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Heterologous expression of pyranose dehydrogenase from *Agaricus bisporus* in *Hypocrea jecorina*

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Abstract

Since its discovery, pyranose dehydrogenase (PDH), a quinone dependent flavoenzyme secreted by several wood degrading fungi, became a potentially powerful tool for food industries for the production of substances like D-tagatose or lactulose. Its wide range of substrates and its ability to oxidize sugars at C-1 to C-4 position makes this enzyme interesting for industry and research.

The objectives of this work were to attempt heterologous expression of pyranose dehydrogenase from *A. bisporus* in *T. reesei* (*H. jecorina*). This was done by designing a construct that contains the *pdh* gene coupled with the *cbh1* promoter on the one hand and a construct that links the *pdh* gene to the *cbh2* promoter and coding sequence and ends with the *cbh2* terminator on other hand. In the second construct that was designed as a fusion protein a thrombin recognition sequence was introduced, in order to facilitate further protein purification. The constructs were transformed into *T. reesei* (*H. jecorina*) wild type by protoplast transformation together with a plasmid carrying a hygromycin resistance gene. Transformants that survived screening were cultivated in liquid Mandels Andreotti medium in shake flasks under expression inducing conditions. No PDH activity could be obtained, whereas levels of CBH I or CBH II, respectively, could be detected by Northern and Western blot analysis. Nevertheless, the presence of *pdh* in the genomic DNA could be verified by PCR screening. Production of active PDH seems to be inhibited on the transcriptional level.

Furthermore, samples of PDH from *A. bisporus* were deglycosylated using either Endo Hf and α-mannosidase or PNGase F. To visualize the PDH-bound carbohydrates dansyl hydrazine staining of these carbohydrates was performed. Some experiments delivered contradictory results.

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1 Introduction

1.1 The genus Trichoderma

Trichoderma, a filamentous fungus, is an omnipresent inhabitant of soils, rotting wood, and vegetable material, even affecting animal products like wool or leather.

It belongs to the division of sac fungi (Ascomycetes), yet hardly any strain of *Trichoderma* has been associated with a sexual state of reproduction (teleomorph), which therefore classifies most of the *Trichoderma* strains as fungi imperfecti (Deuteromycetes)[1, 2].

Tab. 1: Phylogenetic tree of *Trichoderma* / *Hypocrea* [3]

Kingdom	*Fungi*
Phylum	*Ascomycota*
Class	*Sordariomycetes*
Subclass	*Hypocreomycetidae*
Order	*Hypocreales*
Family	*Hypocreaceae*
Genus	*Trichoderma / Hypocrea*

Known for about 200 years but never dealt with in scientific connections for more than a century, *Trichoderma* attracted attention during World War II, when the US Army examined rotting matter in tropical regions. All strains were identified as '*T. viride*' back then. After another 20 years a biological unique isolate was noted as 'QM 6a' and named '*T. reesei*' after its main explorer Elwyn T. Reese [3]. The genome of *T. reesei* with the size of 33 Mb is made up of seven chromosomes.

There is a close genetic relation of *Trichoderma* and *Hypocrea*, which makes some species hard to assign and distinction of species in *Trichoderma*

challenging [1]. *T. reesei* was testified to be the anamorph (means not showing a sexual state of reproduction) of *H. jecorina* [4]. As a consequence, *T. reesei* is now defined as *Hypocrea jecorina*.

1.2 Morphology of T. reesei (H. jecorina)

Colonies grow rapidly, conidiation is slow and scattered widespread in small tufts. Conidia are one-celled, pale green, ellipsoid, sizing 3.0 - 4.5 x 2.3 – 3.0 µm [1]. *T. reesei* is represented in Fig. 1. Conidiation is induced throughout the culture when the mycelium reaches the edge of the Petri dish. In younger colonies brief illumination results in a ring of conidia at the position of the hyphal front [5]. While the exact mechanisms governing conidiation are not fully understood to date, a reaction to environmental stimuli similar to stress response appears to be the principal regulatory mechanism.

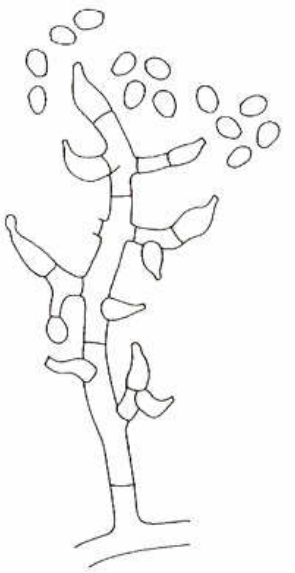

Fig. 1: Magnified appearance of *T. reesei*

1.3 Features of Trichoderma

Trichoderma is considered mostly as not plant pathogenic, except for one strain which induces grave disease of the commercial mushroom (*Agaricus bisporus*) [6].

Some *Trichoderma* species grow on root surfaces of plants and penetrate into the outer cells. Some produce or release various substances that can effect a certain level of resistance [7] or their metabolites can act against other phytopathogenic fungi [8].

Since fungal infections on crops or even stored fruits have become a grave economical problem, and chemical plant disease control seems insufficient or even counterproductive (causing pathogen resistance against pesticides, affecting non-target organisms, etc.), the need for biological control agents is obvious. One way of controlling plant diseases is the use of antagonists of phytopathogenic fungi, which is mostly done by using various strains of *Trichoderma* (most commonly strains of *T. virens*, *T. viride* and particularly *T. harzianum*). The highly competitive nature of *Trichoderma*, its ability to grow under inhospitable conditions, its efficient exploitation of nutrients, its ability of promoting plant growth and defense mechanisms and other attributes contribute to the success of this genus as biological control agent [9].

A specific example for a beneficial use of *Trichoderma* spp. for plants is their ability of consuming toxins like eutypine or 4-hydroxybenzaldehyde, which both are produced by pathogens and cause trunk diseases of grapevine [10]. Thus, the competency of some *Trichoderma* ssp. to oxidize aldehydes, as an example, makes them an effective instrument to combat plant pathogens.

Trichoderma reesei (Hypocrea jecorina) is not suitable for biocontrol of plant pathogens, but it is of great industrial importance, known to produce large quantities of cellulases and xylanases. Cellulases can be used for hydrolysing crystalline cellulose, which is used to obtain glucose as well as for getting rid of loose fibre ends in biofinishing of cotton, in food and feed industries, textile, pulp and paper industries. *T. reesei* produces some endoglucanases (EG) and

two different cellobiohydrolases (CBH I and CBH II). The cellobiohydrolases make up to 85 % of extracellular proteins under inducing conditions [11, 12].
Another industrial application of cellulases is for fuel ethanol production. As this consumes a considerably high amount of agricultural harvests, alternative biomass materials (e.g. by-products from agriculture or forestry) could be used as sugar sources. Since the sugars in these materials are supplied as polysaccharides, an enzymatic conversion into mono-sugars is necessary, which can be done by *Trichoderma* cellulases [13].
Xylan is the most abundant hemicellulose in higher plants, making up to 35 % of dry matter. Xylans are heteropolysaccharides with a backbone consisting of β-1,4-linked xylopyranose units. *T. reesei* (*H. jecorina*) produces two endo-1,4-β-xylanases (EC 3.2.1.8), XYNI and XYNII and an exo-1,4- β-xylosidase (EC 3.2.1.37), BXLI. XYNI and XYNII cause endohydrolysis of 1,4-β-xylosidic linkages in xylans, while BXLI hydrolyzes 1,4-β-D-xylans to successively remove D-xylose from the non-reducing ends. The production of xylanases is taking place during growth of the fungus on macromolecular substrates including xylan and cellulose. Xylanase and cellulase biosynthesis is separately regulated in *T. reesei*. Xylan hydrolyzation is, for example, used for bio-bleaching and in the food industry, as well as in paper-, pulp- and feedstock industry. [14-16]

1.4 Cellobiohydrolases (CBH)

Cellobiohydrolases (CBH, Cellulose 1,4-beta-cellobiosidase, EC 3.2.1.91) hydrolyse 1,4-β-D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose. CBH II cleaves cellobiose from the non-reducing while CBH I (also known as Cel7) cleaves cellobiose from the reducing ends of cellulose chains [11].

T. reesei cellulases are subdivided in two domains, a large catalytic domain and a small cellulose-binding domain (CBD). The domains are connected through a glycosylated linker protein.

CBH I is dominantly expressed, which indicates its possession of a strong promoter. On this account, the *cbh1* promoter is highly exploited for either overproduction of native CBH I through mutant *Trichoderma* strains, or for recombinant protein production by fusing the gene of interest to the *cbh1* gene and expressing it heterologously.

1.5 Regulation of cellulase biosynthesis in T. reesei (H. jecorina)

Cellulase biosynthesis and secretion by *T. reesei* is known to be adaptive, which means that it is strongly induced by the presence of cellulose (as well as sophorose), but repressed by (sole) carbon sources that can easily be metabolized, like glucose or glycerol. (Light induction is also occurring through cellobiose and lactose.)

It was shown that the regulation of cellulase production in *Trichoderma reesei* is depending on the inducer rather than on the repressor, because low synthesis rates seem to be due to the lack of an inducer and not to carbon source repression.

However, it is not known exactly if there is a complete repression of cellulase biosynthesis by particular carbon sources; low levels of cellulase may be

secreted constitutively. The regulation of cellulase synthesis is believed to take place mainly at the transcriptional level [17].
By the use of some cheap undefined media that induce CBH I expression extracellular proteases might also be induced [18]. Therefore, attention to the choice of media has to be paid.

In laboratory practice, both homologous and heterologous protein production in *T. reesei* is usually done by primarily growing it in the presence of repressing glucose levels, subsequently changing to inducing conditions by using cellulose or lactose as exclusive carbon source.
It is also possible to combine the carbon sources glucose and sophorose to gain as much productivity as with lactose.

1.6 Heterologous production of proteins in T. reesei (H. jecorina)

Species of filamentous fungi are known to be good and safe producers of either native or recombinant proteins. The advantages of cultivation in cheap and simple media and of natural secretion into the medium in remarkable quantity are evident.

Although *T. reesei* is being used for production of mammalian proteins (e.g. calf chymosin or interleukin-6) the production of heterologous fungal proteins is considered to be more effective. The closer the relationship between two species the more likely a protein of interest is expressed the way it is supposed to be (concerning appropriate folding, glycosylation, etc.).
Using parts of the cellulase CBH I for heterologous protein expression in *T. reesei* has turned out to be a powerful tool to obtain high production rates. The gene locus of *cbh1* seems to be beneficial for expression, and as already mentioned, the *cbh1* promoter is a very strong promoter. Heterologous expression of *Melanocarpus albomyces* laccase in *T. reesei* under the *cbh1*

promoter led to activities of 230 mg / L in shake flasks and 920 mg / L during fed-batch fermentation [19]. Expression of a *T. harzianum* endochitinase in *T. reesei* yielded an activity of 130 mg / L in shake flask cultivation, which was 20 times more than through native protein synthesis [20]. This high yield makes *T. reesei* an interesting tool for profitable protein production.

