms. However, results seem to be different and even contradictory depending on the organism and proteins studied.

In the filamentous fungus Aspergillus awamori, it has been shown that increasing the levels of BiPA has a strong effect on the production of the heterologous sweet protein thaumatin, but it has little effect on homologous protein secretion suggesting the existence of different limiting factors in the secretion of each protein analysed (M. Lombraña, F. Moralejo, R. Pinto and J.F. Martín, submitted for publication).

The requirement of BiPA for folding and secretion of thaumatin in the overproducing A. awamori TGP-3 strain was confirmed by attenuation of the endogenous bipA gene expression using an antisense RNA cassette. The decrease in bipA expression reduced significantly the amount of secreted thaumatin without affecting the secretion of homologous alpha-amylase and glucoamylase proteins.

BiPA-assisted folding is therefore very important for secretion of some heterologous proteins such as thaumatin in A. awamori.

VIIIp-32

USING INSERTIONAL MUTAGENESIS TO IMPROVE THE PRODUCTION OF CELLULOLYTIC ENZYMES IN *TRICHODERMA REESEI*

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Insertional mutagenesis has been used extensively in many organisms to link phenotypes with specific genotypic alterations. An insertional mutagenesis method for use in *Trichoderma reesei* was developed. Use of this method allowed the isolation of large numbers of stable transformants expressing the selectable marker. Using Southern analysis, integration of the marker was shown to occur by nonhomologous recombination and at a low copy number. Large mutant libraries were screened using selective plate screens allowing the direct selection of mutants with improved cellulase production. Improved strains were examined in shake flask and fermentors. Several strains were found to have increased yield and/or productivity.

THERMAL STABILITY OF CBH FROM GLYCOSYL HYDROLASE CEL7 FAMILY MEMBERS AND VARIANTS

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Genencor International has been working under a subcontract from the Office of Biomass Program, within the DOE Office of Energy Efficiency and Renewable Energy, for cellulase cost reduction for biomass conversion to fermentable sugars. The goal of this three-year program was to reduce the cost of cellulase by ten-fold. Reaching this aggressive target needs improvements in both the production, and in the specific performance, of the cellulase mixture. This has required a large, multi-disciplinary and integrated research effort, involving workers in Europe and the USA, focused on improved production of improved cellulases. Biomass conversion is performed in nature by a complex mixture of cellulolytic enzymes. Within the cellulase mixture produced by *Trichoderma reesei,* CBHI (*Hypocrea jecorina* Cel7A) is ~50% of the secreted protein and is an essential enzyme for cellulose degradation. To improve this molecule, a program has been started to identify mutants with changes in activity and in thermal stability. In addition, many previously identified CBHI homologs have been cloned, sequenced and expressed. Genes have been obtained by either specific or homologous cloning approach, using *Aspergillus niger var. nidulans* as a heterologous expression host. The enzymes have been purified and the thermal stability has been was evaluated by circular dichroism.

GPI-ANCHORED CELL SURFACE PROTEIN OF ASPERGILLUS KAWACHII

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The cell wall of a microbe is in contact with the environment and contains many proteins involved in the incorporation of external information or materials, intercellular recognition and other functions. The cell wall also has an important role in practical applications such as brewing or fermentation, because the cell wall has direct access to the cultivation medium. However, there is little knowledge of the cell wall proteins of filamentous fungi such as Aspergillus. We obtained four candidates of GPI (glycosylphosphatidylinositol)-anchored cell surface proteins (presumed mannnoprotein: CwpA and CwpB, presumed phosphatase: PhoB and PhoC) from white koji mold (A. kawachii IFO4308) and analyzed their functional characteristics.

CwpA and CwpB were obtained incidentally in the process of cellulase cloning, because these proteins were T/S rich and cross-hybridized with the linker region of cellulase. As signal peptides were recognized in the N-terminus of these proteins and the hydrophobic regions were also recognized in the C-terminus, these proteins were considered as GPI-anchored cell surface proteins.

In order to examine the localization in more detail, HA-Tag was added to protein CwpA. After the fractionation of mycelia and cultivation into cell wall, membrane and secreted-protein fractions, the localization of HA-CwpA fusion protein was analyzed, and HA-CwpA was found to exist in the membrane fraction. Protein HA-CwpA was liberated by PIPLC (phosphatidylinositol specific phospholipase C) treatment. Next, a stop-codon was inserted artificially in front of the GPI-signal to examine the changes in localization, and it was shown that this truncated protein was secreted into the medium. These results show that CwpA is a GPI-anchored cell-surface protein, and is anchored to the cell membrane but not to the cell wall. In Saccharomyces cerevisiae, two types of GPI-anchored cell surface protein are known; viz., the cell membrane-anchored protein (e.g. Gas1) and the cell wall-anchored cell-surface protein (e.g. Sed1). CwpA of A.kawachii is considered to be a Gas1-like cell wall-anchored cell-surface protein. We are now analyzing the location of the other candidates and their functions.