Nevertheless, other loci of the *T. reesei* genome can be used for profitable protein production [18]. A good selection of the promoter is crucial.
Even if protein biosynthesis is effective in regard to folding and secretion, host proteolysis is certainly an issue to consider. Employing strains lacking particular proteases is one way of dealing with this problem.

Transformation techniques are manifold, e.g. polyethylene glycol (PEG) transformation of protoplasts or using *Agrobacterium tumefaciens* for introduction of DNA into hyphae or spores, just to mention two methods.

1.7 Fusion proteins and tags

Fusion proteins are constructions of a protein of interest combined with a (or parts of a) protein with usually known characteristics. Fusion proteins are useful on the one hand to exploit beneficial properties of the chosen fusion partner (e.g. using a strong promoter for gene expression, efficient secretion etc.), on the other hand to be able to detect whether and where expression is taking place. Construction of fusion proteins is a successful method for production of heterologous proteins which shall improve mRNA stability. In addition, the heterologous part should be protected from enzymatic degradation.
The primary object in generating a fusion protein is to maintain the characteristics of the protein of interest. In addition, the activity or other properties of the fusion partner can be used to detect the fusion protein [21].
The use of fusion proteins is not only useful for detection, it can also be auxiliary for further purification steps or beneficial for solubility of a fusion

participant. Because it is much more efficient to rely on well-established purification methods rather than to establish appropriate methods for each new enzyme and for fusion proteins, labeling with tags is very popular.

Commercially available tag systems include maltose-binding protein (MBP), glutathione-S-transferase (GST), cellulose binding domain (CBD), His_6-tag and many others. Several tags are used to indicate correct folding of proteins, particularly the Green Fluorescent Protein (GFP).

1.8 Pyranose dehydrogenase (PDH)

Quinone-dependent pyranose dehydrogenase (PDH, pyranose:acceptor oxidoreductase, EC 1.1.99.29) is a monomeric flavoglycoprotein, able to catalyze (di)oxidation of free, non-phosphorylated sugars in pyranose form into their particular aldo(di)ketoses [22-26]. Besides formation of 2- and 3-ketosugars, described in [23], oxidations on positions C-1 and C-4 were also reported [27, 28]. The reaction selectivity is dependent on the sugar substrate, as well as on the enzyme source [26].
The physiological function of PDH is so far barely known; it seems to be involved in lignocellulose degradation since it shows activity with almost every sugar that appears in wood polysaccharides. It may connect ligninolysis with degradation of cell-wall-polysaccharide components [29].

PDH from *Agaricus bisporus* was isolated and characterized in 1997, showing a molecular weight of ca. 75 kDa (as defined by SDS-PAGE) [29]. PDH from other species have been characterized since then, including *Macrolepiota rhacodes* [24], *Agaricus subperonatus* [30] and *Agaricus xanthoderma* [31]. PDH derived from *A. bisporus* was originally identified as pyranose 2-dehydrogenase, due to the fact of predominant oxidation of the C-2 site of the carbon source.

In 2001, 76 strains of different wood degrading basidiomycetes were screened for PDH, resulting in 37 PDH-positive strains, belonging to seven (phylogenetically) closely related genera, most of them *Agaricales*. All tested *Agaricus* species (20) were positive for PDH, other positive strains belonged to six other genera, *Calvatia*, *Coprinus*, *Leucogaricus*, *Leucocoprinus*, *Lycoperdon* and *Macrolepiota*.

Macrolepiota proved to produce a PDH with similar properties as PDH from *Agaricus bisporus*. The glycosylation rate of the enzyme was estimated to be about 17.3 %. (Recent studies of *A. xanthoderma* speak of a glycosylation of about 5 %.)

A new assay for measuring PDH activity using ferrocenium as an electron acceptor turned out to be more sensitive than the formerly used benzoquinone assay [24].

For many years it remained unclear if the pyranose dehydrogenase might be a phylogenetic variant of the well-studied pyranose oxidase (P2Ox, pyranose:oxygen 2-oxidoreductase, EC 1.1.3.10), an enzyme that is widespread among basidiomycetes. However, serious doubts are based on many distinguishing factors:
PDH is synthesized as a highly glycosylated, monomeric polypeptide, whereas P2Ox is not known to be glycosylated, independent of its source, and known to consist of four subunits. (PDH can utilize a large variety of substrates, which is not the case for the P2Ox.) PDH is actively secreted into the environment, whereas P2Ox features an uncharacteristic signal sequence and remains associated with the mycelium. P2Ox converts molecular oxygen into hydrogen peroxide, while PDH is not known to react with oxygen. Finally, amino-acid sequences of both enzymes show only distant similarities.
Pyranose dehydrogenase has just recently been given a separate EC number (approved by the IUBMB, the International Union of Biochemistry and Molecular Biology), due to distinctions from pyranose oxidase.

1.9 Industrial relevance of PDH

By hydrolysing various mono- and disaccharides PDH can deliver a range of substances potentially interesting for food industries. D-galactose can be converted by PDH to 2-keto-galactose, which presents an intermediate for the production of D-tagatose. D-tagatose is used in food technology as a sweetener, with a level of sweetness comparable to sucrose, but having only a third of the calories. It is only partially absorbed by the body, resulting in reduced energy value. Most of the D-tagatose reaches the colon unabsorbed and is metabolised there by the colonic microflora. D-tagatose is a non-cariogenic sugar with

prebiotic qualities, which means it positively affects the intestinal flora. D-tagatose is also known to be a flavor enhancer.
D-tagatose is usually produced commercially from lactose, a bulk by-product from dairy production, which is first enzymatically hydrolyzed to glucose and galactose, then isomerized under alkaline conditions to D-tagatose by calcium hydroxide [32]. The second, chemical step in this procedure can be replaced by reactions described above, but also by a single enzymatic step, using L-arabinose isomerase [33].

Another possibility to valorize lactose is the conversion of lactose to lactulose. A conversion of lactose to 2-dehydrolactose can be done with the aid of PDH. 2-dehydrolactose, therefore, serves as an intermediate in the (chemo)enzymatic process of isomerization to lactulose [26]. Lactulose is a synthetic sugar used in treatment of constipation and hepatic encephalopathy (a disease of the liver). Since no human enzyme capable of hydrolysing lactulose exists, it is entirely metabolised in the colon by enteric bacteria. Lactulose is produced chemically at the moment, through isomerization of the glucose fraction to fructose.
By application of full enzymatic synthesis, more specific reactions with higher yield and potentially higher economic profits could be achieved.

2 Objectives

Main aim of this thesis was the attempt of heterologous expression of pyranose dehydrogenase (PDH) from the basidiomycete *Agaricus bisporus* in the ascomycete *Trichoderma reesei* (*Hypocrea jecorina*). Starting from transformants that contained the *pdh* gene under the control of the strong *cbh1* promoter these clones had to be cultivated in order to express PDH. Additionally, a construct of the *pdh* gene linked to the *cbh2* promoter had to be constructed, transformed into *T. reesei* (*H. jecorina*) and resulting progeny tested for PDH activity. The expression of PDH should be monitored through quantification of *pdh* encoding mRNA by Northern blot analysis on the one hand and through PDH activity tests of the culture supernatant on the other hand. Another task was to perform deglycosylation experiments on PDH. This should be done along with staining of PDH-bound carbohydrates using the dansyl hydrazine method.

3 Material and Methods

3.1 Material

3.1.1 Chemicals

Common laboratory chemicals were obtained from SIGMA (St. Louis, USA), MERCK (Darmstadt, Germany), FLUKA (Buchs, Schweiz) and ROTH (Graz, Austria), of the highest available quality. Substances used for culture media were obtained from SIGMA, MERCK and FLUKA.

If not otherwise stated, enzymes, DNA size standards and buffers used for molecular biological work were purchased from BIORAD (Richmond, USA), MBI FERMENTAS (Vilnius, Lithuania), NEW ENGLAND BIOLABS (Ipswich, MA, USA), PROMEGA Corp. (Madison, WI, USA) or from SIGMA (St. Louis, USA).

3.1.2 Kits for molecular biological work

Tab. 2: Kits used throughout the experiments

PURPOSE	KIT IDENTIFICATION	MANUFACTURING COMPANY
Plasmid DNA Miniprep	GeneElute Plasmid Miniprep Kit	SIGMA (St. Louis, USA)
Plasmid DNA Miniprep	Pure Link Plasmid Miniprep Kit	INVITROGEN (Carlsbad, CA)
Plasmid DNA Midiprep	Pure Yield Plasmid Midiprep Kit	PROMEGA (Madison, WI)
RNA Isolation	SV Total RNA Isolation System	PROMEGA (Madison, WI)
DNA Purification / Elution from gel	Wizard SV Gel and PCR Clean-Up System	PROMEGA (Madison, WI)
cDNA Synthesis	First Strand cDNA Synthesis Kit	MBI FERMENTAS (Vilnius, Lithuania)
DNA blunt end Cloning	Zero Blunt TOPO PCR Cloning Kit	INVITROGEN (Carlsbad, CA)

3.1.3 Organisms

Trichoderma reesei strain QM9414 was obtained from Vienna University of Technology, Institute of Chemical Engineering, Division of Gene Technology and Applied Biochemistry.

For cloning experiments in *E. coli* cells of the strains DH5α, BL21 Star or TOP 10 were obtained from Invitrogen (Carlsbad, USA).

3.1.4 Media

3.1.4.1 Media preparation

The weighed media constituents were solved either in tap water or in distilled water and autoclaved at 121 °C for 15 min. Agar containing media were tempered at approx. 55 °C in a water bath before being poured aseptically into Petri dishes. This was done in laminar flow workbench.

3.1.4.2 Cultivation Media for *Trichoderma reesei* (*Hypocrea jecorina*)

3.1.4.2.1 Potato dextrose agar (PDA)

PDA powder	39 g / L
filled up to 1 L with H_2O; autoclaved	

3.1.4.2.2 Malt extract agar (MEA)

malt extract	30 g / L
agar	20 g / L
filled up to 1 L with H2O, autoclaved	

3.1.4.2.3 Minimal medium (MM)

$(NH)_4SO_4$	5 g/L
KH_2PO_4	15 g/L
$MgSO_4$	0.6 g/L
$CaCl_2$	0.1 g/L
Potassium hydrogenphthalate	10 g/L
Lactose	40 g/L
Peptone from soy	2 g/L
trace element solution (100-fold)	10 mL / L
100-fold Trace element solution	
$FeSO_4$ x 7 H_2O	0.5 g/L
$MnSO_4$ x H_2O	0.16 g/L
$ZnSO_4$ x 7 H_2O	0.14 g/L
$CoCl_2$	0.2 g/L
pH = 6	

3.1.4.2.4 Mandels Andreotti Medium (MA)

Na2HPO4 x 2 H2O (0.1 M)	17.799 g / L
500 mL of the phosphate buffer were prepared, pH = 5 adjusted with citric acid then filled up to 900 mL, addition of:	
$(NH_4)2SO_4$	1.4 g / L
KH_2PO_4	2.0 g / L
$MgSO_4$ x 7 H_2O	0.3 g / L
$CaCl_2$ x 2 H_2O	0.4 g / L
50-fold trace element solution	20 mL / L
carbon source (lactose)	10 g / L
peptone from casein, pancreatic digest	1 g / L
urea (5 mM)	0.3 g / L
Tween 80 (just for lactose as carbon source)	0.5 g / L
filled up with water to 1 L, pH adjusted; autoclaved	
50-fold trace element solution	
$FeSO_4$ x 7 H_2O	0.25 g / L
$MnSO_4$ x H_2O	0.08 g / L
$ZnSO_4$ x 7 H_2O	0.07 g / L
$CoCl_2$ x 2 H_2O	0.1 g / L
pH = 2, adjust with sulfuric acid	

3.1.4.3 Cultivation media for *E. coli*

3.1.4.3.1 Luria broth (LB) (/ LB agar)

peptone from meat	10 g / L
yeast extract	5 g / L
NaCl	5 g / L
(agar	20 g / L)

3.1.4.3.2 LB + kanamycin

50 µg / mL kanamycin were added to the autoclaved LB medium through a sterile filter, after it was cooled down to approx. 50 °C.