VIIIp-35

EXPRESSION AND SECRETION OF MULTIPLE FORMS OF THE DICTYOGLOMUS THERMOPHILUM XYLANASE B (XYNB) IN DIFFERENT TRICHODERMA REESEI STRAINS

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Trichoderma reesei is one of the most powerful secretors of extracellular proteins and several high protein secreting mutant strains were tested as expression hosts for the production of the thermophilic bacterial xylanase, XynB. XynB is apparently not glycosylated in the original bacterial host but has three potential N-linked and a number of potential O-linked glycosylation sites. Earlier reports have indicated that N-glycosylation of cellulases of T. reesei is not to obligatory for enzyme activity and secretion but has an effect on their resistance to proteolysis. Expression studies of XynB in T. reesei strains VTT-79125 (normal protease), RutC30, A2221 and HEP1 (low protease strains) produced different sized but active multiple forms of XynB depending on whether all three potential N-linked glycosylation sites were removed or left intact. In the low protease mutant RutC30, production of XynB revealed up to four different sized active forms. There was no obvious difference in total XynB enzyme activity in the RutC30 transformants with XynB having all three potential N-linked glycosylation sites removed compared to transformants harbouring the xynB gene which all glycosylation sites left intact. However, there was a clear difference in the XynB secretion profile pattern in the different transformants suggesting that some post-translational modifications may have occurred.

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VIIIp-36

STUDY OF THE *pgi* DELETION IN Trichoderma reesei.

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T. reesei is widely used for industrial production of proteins. We have studied the role of pentose phosphate pathway (PPP) in *T.reesei* by deleting phosphoglucose isomerase (PGI) gene in the strain RutC30. This strain has also at least a partial inactivation in the glucose repressor gene *cre1*. *Pgi* mutants are unable to convert glucose-6P into fructose-6P and have the glycolysis blocked. PGI activity was absent in the knock-out mutants. The mutants showed morphological changes such as swolled, short and highly branched hyphae.

Pgi mutants were able to grow in minimal medium (MM) with 1% glucose indicating that the PPP is active in Trichoderma in this mutant background. Cellulase activity was found to be higher in the *pgi* mutants on MM + 1% glucose in shake flasks than in RutC30, which did not produce cellulase activity. However, in a bioreactor on glucose, the maximum cellulase activity was 4-fold higher in RutC30 than in the *pgi* mutant. Endoglucanase 1 (egl1) mRNA was highly expressed during the exponential phase and at very low levels during the stationary phase in the RutC30 strain but was low in the mutant throughout the fermentation.

Glucose consumption under this fermentation condition were 0.18 glu/h/g biomass and 0.0097 g gluc/h/g biomass for RutC30 and the *pgi* mutant, respectively. Cellulase activity produced per glucose consumed was higher in the mutant than in RutC30 (0.667 nkat/ml /g gluc and 0.129 nkat/ml /g gluc, respectively).

DEVELOPMENT OF OPHIOSTOMA AS A NOVEL FUNGAL EXPRESSION HOST

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Ascomycete Ophiostoma spp. comprise common sap-staining fungi that invade wood via the parenchyma cells. Some albino variants of O. floccosum have been used as biological control agents to prevent sap-staining. The ability to invade wood and secretion of enzymes outside the growing mycelium in order to utilize the non-structural components of sapwood provide an excellent basis for the development of a fungal system both for the in situ delivery of selected gene products into wood tissue as well as their expression and production on a larger scale in liquid cultivation. We have improved the overall protein secretion of Ophiostoma by repeated rounds of UV-mutagenesis, identified effectively expressed proteins (protease and glucoamylase) for the isolation of the corresponding strong promoters for heterologous gene expression, and identified suitable antibiotic selection markers for efficient transformation by particle bombardment. We have also optimised the cultivation conditions to promote mycelial growth essential for efficient protein secretion.

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HETEROLOGOUS EXPRESSION OF CELLULASES IN TRICHODERMA REESEI

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Biomass conversion for the production of inexpensive sugars for use as feedstock for chemical and bioethanol production has been a long-term goal. However the cost of cellulase enzymes has been a major impediment of economic bioconversion. Trichoderma reesei has been used for industrial production of cellulase enzymes for some decades. The best strains are arguably the most productive protein-producing microorganisms. T.reesei cellulase production has been improved over many years by classical mutagenesis, screening, selection and development of highly refined, large-scale inexpensive fermentation conditions. But, while the multi-component cellulase system of T.reesei is able to hydrolyze cellulose to glucose, there are cellulases from other microorganisms that have preferred properties for efficient hydrolysis. Thermophilic cellulases have been described, their genes cloned, but they have often lacked efficient production systems. Cost effective T.reesei expression of thermophilic cellulases from bacteria and fungi and the effect upon host productivity will be described.