3.1.4.3.3 SOC medium

tryptone water	20 g / L
yeast extract	5 g / L
NaCl	0.58 g / L
KCl	0.18 g / L
$MgCl_2$	2 g / L
$MgSO_4$	2.4 g / L
glucose	3.6 g / L

3.1.4.4 Solutions for generation and transformation of protoplast cells

3.1.4.4.1 Solution A

Sorbitol	1.2 M	21.84 g
KH_2PO_4	100 mM	1.36 g
H_2O		up to 90 mL
pH = 5.6 (adjust with KOH; add H_2O up to 100 mL) autoclave and store at room temperature		

3.1.4.4.2 Solution B

Sorbitol	1.0 M	18.2 g
Tris / HCl	10 mM	1 mL of Solution C
$CaCl_2$ (anhydrous)	50 mM	0.56 g
H_2O		up to 90 mL
pH = 7.5 (adjust with HCl, if necessary; add H_2O up to 100 mL) autoclave and store at room temperature		

3.1.4.4.3 Solution C

Tris / HCl	1 M	6.06 g
H_2O		up to 40 mL
pH = 7.5 (adjust with HCl; add H_2O up to 50 mL)		

3.1.4.4.4 Protoplasting solution

Lysing enzymes from *Trichoderma harzianum*	100 mg
Solution A	up to 20 mL
filter sterilize using a 0.2 µm filter	

3.1.4.4.5 PEG (polyethylene glycol) – solution

PEG 6000	25 %	12.5 g
$CaCl_2$ (anhydrous)	50 mM	0.2775 g
H_2O		up to 50 mL
filter sterilize using a sterile syringe and a 0.2 µm filter		

3.1.4.4.6 PDA + hygromycin

After cooling the autoclaved PDA medium to 50 °C the antibiotic was added to obtain final concentrations of 50, 75 or 100 µg / mL (depending on the purpose). For *T. reesei* transformation either MM or PDA with 2 % agar were used as bottom media. 16 mL of bottom agar were poured into Petri dishes. In this case, the antibiotic concentration was calculated for the final concentration when topped up to 20 mL with overlay agar.

3.1.4.4.7 Overlay agar

The overlay agar for transformations of protoplasts was prepared as MM (see 3.1.4.2.3) with the addition of 1.0 M Sorbitol (182.17 g / L), 0.1 % Triton X-100 (1 mL / L) and 1 % agar. After autoclaving the medium it was aseptically filled in eprouvettes, 4 mL each, and closed with a metal cap.

This overlay medium was added to 16 mL of bottom medium, as described in 3.2.18.4.

3.1.5 List of utilized primers for vector constructions

3.1.5.1 Primers used for *cbh1* vector construction

Tab. 3: Primers for *cbh1* vector construction

primer name	Tm [°C]	sequence	purpose
BspTrichfw1 (containing *Bam*HI site)	69.9	GGA TTC AGG TTG GGA TCC GTG CCA TCA	**amplification of the *pdh* coding sequence**
BspSachrv1 (containing *Nde*I site)	52.3	GGA AGA CGC ATA TGA GCA GG	
BspTrichfw2 (containing *Apa*I site)	75.2	ATG CCG ACC GCG GGG CCC ACT TAA CG	**amplification of the *hph* terminator**
BspASrv2 (containing *Apa*I site)	73.6	ATG CCT GGG GCC CGA GTG GAG ATG TGG	
pAMH1100fw2	45.5	CTC ATC AAC TCA GAT CCT CC	**lies on the *cbh1* promoter**
pAMH1100rv2	61.6	AGC CAA TAC CGC CGC ACT GG	**lies on the *cbh1* terminator**

3.1.5.2 Primers used for *cbh2* vector construction

Tab. 4: Primers for *cbh2* vector construction

primer name	Tm [°C]	sequence	purpose
cbh2fw1	59.3	TGA GGC AAT AAG ACG CAT CTC AGG	**amplification of the *cbh2* gene**
cbh2rv1	66.0	CAT GTG GCA GCA GGC CAA TCG G	
cbh2fw4 (containing *NdeI* site)	72.0	TTG CAT ATG GGC TTT CGT GAC CGG GCT TC	**amplification of the *cbh2* terminator**
cbh2rv4 (containing *NotI* site)	81.0	TCG CGG CCG CCT GCT GCC GAT GGG TTA TCC	
cbh2rv2 (containing *ApaI* site)	87.8	TT GGG CCC GCT GCC GCG CGG CAC CAG CAG GAA CGA TGG GTT TGC G	**contains a thrombin site as linker between *cbh2* and *pdh***
BspSachrv1 (containing *NdeI* site)	52.3	GGA AGA CGC ATA TGA GCA GG	**amplification of the *pdh* coding sequence**
cbh2fw3	56.1	AGT CAT CAA TTC GCC TCA GAT CC	

3.1.6 List of utilized plasmids and fragments for vector construction and analysis

3.1.6.1 Plasmids used for *cbh1* vector construction

pAN7.1 plasmid holding *hph* gene [34]

pAN8 pBluescreen II backbone (3 kb) with *hph* cassette (4 kb)

pAMH110 primal plasmid for *T. reesei* transformation

pDF38 pAN8 without *hph* terminator (*BamH*I and *Xba*I restriction sites)

pDF40 pAMH110 with multiple cloning site (MCS) cut out

pDF41 pDF40 carrying the *pdh* gene

pDF42 pDF38 with *hph* terminator relocated

3.1.6.2 Plasmids and fragments used for *cbh2* vector construction

pWS1 4 kb large region out of the *cbh2* gene cloned into the TOPO blunt vector

pWS1.1 *cbh2* promoter – cds *cbh2* – thrombin – *Apa*I construct

pWS2 *cbh2* terminator

pWS3 pWS1.1 and pWS2 ligated and cloned into the TOPO blunt vector

pWS4 pWS3 ligated with *cbh2* terminator → template for transformation

3.1.6.3 Primers used for vector analysis

Tab. 5: Primers for probe labeling

	primer name	sequence	purpose
***pdh* probe "pDF3"**	**BspPDHfw1**	for details cf. [35]	**~ 2.1 kb fragment out of genomic *A. bisporus* DNA used for *pdh* probe labeling**
	AmePDHctrv1		
***cbh1* probe**	**cbh1fw1**	5′ – GCA CTC TCC AAT CGG AGA CT – 3′	**~ 1.6 kb fragment out of genomic *T. reesei* DNA used for *cbh1* probe labeling**
	cbhrv1	5′ – AGG CAC TGA GAG TAG TAA GG – 3′	
***cbh2* probe**	**cbh2fw7**	5′ – CAC ATG CGT CTA CTC CAA CG – 3′	**~ 1.2 kb fragment out of genomic *T. reesei* DNA used for *cbh2* probe labeling**
	cbh2rv7	5′ – TGG CTT GAC CCA GAC AAA CG – 3′	

Tab. 6: Primers for PCR screening of transformants

primer name	sequence	purpose
pAMH1100fw2	5′ – CTC ATC AAC TCA GAT CCT CC – 3′	**used for PCR screening of transformants with the *cbh1* construct**
BspPDHrv3	5′ – ATA GAC AAC GTT CCC ACA CCA TGC – 3′	
cbh2fw6	5′ – TCA GCG CAC GGA AGT CAC AG – 3′	**used for PCR screening of transformants with the *cbh2* construct**
cbh2rv7	5′ – TGG CTT GAC CCA GAC AAA CG – 3′	
cbhfw6	5′ – TCA GCG CAC GGA AGT CAC AG – 3′	
BspPDHrv6	5′ – CTG AGA CGA GAG GCC ACA AC – 3′	

3.2 Methods

3.2.1 Determination of the protein content

The overall protein content was determined photometrically at 595 nm at room temperature according to the dye binding method by Bradford [36], using the BioRad protein assay dye reagent. The measurement was done after in incubation of 15 min. Bovine serum albumin (BSA) was used as standard protein (0.1 – 1.0 mg / mL).

3.2.2 Determination of DNA or RNA concentrations

Samples were drawn and the optical density was measured at 260 nm. The following formula gives the total RNA concentration:

$c = OD_{260} \times DF \times F$ [µg DNA / RNA per mL]

OD_{260}	optical density at 260 nm	
DF	dilution factor	
F	specific factor:	50 for double-stranded DNA
		40 for RNA
		37 for single stranded DNA

In addition to photometric measurement at 260 nm, the absorption at 280 nm was measured. The ratio of OD_{260}/OD_{280} – results provides information about the purity of the sample solution. A ratio value between 1.8 and 2.0 suggests a pure sample, whereas lower values indicate protein impurity.

3.2.3 Agarose gel electrophoresis

For determination of DNA fragment sizes and in order to estimate the amount of DNA, agarose gel electrophoresis was performed.
0.8 % (w/w) agarose was melted in a microwave oven with Tris–Borate–EDTA buffer (10 x TBE buffer: 0.89 M Tris / HCl, 0.89 M Boric acid, 0.02 mM EDTA-Na2-salt). After cooling the homogenous solution down to 50 - 60 °C 0.1 % (v/v) ethidium bromide was added. The solution was mixed carefully (avoiding air bubbles), and immediately poured into the selected device.
For sample application 6 x loading dye (0.03 % bromphenol blue, 60 % glycerol, 60 mM EDTA-Na-salt) was added at a ratio of 1:6 (1 volume share loading dye and 5 volume shares sample). Sample and loading dye were thoroughly mixed and applied on the gel. Electrophoresis was usually performed at 90 V.

3.2.4 PDH standard activity assay

The assay is based upon the D-glucose-dependent reduction of the blue-coloured ferrocenium ion ($[Fe(C_5H_5)_2]^+$, provided as its $[PF_6]^-$ salt: $FcPF_6$) to ferrocene ($Fe(C_5H_5)_2$; Fc). It is monitored at 300 nm and observed for 3 min. Only the linear range of the decrease is used for activity calculations. The assay is performed at 30 °C (the reagents pre-heated to 30 °C in a water bath), at pH 7.5. The molecular extinction coefficient is $\varepsilon = 4300\ M^{-1}\ cm^{-1}$.
One unit (U) is defined as the amount of enzyme necessary to reduce 2 µmol of $FcPF_6$ in one minute under standard conditions.

All reaction components except for glucose were pipetted in a 1 mL measuring cuvette. The reaction was started by addition of glucose and measured photometrically immediately. The $FcPF_6$ solution had to be prepared freshly for each individual test series.

Tab. 7: PDH assay scheme

Reagent	Quantity [µL]
100 mM KPP (potassium phosphate buffer), pH 7.5	770
1mM $FcPF_6$, solved in 5mM HCl	200
sample	20
2.5 M D-glucose, solved in 100 mM KPP, pH 7.5	10

3.2.5 PCR – Polymerase Chain Reaction

By the use of this method it is possible to amplify any sequence of a DNA template within a short time to manifold copies. There are many ways to vary the reaction conditions in order to achieve the optimal performance.

Especially at the beginning of the PCR mishybridization of primers or primer dimers can occur. This leads to poor yield and unspecific products. One thing to prevent things like that is the so-called 'hot start'. In this case the chain reaction does not get started before the reaction mixture is at a high temperature (ideally higher than the melting temperature of the DNA). After that the polymerase is added. Manually pipetting at this stage is very impractical, so there are other strategies using commercially available products like polymerases trapped in wax globules that melt at the right temperature.

Some kind of 'hot start' was attained by putting the whole PCR mixture (including the polymerase) in the PCR machine when the temperature was already high.

If it is necessary to get a product free from mutations, the use of *Taq* polymerase may not be the right choice. For the construction of the vectors for the *Trichoderma* transformation iproof DNA polymerase was used. This polymerase is 52-fold more accurate than the *Taq* polymerase and its high processivity drastically reduces extension time. By the use of iproof polymerase it is possible to amplify fragments up to 37 kb.

Tab. 8 shows certain characteristics of both the enzymes, Tables 9 – 12 supply information about applied reaction specifics.

Usually a negative control was performed by addition of all the ingredients except for the template. This was done in order to monitor if any of the reaction components were contaminated, if primer dimers were forming etc.

Tab. 8: Characteristics of used polymerases

Enzyme	Performance	Produced ends on DNA	3'-5'-exonuclease activity (proofreading)
Taq polymerase	~ 1 kb / min.	TA-ends	no
iproof polymerase	~ 2 – 4 kb / min.	blunt-ends	yes

Tab. 9: Temperature cycle of the PCR using *Taq* polymerase

	Temperature [°C]	Time [sec.]	
initial strand separation	95	120	
strand separation	94	40	32 – 35 repeats
annealing	depending on primers	60	
elongation	72	depending on template length	
elongation	72	360	
end	4	until machine shut off	

Tab. 10: Temperature cycle of the PCR using iproof polymerase

	Temperature [°C]	Time [sec.]	
initial strand separation	98	120	
strand separation	98	40	30 repeats
annealing	depending on primers	30	
elongation	72	depending on template length	
elongation	72	300	
end	4	until machine shut off	

Tab. 11: Preparation scheme for *Taq* PCR

Reagent	Quantity [µL]
5 x GoTaq buffer	10
Primer forward	2
Primer reverse	2
dNTP-mix (10 mM)	1
template	1
Taq polymerase	0.25
water	33.75

Tab. 12: Preparation scheme for iproof PCR

Reagent	Quantity [µL]
5 x iproof buffer	10
Primer forward	2
Primer reverse	2
dNTP-mix (10 mM)	1
template	1
iproof polymerase	0.5
water	33.5

The reaction mixture was pipetted together, adding the polymerase as the last component. Tab. 11 and Tab. 12 show standard compositions of elements for the PCRs. Template quantities were frequently changed, differences in volume were compensated with water, always providing a total volume of 50 µL.

3.2.6 Cloning of blunt-end PCR products

For the insertion of blunt end PCR products (produced by iproof polymerase) into a vector for plasmid multiplication in *E. coli* the 'Zero Blunt TOPO PCR Cloning kit' was used. This kit allows direct insertion of the blunt end template into the vector by the use of topoisomerase I. This polymerase binds specifically to duplex DNA and cleaves the phosphodiester backbone at a certain site. The energy derived is used for the formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue of topoisomerase I. This bond can then be attacked by the 5' hydroxyl of the original cleaved strand, consequently reversing the reaction and releasing topoisomerase again (Fig. 2).

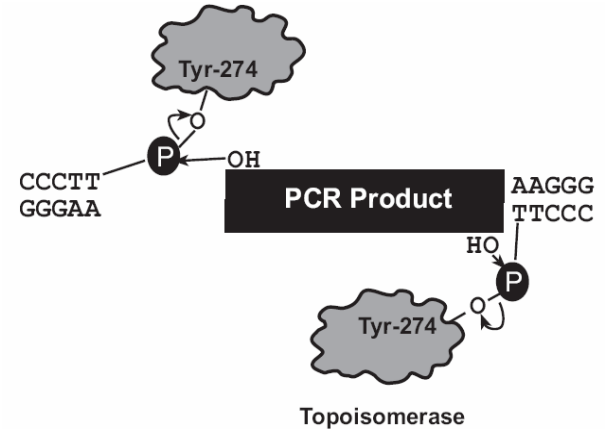

Fig. 2: Reactions of topoisomerase I

The produced plasmid can be transformed into chemically competent or electrocompetent *E. coli* cells. Direct screening for recombinant individuals is possible by disruption of a lethal *E. coli* gene, which is located on the vector. Cells containing the non-recombinant vector are killed upon plating. The vector also contains resistance genes for kanamycin and zeocin. We plated the *E. coli* cells on LB medium containing kanamycin (50 µg / mL).

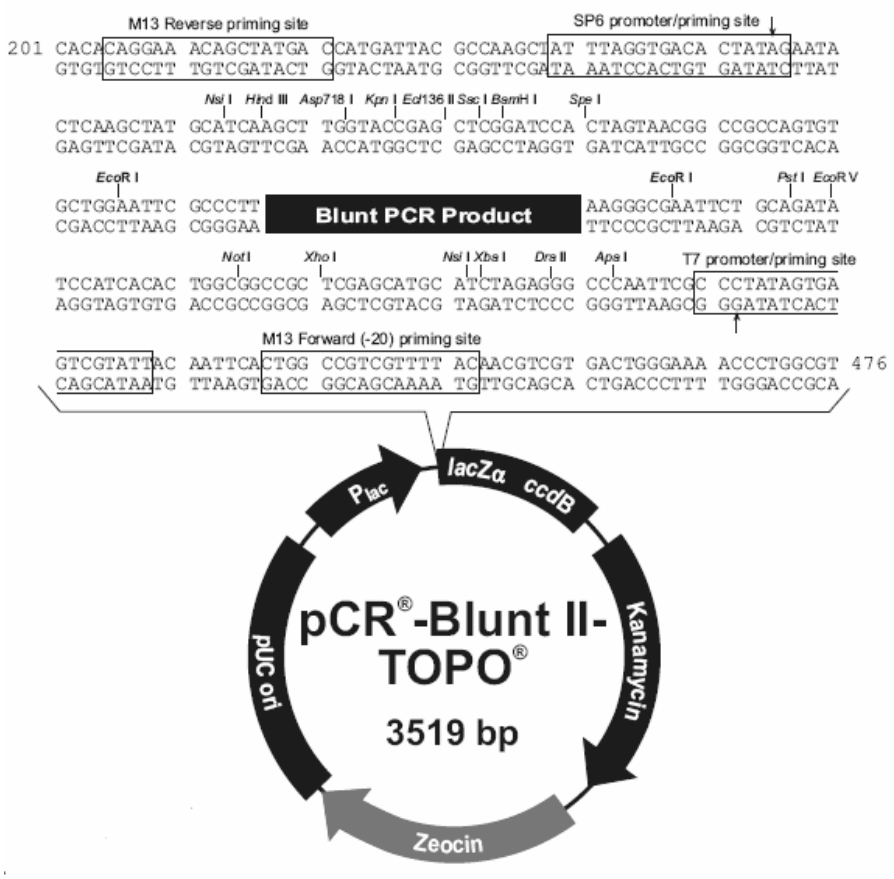

Fig. 3: Map of the pCR-Blunt II-TOPO vector

3.2.7 Plasmid transformation and multiplication in *E. coli*

After cloning into the pCR-Blunt II-TOPO vector the plasmids were either transformed chemically or electroporated into *E. coli* cells, following the 'Zero Blunt TOPO PCR Cloning Kit' manual, except when other cells as described were used.
E. coli cells used for electroporation were DH5α or BL21 Star cells, chemical competent cells used were TOP10 cells. Transformed cells recovered by addition of 250 µL pre-warmed SOC medium at 37 °C on the horizontal shaker for at least one hour. 50 µL were plated on pre-warmed LB + kanamycin agar plates for overnight incubation. Single colonies were picked for incubation in liquid LB medium containing kanamycin until dense growth. Those colonies could be used for plasmid preparations.

3.2.8 Restriction digest of PCR products

Restriction digest of DNA was done using restriction enzymes from Fermentas (Vilnius, Lithuania), following the conditions suggested on the company homepage.
The restriction digests were usually done at 37 °C for at least two hours.

3.2.9 Ligation of PCR products

T4 DNA ligase is encoded by bacteriophage T4, and is produced upon infection of *E. coli* cells. It can carry out both blunt- and sticky-end ligations and requires ATP. The ligation of fragments for engineering of the *cbh2* – thrombin – *pdh* construct was done using T4 DNA ligase and corresponding buffer. The reaction volume was chosen as 10 µL or 30 µL, using 1 µL of T4 ligase for each reaction.

3.2.10 SDS polyacrylamide gel electrophoresis (PAGE)

SDS (sodium dodecyl sulfate) is used to apply negative charge on the protein and to get the protein in a state of only containing primary structure, so that a proper migration in a separating gel towards the positive pole is assured.

The gels were produced using the components described in Tab. 13 and Tab. 14. The work was done in the extractor hood with great caution, due to the fact that acrylamide is very toxic. The mentioned ingredients were for one 10 x 15 cm polyacrylamide gel used for SDS-PAGE in a PeqLab and a BioRad device.

The technique that was applied was a discontinuous SDS-PAGE, which refers to the use of two gels poured on top of one another. The upper gel that the proteins enter first is called stacking gel, the lower one separating gel. The purpose of the stacking gel is to densify the protein into a compact band before running it through the separating gel. The resolution increases by the use of this method. The stacking effect is based on the lower concentration of polyacrylamide in the stacking gel. The proteins move fast through the stacking gel, giving proteins at the top of the gel time to catch up with the proteins at the bottom, before all the proteins enter the separating gel. After the separating gel was poured it was covered with butanol to ensure an even surface.

Tab. 13: Preparation scheme for 5 mL STACKING GEL (4 % acrylamide)

component	amount
H_2O	2.67 mL
30 % acrylamide / 2.7 % bisacrylamide (stock solution)	1.00 mL
10 % SDS	0.05 mL
4 x stacking buffer (1 M Tris/HCl pH 6.8)	1.25 mL
degas for at least 20 min.	
10 % APS	40 µL
TEMED	5 µL

Tab 14: Preparation scheme for 11.5 mL SEPARATING GEL (10 % acrylamide)

component	amount
H_2O	4.6 mL
30 % acrylamide / 2.7 % bisacrylamide (stock solution)	3.83 mL
4 x resolving buffer (1 M Tris pH 8.8)	2.88 mL
10 % SDS	0.12 mL
degas for at least 20 min.	
10 % APS	92 µL
TEMED	9 µL

3.2.11 SDS polyacrylamide gel electrophoresis (PAGE) using the PHAST System

Besides large self-made polyacrylamide gels small commercially available PAGE gels were used, utilizing the Phast System by GE Healthcare (Wisconsin, USA). Gradient 8-25 SDS PAGE gels together with SDS PAGE buffer strips (GE Healthcare) were used.

The samples had a protein content of approx. 1.0 mg/mL. 10 µL of sample were mixed with 10 µL of Laemmli buffer (BioRad, Richmond, USA), then the mixture was boiled at 99 °C for 2 minutes in a thermocycler. The molecular mass of the protein samples were estimated with the aid of the 'Precision Plus

Dual Color Standard' (BioRad). 4 µL of each sample and the standard were applied onto the gel via a sample comb. The gel was run at a fixed voltage until the blue buffer dye almost reached the end of the gel.

Staining of the gel was done in the developer chamber of the Phast System, using the silver or coomassie staining method. The program cycles are described in the Phast System manual (https://www.gelifesciences.co.jp/tech_support/manual/pdf/elpphast/80132015ai.pdf).

Silver nitrate as well as the developer were prepared freshly.

3.2.12 Coomassie staining of polyacrylamide gels

Coomassie Blue staining solution

Coomassie Brilliant Blue R 250	0.5 g / L
methanol	500 mL / L
distilled H_2O	400 mL / L
acidic acid	100 mL / L

Destaining solution

methanol	50 mL / L
acidic acid	70 mL / L
distilled H_2O	880 mL / L

Procedure:

- The gel was agitated for 40 min. in the Coomassie Blue staining solution on a shaker.
- The Coomassie Blue staining solution was carefully poured back into the storage bottle. Destaining solution was added, and the gel agitated for 20 min. The gel was stored at 4 °C overnight in 1:2 diluted destaining solution.
- On the next day the destaining solution was replaced and the gel destained until protein bands could be seen.
- For documentation of the work gels were dried or scanned.

3.2.13 DNA Miniprep – rapid method for fungal DNA

Isolation of genomic DNA was performed at Miniprep level as well as at Midiprep level using the mentioned kits, following the instruction manuals. Additionally, a quick method for fungal DNA Mini-Preparation was carried out successfully according to [37].

The working procedure was the following:

- 500 µL lysis buffer (400 mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1 % sodium dodecyl sulfate) were provided in a 1.5 mL Eppendorf tube. A small lump of mycelia was added with a sterile toothpick, which thereupon was used for disruption of the mycelia. The tube was left for 10 min. at room temperature.

- After addition of 150 µL of potassium acetate (pH 4.8; made of 60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of distilled water) the tube was vortexed briefly and centrifuged at > 10000 g for 1 min.

- The supernatant (containing the DNA) was transferred to another 1.5 mL Eppendorf tube and again centrifuged at > 10000 g for 1 min. Once again the supernatant was transferred to a new 1.5 mL Eppendorf tube, and an equal volume of isopropyl alcohol was added. The tube was mixed briefly by inversion.

- The tube was centrifuged at > 10000 g for 2 min., and the supernatant was discarded. The pellet of DNA was washed in 300 µL of 70 % ethanol. After centrifugation of the pellet at 10000 g for 1 min. the supernatant was again discarded.

- The DNA pellet was air-dried and dissolved in 50 µL of 1 x Tris-EDTA or (varying from the standard protocol:) in 50 µL of double distilled water.
- 1 µL of the purified DNA was used for 25 or 50 µL of PCR mixture.

The method proved to be suitable for isolating *Trichoderma reesei* (*Hypocrea jecorina*) DNA by loading amounts of the DNA preparations onto agarose gels, where a clear band could be seen.

3.2.14 RNA isolation

RNA isolation was done by using the SV Total RNA Isolation System by PROMEGA (Madison, WI).
Frozen mycelium of *T. reesei* (*H. jecorina*) from a liquid culture was ground into fine powder in the presence of liquid nitrogen, always paying attention that the mycelium did not thaw. Furthermore, the instructions from the manual were followed.

3.2.15 Transcription of mRNA into cDNA

Reverse transcriptase is, in presence of a suitable primer and the four desoxynucleosidtriphosphates (dNTPs), able to transcribe RNA into DNA that is complementary to the sequence (complementary DNA, cDNA). This piece of cDNA can then be amplified by PCR.
The 'First Strand cDNA Synthesis Kit' by Fermentas (Vilnius, Lithuania) was used to transcribe mRNA into cDNA. The amount of RNA used for reverse transcription depended on the concentration, a volume containing 0.1 – 5 µg RNA could be applied.

Procedure:

- 1 µL of an oligo dT anchor primer (5'-GGC CAC GCG TCG ACT AGT ACT TTT TTT TT-3') was added to a certain amount of RNA, DEPC water was used to top up the volume to 11 µL.
- After gentle mixing the solution was incubated at 70 °C for 5 min., then immediately put on ice and shortly centrifuged.
- µL of 5 x buffer, 1 µL of ribonuclease inhibitor and 2 µL of 10 mM dNTP mix were added, the solution was mixed and shortly centrifuged again.
- The mixture was left at 37 °C for 5 min., then 2 µL of reverse transcriptase were added.
- The mixture was incubated at 37 °C for one hour, then at 70 °C for 10 min. to stop the reaction by deactivating the enzyme.

3.2.16 Plasmid isolation

Plasmid DNA was isolated using the GeneElute Plasmid Miniprep Kit (SIGMA, St. Louis, USA) or the 'Pure Link Plasmid Miniprep Kit' (INVITROGEN, Carlsbad, CA). Picked bacterial colonies were incubated in a shaking culture in approx. 3 mL of liquid LB medium overnight, in the presence of the adequate antibiotic.

3.2.17 DNA elution from agarose gels / DNA Purification

This was done with the 'Wizard SV Gel and PCR Clean-Up System' by PROMEGA. Membrane binding solution was added to DNA cut out of an agarose gel or DNA from PCR, then the mixture was applied to a column. DNA bound to the column and impurities were removed by the use of a wash solution. The DNA was then eluted in nuclease-free water and could be used for further applications.

3.2.18 Protoplast transformation of *T. reesei* (*H. jecorina*)

Since sterile work was inevitable every step was done carefully at a workplace cleaned with ethanol or in the laminar flow, using gloves and sterile instruments.

3.2.18.1 Growing mycelium on cellophane-covered PDA plates

Five cellophane sheets were cut to Petri dish size and wrapped in moistened paper towels. The paper towels were put in aluminium foil and autoclaved 20 min. at 121 °C.

The agar surface of five PDA plates was covered with the sterile cellophane sheets. 100 µL of spore suspension of *T. reesei* wild type (in 0.8 % NaCl, 0.1 % Triton X-100), containing roughly about 5×10^6 spores / mL was pipetted on each cellophane sheet and spread using a Drigalski spatula. The PDA plates were incubated at 30 °C overnight.

Two glass funnels (one of them as a backup) were filled with glass wool, put in a glass beaker together with a 50 mL Greiner test tube, each wrapped in aluminium foil and sterilized overnight at 80 °C.

3.2.18.2 Generation of protoplast cells

After incubation of the five PDA plates until mycelium growth all over the cellophane surface was visible the first cellophane sheet was picked up with forceps and put in a Petri dish containing 2-3 mL of protoplasting solution (as described in 3.1.4.4.4). The cellophane sheet was covered with another 2-3 mL of protoplasting solution. Another cellophane sheet was put on top and again covered with 2-3 mL protoplasting solution. This was done with all the five

cellophane sheets, the last one was covered with the rest of the 20 mL solution. The Petri dish was closed and incubated at 30 °C for 1-2 hours, shaking it every once in a while.

The cellophane was taken out with forceps and the mycelium was then washed off the sheets by pipetting the suspension onto the surface several times with a large tip cut at the front end to avoid shear forces on the mycelium.

From here on every working step was done chilled on ice. The suspension containing the protoplasts was immediately filtered through the glass wool into the Greiner test tube (sterilized the day before). In case of blockage of the glass wool by particles of mycelium the second funnel was used for filtration. The solution containing the protoplasts was centrifuged for 10 min. at 2000 rpm and 4 °C. The supernatant was discarded and the pellet suspended carefully in 4 mL of cooled Solution B. The suspension was again centrifuged for 10 min. at 2000 rpm and 4 °C. The supernatant was again discarded and the protoplast pellet was suspended carefully in 1-2 mL Solution B. An aliquot of the suspension was used for observation and counting of the protoplasts.

3.2.18.3 Counting of protoplast cells using the Thoma counting cell chamber

For the following transformation it was necessary to estimate the amount of protoplasts. This was done by counting the protoplasts in the Thoma counting cell chamber under a light optical microscope. The chamber was washed well with 70 % ethanol, and the cover glass was set on the bridges, leaving a 0.100 mm space between the counting area and the cover glass. The cover glass was moved back and forth until Newtonian rings could be observed, which assured a tight fit (Fig. 4).
The chamber was filled with a pipette by letting the liquid sample flow under the cover glass from one side.

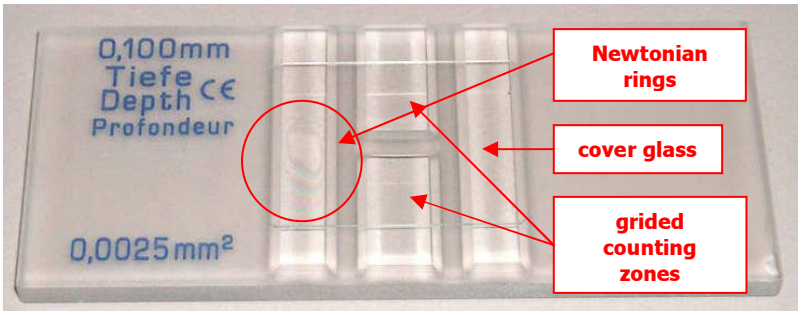

Fig. 4: Assembly of a Thoma chamber

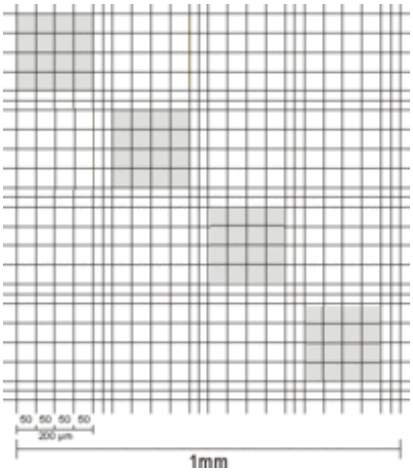

Fig. 5: Counting squares in the Thoma chamber

64 squares were counted, counting four times 16 small squares, going from top left to bottom right (indicated by grey squares in Fig. 5). The cell count was calculated using the following formula:

cells per mL = (n x 400 x 10^5) / f

n … number of cells counted
f … number of counted squares

After counting the protoplast cells the suspension was diluted if the cell density was too high or centrifuged again and suspended in less volume of Solution B if the density was too low. A concentration between 5 x 10^7 and 5 x 10^8 protoplast cells per mL is desirable.

3.2.18.4 Transformation of protoplast cells

The transformation was performed as a co-transformation, meaning transforming the plasmid containing the designed expression cassette (or only the insert, respectively) along with a plasmid carrying the selection criterion (in this case the gene for hygromycin resistance).

The plasmids were applied at ratios of about 4:1 (plasmid with insert : plasmid with hygromycin resistance), in a total volume of 10 µL.

200 µL of chilled protoplast solution were pipetted into a 1.5 mL Eppendorf tube (on ice). The 10 µL of plasmid DNA mix was added, as well as 50 µL chilled PEG-solution. The mixture was left on ice for 20 min.
Another 2 mL of PEG solution was added, and the reaction mix was left for 5 min. at room temperature.
4 mL of Solution B (room temperature) was added, and the solution was mixed by gently turning upside down.
As a blank sample, 10 µL of TE buffer was treated the same way as the DNA mix, in order to monitor the success of the transformation.

Between 200 and 500 µL mL of the resulting solution was added to tempered (48 – 50 °C) overlay agar, vortexed briefly, poured on a PDA bottom agar plate (16 mL, containing hygromycin) quickly and spread over the surface by rotating it carefully.
The agar plates were incubated at 30 °C until until slight growth was visible (2 – 4 days).

Along with the plasmids a positive sample (containing protoplasts and TE buffer on PDA without hygromycin) and a negative sample (containing protoplasts and TE buffer on PDA with hygromycin) were incubated to assure the success of the transformation.

3.2.19 Calculation of the transformation rate

For calculation of the transformation rate the number of transformed protoplasts was set in relation to the applied plasmid amount.
Colonies grown on all plates containing the transformation preparation were counted and this number was divided through the applied plasmid DNA amount (per mg plasmid DNA). This ratio gives the transformation rate.

3.2.20 Cultivation of *T. reesei* (*H. jecorina*) in expression medium

Aliquots of 100 µL dark green spore suspension of recombinant and wild type *T. reesei* (in 0.8 % NaCl, 0.1 % Triton X-100) was added to 50 mL of MA medium (containing lactose) in a shake flask. The flasks were incubated at 28 or 30 °C while shaking at max. 200 rpm. Samples were drawn daily during the first three days. The samples were centrifuged, the supernatant was used for Western blot analysis and the mycelium was used for Northern blot analysis. The water was squeezed out, the mycelium was quickly frozen in liquid nitrogen and kept at -20 °C until further work.

3.2.21 Labeling a DNA probe

A fragment of DNA served as PCR template for a non-radioactive labeled probe as product. The DIG labeling kit by ROCHE DIAGNOSTICS was used. The DIG labeling system provides a non-radioactive method for labeling DNA probes. DIG (digoxigenin) is a plant steroid that can be covalently linked to nucleotides like dUTP. DIG dUTP can be integrated into the nucleotide sequence of a PCR product by the *Taq* polymerase. The resulting labeled probe can then be used for hybridization.

An antibody that binds specifically to DIG is used for detection. Alkaline phosphatase is attached to the antibody, which reacts with the chemoluminescent substrate CSPD and sets free photons. This can be visualised by exposure on an x-ray film, delivering a signal proportional to the content of digoxigenin.

Procedure:

Tab. 15: Pipetting scheme for DNA probe marking

sample		**blank sample**	
reagent	amount	reagent	amount
5 x *Taq* buffer	10 µL	5 x *Taq* buffer	10 µL
Taq polymerase	0.25 µL	*Taq* polymerase	0.25 µL
dNTP mix (including DIG-dUTP)	5 µL	dNTP mix (normal)	5 µL
Primer forward [10 pmol / µL]	2 µL	Primer forward [10 pmol / µL]	2 µL
Primer reverse [10 pmol / µL]	2 µL	Primer reverse [10 pmol / µL]	2 µL
template	1 µL	template	1 µL
H2O	29.75 µL	H2O	29.75 µL
Total Volume	50 µL	Total Volume	50 µL

3.2.22 Immuno-blotting

Western blot analysis and Northern blot analysis were performed as described in the DIG application manual by ROCHE DIAGNOSTICS.

3.2.23 PDH deglycosylation

3.2.23.1 PDH deglycosylation with α-mannosidase

α-mannosidase cleaves all α(1-2, 3, 6) linked mannose residues. 0.2 mg α-mannosidase / mg PDH is needed.

Tab. 16: Pipetting scheme for α-mannosidase deglycosylation

PDH (2 mg / mL)	10 µL
denaturing buffer	5 µL
H_2O	35 µL
10 x sodium citrate buffer (0.5 M)	6 µL
$ZnCl_2$ (0.2 M)	0.5 µL
α-mannosidase	1 µL

As a reference 10 µL of ovalbumin were used for the reaction. The reaction was incubated at 37 °C for several hours, samples were drawn after two and four hours. Unglycosylated PDH and ovalbumin were used as reference material on an SDS gel.

3.2.23.2 PDH deglycosylation with α-mannosidase and Endo Hf

α-mannosidase cleaves α(1-2, 3, 6) linked mannoses whereas Endo Hf cleaves only high-mannose structures. α-mannosidase needs zink-ions for activity.
For deglycosylation of the PDH 0.2 mg α-mannosidase / mg PDH and 10000 U Endo Hf / mg PDH are required. As a reference CDH (cellobiose dehydrogenase) was also deglycosylated.

Tab. 17: Pipetting scheme for α-mannosidase and Endo Hf deglycosylation

	PDH deglycosylation	**CDH** deglycosylation (reference)
PDH (2 mg / mL)	62.5 µL	-
CDH (10 mg / mL)	-	100 µL
50 mM sodium citrate, pH = 5.5	181 µL	849 µL
2 M $ZnCl_2$	0.25 µL	1 µL
α-mannosidase (5 mg / mL)	5 µL	40 µL
Endo Hf (1000 U / mL)	1.25 µL	10 µL
total volume	250 µL	1000 µL

The reaction mix was incubated at 37 °C for several hours, samples were drawn for visualization on SDS gels.

3.2.23.3 PDH deglycosylation with N-Glycosidase F (PNGase F)

PNGase F cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins.

Tab. 18: Pipetting scheme for PNGase F deglycosylation

PDH (2 mg / mL)	10 µL
10 x denaturing buffer	5 µL
H_2O	35 µL
denature 10 min. at 100 °C	
G7 buffer (sodium phosphate, 0.5 M, pH = 7.5	6 µL
PNGase F	1 µL

The reaction was incubated at 37 °C for one hour. As a control, ovalbumin (2 mg / mL stock solution) was incubated as well.

3.2.24 Dansyl hydrazine staining of PDH-bound carbohydrates

For the detection of glycoproteins after polyacrylamide gel electrophoresis this sensitive method was used. Sugars are oxidized to aldehydes by treatment with periodic acid and acidic acid. The aldehydes are condensed to hydrazones with dansyl hydrazine, then reduced to hydrazine derivatives [38].

Working procedure:

- **fixation of the gel in 40 % ethanol and 5 % acidic acid**

200 mL were made for a 10 x 15 cm gel. The fixation was done at least one hour or overnight in a plastic tray. All the incubation steps were done on an orbital shaker.

- **oxidation of sugars to aldehydes**

A solution containing 0.7 % (w/v) periodic acid and 5 % acidic acid was produced (100 mL). The gel was incubated for one hour and afterwards washed with distilled water.

- **destruction of periodic acid excess**

A solution containing 0.5 % sodium disulfite and 5 % acidic acid was made (100 mL). The incubation time was 0.5 - 0.75 hours, until the gel was uncolored.

- **condensation of the aldehydes with dansylhydrazine to hydrazones**

50 mL of acetic acid / DMSO (dimethyl sulfoxide) - solution (0.6 mL HCl / L DMSO) were blended with 50 mL of dansyl hydrazine solution (2 mg dansyl hydrazine / mL DMSO), and the gel was incubated in this solution for one hour at 60 °C.

- **reduction to hydrazine derivatives**

A solution of KBH_4 in DMSO was made (0.2 mg KBH_4 / mL DMSO), 100 mL were produced. The gel was incubated in the solution for 30 min. at room temperature.

The gel was washed with water and subsequently destained for 5 min. in 1 % acidic acid.

After destaining the gel in acidic acid the fluorescent-labeled glycoproteins can be visualized with a long-range uv light source.

For staining Phasts gel with the dansyl hydrazine method the volumes as well as the incubation times were halved.

4 Results

4.1 Construction of vectors for T. reesei (H. jecorina) transformation

4.1.1 Construction of a *cbh1*-vector for PDH

For the construction of vectors for transformation containing parts of the *cbh1* gene three different strategies were mapped out, two of them had to be dropped, one strategy was pursued. *cbh1* vector constructions as well as creation of *pdh* containing plasmids pDF18, pDF38, pDF40, pDF41, and pDF42 were performed by Christoph Sygmund (unpublished data) and Dorothea Fragner [35].

4.1.1.1 Strategy I

The first strategy was to use two different multiple cloning sites (MCS) of the plasmid pAMH 110 (T. Pakula, VTT Biotechnology, Finland) for insertion of a hygromycin resistance cassette in one and the *pdh* coding sequence in the other MCS (after the *cbh1* signal sequence, between the *cbh1* promoter and the *cbh1* terminator sequence). The vector should then carry both the antibiotic resistance gene and the *pdh*. The *pdh* and *hph* gene should be integrated ectopically, which means somewhere in the genome, randomly.

pdh from *A. bisporus* was amplified through PCR by the use of the Primers Trichfw1 (forward, introducing a *BamH*I restriction site) and BspSachrv1 (reverse, introducing a *Nde*I restriction site), with the plasmid pDF18 (containing the *pdh* cDNA) as a template. The annealing temperature was 50 °C and the extension time 2 min. (product: ca. 1.8 kb long 'PCRTRICH1').

A part of one MCS of pAMH110 was excised by digestion with the restriction enzymes *Kpn*I and *Stu*I. The linearized plasmid was blunted with the Klenow fragment and the ends ligated at 16 °C overnight ('pDF40').
The DNA fragment containing the *pdh* gene was ligated into the modified pAMH110 vector. Therefore PCRTRICH1 and pDF40 were digested with *BamH*I and *Nde*I and ligated ('pDF41').
In the last step the hygromycin phosphotransferase (hph / hpt / aphIV; enzyme detoxifies hygromycin B) expression cassette from the vector pAN7.1 [34] should be ligated into pDF41. pAN7.1 was digested with *Bgl*I and *Xba*I, the ends of the digestion product were blunted. pDF41 was digested with Sac, the ends blunted and dephosphorylated with shrimp alkaline phosphatase (SAP). Since the final ligation failed to work out this strategy was dropped.

4.1.1.2 Strategy II

The second attempt was to produce a construct that carries the *pdh* gene between the *cbh1* promoter and the *hph* terminator and the *hph* gene between the *hph* promoter and the *cbh1* terminator. This construct should integrate itself into the genome by homologous recombination.

The *hph* expression cassette was cloned into the vector pBluescript II, which led to the vector 'pAN 8'. The *hph* terminator was cut out by digestion of pAN 8 with *Bam*HI and *Xba*I, production of blunt ends and ligation ('pDF38').
The *hph* terminator was amplified with PCR using the primers BspTrichfw2 and BspASrv2 with pAN7.1 as template ('PCRTERM'). The annealing temperature was 65 °C and the elongation time 1 min.
PCRTERM and pDF38 were digested with *Apa*I and ligated ('pDF42'). The correct orientation was verified through sequencing.
The modified *hph* expression cassette should at last be ligated in pDF41 (4.1.1.1). Therefore pDF41 was digested with *Nde*I and blunt ends were produced. pDF42 was digested with *Not*I and *Kpn*I to gain a 4 kb fragment, which was blunted and treated with SAP. Since the ultimate ligation could not

be performed successfully, like in strategy I, this approach had to be abandoned as well.

4.1.1.3 Strategy III

The third strategy was a co-transformation of a plasmid containing the *pdh* gene between the *cbh1* promoter and terminator (pDF41) and a plasmid carrying the hygromycin resistance gene (pAN8).

The transformation was done successfully and was performed as described in 3.2.18.

4.1.2 Construction of a *cbh2*-vector for PDH

Just like for the third and effective attempt for the transformation vectors here again a co-transformation was performed to introduce the *pdh* gene on the one hand and the *hph* gene on the other hand. The difference was that a *pdh* construct was made that was linked to the *cbh2* expression cassette instead of the *cbh1* cassette. Additionally, a thrombin site was introduced for detection and purification of the PDH.

Initially, the DNA sequence of the *cbh2* gene was amplified by iproof PCR by the use of the primers cbh2fw1 and cbh2rv1. A ca. 4 kb long was obtained that was subsequently cloned into the pCR-Blunt II-TOPO vector ('pWS1').
A fragment containing the *cbh2* promoter and the coding sequence of *cbh* was produced via iproof PCR, using the primers cbh2fw1 and cbh2rv2. pWS1 was used as template and a ca. 2.8 kb long fragment was obtained. The reverse primer cbh2rv2 was designed to introduce an *Apa*I restriction site as well as a thrombin recognition site. The amplified fragment was digested with *Apa*I, leading to a 5' blunt end and a 3' *Apa*I end.

The *pdh* cDNA from *A. bisporus* was amplified by iproof PCR with the primers cbh2fw3 (containing an *Apa*I restriction site) and Bspsachrv1 (containing a *Nde*I restriction site). A ca. 1.9 kb fragment was obtained that was digested with *Apa*I, leading to a 5' *Apa*I end and a 3' blunt end. The two digested fragments were ligated and cloned into the pCR-Blunt II-TOPO vector ('pWS3'). After transformation into *E. coli* positive transformants were screened by PCR using the primers cbh2fw1 and BspPDHrv3.

Amplification of the *cbh2* terminator was done by iproof PCR with the primers cbh2fw4 (carrying a *Nde*I restriction site) and cbhrv4 (carrying a *Not*I restriction site), providing a ca. 1 kb long fragment ('pWS2'). pWS2 and pWS3 were digested with *Nde*I and *Not*I. Finally, pWS2 and pWS3 were ligated, resulting in pWS4.

pWS4 was used for co-transformation in *T. reesei* together with the vector pAN7.1 and pLH1 respectively, which carried the *hph* cassette. Three different templates of pWS4 were used for transformations, pWS4 undigested, pWS4 digested with *Kpn*I and pWS4 digested with *Kpn*I and *Not*I.

4.2 cbh2 fusion protein

Fig. 6 shows an overview over the final *cbh2* / *pdh* construct that was used for *T. reesei* (*H. jecorina*) transformation and expression attempts. It consists of the *cbh2* promoter and signal sequence, the *cbh2* coding sequence (containing introns), a 18 nucleotide thrombin recognition site, the *pdh* coding sequence (without introns) and the *cbh2* terminator. The thrombin site should be used to facilitate PDH purification steps after expression of the protein. The size of the whole construct was 5.6 kb. Each individual component was amplified by iproof PCR using the primers stated in Tab. 4, the components were cloned into the pCR Blunt II TOPO vector and the plasmids transformed into and expressed in *E. coli*, as described in 3.2.7. After restriction digest the components were ligated together using T4 ligase.

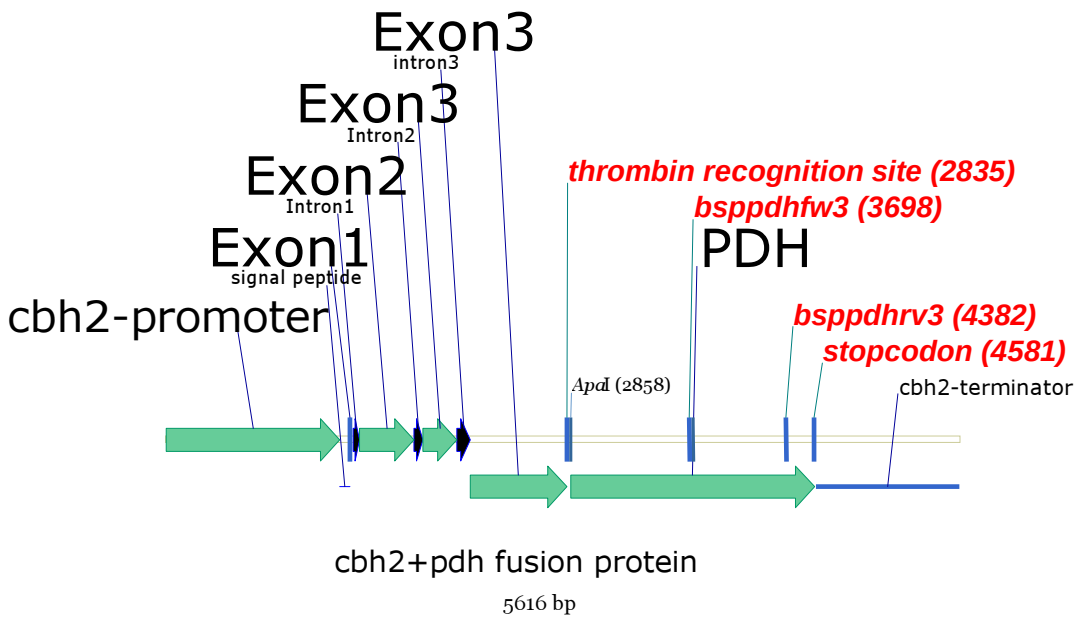

Fig. 6: *cbh2* / *pdh* construct

4.3 Screening for positive transformants

The transformants were grown 3 times alternating on selective medium (PDA with hygromycin) and normal medium (PDA without hygromycin) and back on normal medium at last.

Genomic DNA was isolated using the rapid mini-preparation method (3.2.13), the integration of the construct was checked with the use of PCR.
Spores of the clones were grown under inducing conditions in Mandels Andreotti liquid medium containing lactose. Samples were drawn after 24, 48 and 72 hours, each with 3 x 1 mL supernatant and mycelium.

A Western blot was made, using a *cbh2* antibody for detection. Samples of the day delivering the strongest signal were used for RNA isolation.
A Northern blot was made with the isolated RNA and 2 RNA gels were made, one for detection with a *pdh* probe and one for detection with a *cbh2* probe.

4.4 Transformation of the cbh1 vector into T. reesei (H. jecorina) and analysis of transformants

Transformation of the *cbh1* vector was performed with pDF41 as the *pdh* gene containing construct and pAN7.1 holding the *hph* expression cassette. The transformants were screened by 3 times alternating selective / normal medium cultivation. Genomic DNA from clones that survived the hygromycin screening was isolated using the rapid mini-preparation method (3.2.13). The integration into the genome was checked by two different PCRs with the primers pAMH1100fw2 and BspPDHrv3 on the one hand, and BspPDHfw3 and pAMH1100rv2 on the other hand, leading to fragments of 1.75 kb and 0.9 kb length.

Out of 30 screened transformants finally six positive clones remained: T4, T5, T6, T7, T8 and T9. Fig. 7 shows the result of a PCR screening of the positive clones and the wild type.

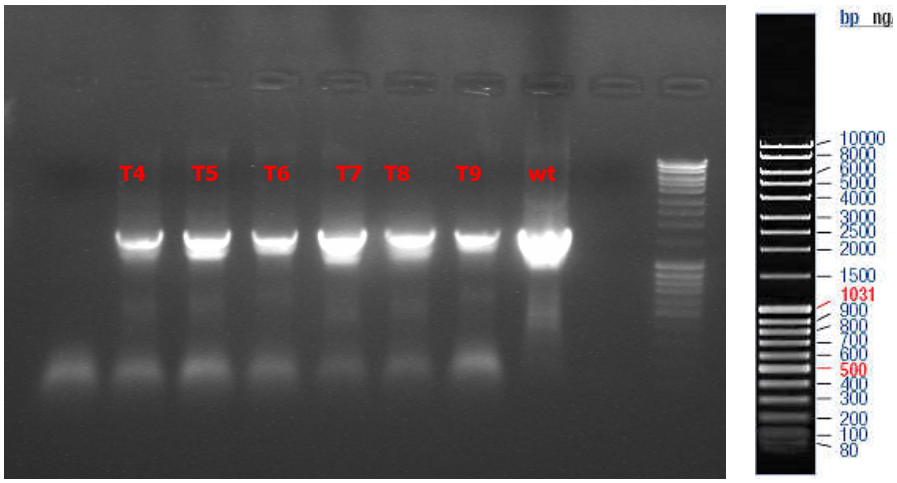

Fig. 7: PCR-screening of transformants with primers pAMH1100fw2 and BspPDHrv3

After determination of the integration into the genome positive clones were grown on MA medium under inducing conditions as described in 3.2.19, the wild type was also cultivated. Samples were drawn after 24, 48 and 72 hours, taking out 3 x 1 mL. Supernatant and mycelium were separated through centrifugation.

A Western blot analysis was performed with samples of the wild type and one positive clone, the detection was made with *Cel16*, a *cbh2* antibody. RNA was isolated from all clones from one cultivation day. Two RNA gels were made and a Northern blot analysis was made. The detection was done using a *pdh* probe and a *cbh1* probe.

4.4.1 Western blot analysis

As it can be observed in Fig. 8 CBH II is expressed from the second day of cultivation on, at the highest level after three days. RNA was isolated from the third day sample, and used for a Northern blot in order to visualize CBH I and PDH on the RNA level.

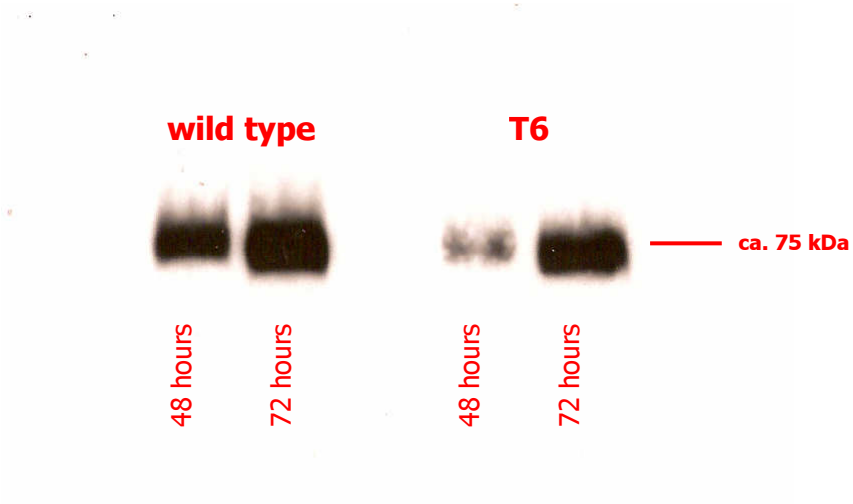

Fig. 8: Western blot of wild type and transformant T6 detected with *Cel16* antibody

4.4.2 Northern blot analysis

The x-ray film shows significant levels of the *cbh1* probe for the wild type and clones T4, T5 and T6. T9 shows a weak signal, whereas T7 and T8 provide absolutely no signal. The *pdh* probe could not be detected in all of the six clones.

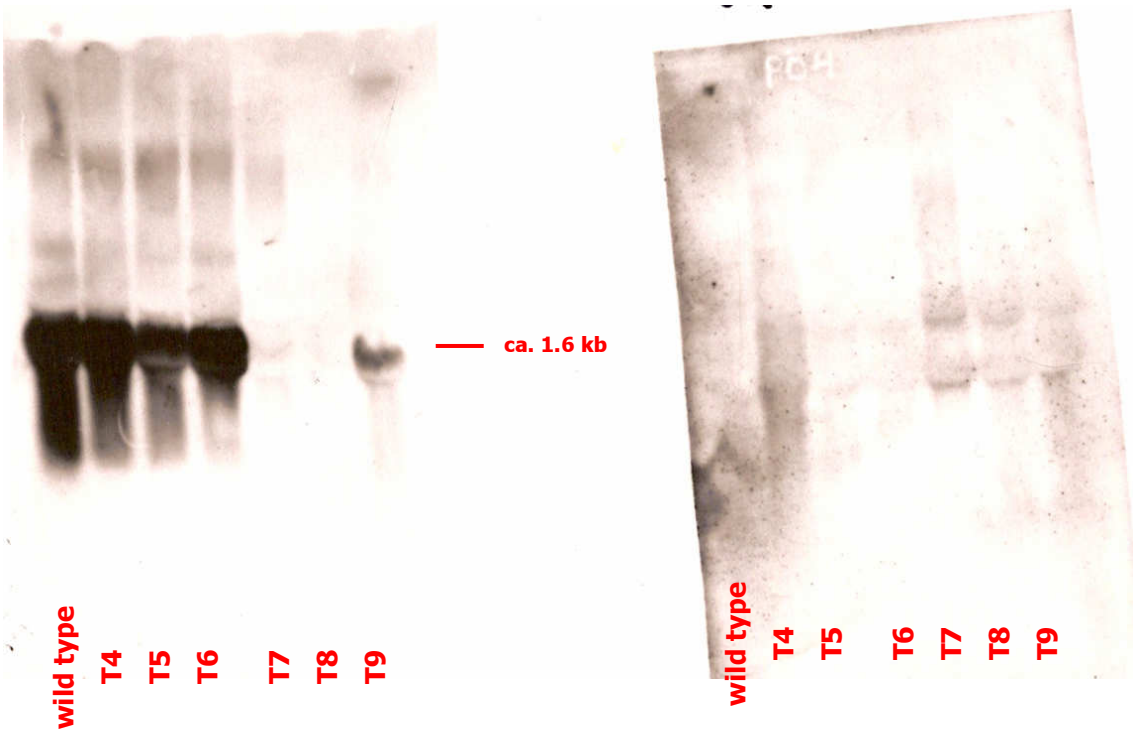

Fig. 9: Northern blot of a DIG labeled *cbh1* probe (left) and a DIG labeled *pdh* probe (right)

4.5 Transformation of the cbh2 vector into T. reesei (H. jecorina) and analysis of transformants

Contrary to former manuals PDA as bottom medium in combination with MM as overlay medium was used. Trials with MM + sorbitol bottom and overlay were less successful. Production of protoplasts was done several times, providing protoplast numbers between 8.8×10^6 and 1.2×10^8 per mL and so yielding an average of 6.9×10^7 protoplasts per mL, which was satisfying and corresponded to literature values.

Tab. 19: Protoplast counts from *T. reesei* transformations with the *cbh2* construct

transformation attempt no.	date	protoplast count
1	27.06.2006	1.2×10^8
2	20.07.2006	5.4×10^7
3	29.08.2006	5.3×10^7
4	25.08.2006	8.8×10^6
5	03.11.2006	1.2×10^8
6	20.11.2006	5.8×10^7

Transformations 1 – 5 were done with pAN7.1 containing the hygromycin resistance gene and pWS4, but the transformations did not did not result in positive transformants. Transformation 6 was done successfully using the plasmid pLH1 (also containing the *hph* cassette) and three different versions of the constructed plasmid:

'1' pWS4
'2' pWS4 cut with *Kpn*I (linear)
'3' pWS4 cut with *Kpn*I and *Not*I (→ only the insert)
(Therefore, clones from the different transformations were called 1/x, 2/y or 3/z.) For the co-transformation 2 µL of pLH1 (conc. ca. 0.5 µg / µL) and 8 µL of '1', '2' or '3' (conc. ca. 0.9 µg / µL) were applied.

Initially, 66 colonies grew on PDA containing 75 µg / mL hygromycin, 20 of them survived further hygromycin screening series and sporulated on PDA plates. Genomic DNA was isolated with the rapid method (3.2.13), the integration into the genome was tested by two PCRs, one with the primers cbh2fw6 and cbh2rv7, the other with cbh2fw6 and BspPDHrv6, resulting in ca. 1.4 kb long and ca. 1.6 kb long fragments.

Eight out of the 20 colonies were screened positively for integration of the constructed vector by PCR, those were the clones **1/3**, **1/8**, **1/11**, **1/12**, **2/16**, **2/19**, **2/25** and **3/7**. Fig. 10 and Fig. 11 show the result of the PCR screening.

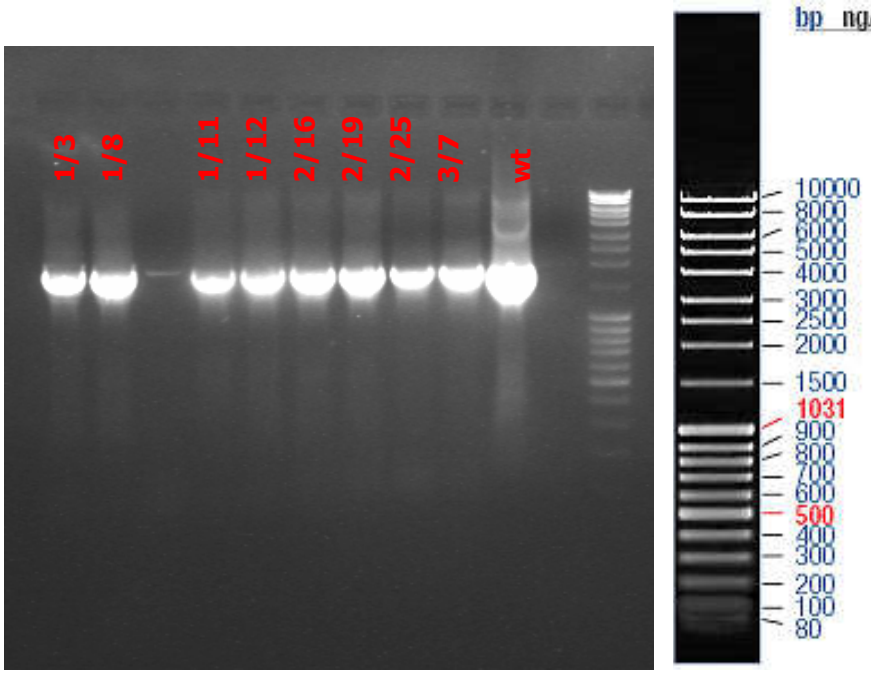

Fig. 10: PCR-screening of transformants and wild type with primers cbh2fw6 and BspPDHrv6

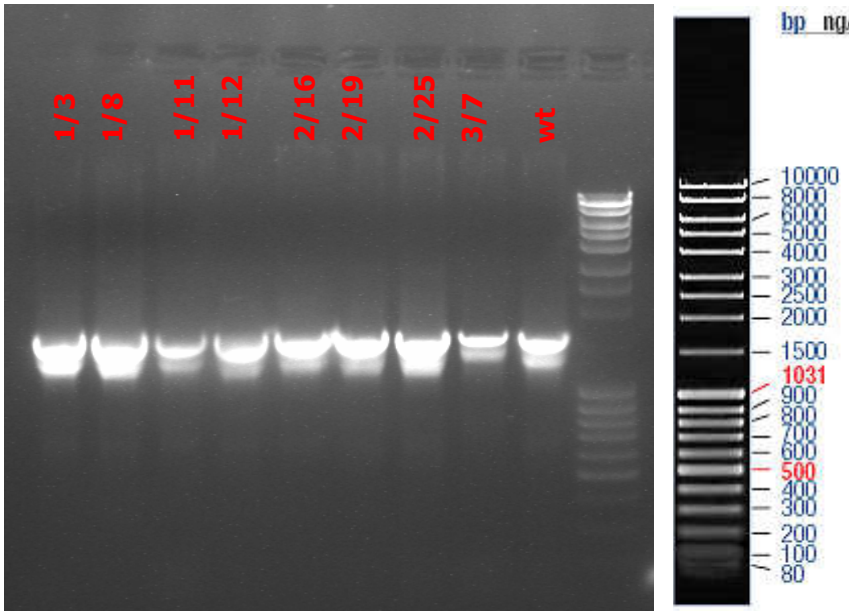

Fig. 11: PCR-screening of transformants and wild type with primers cbh2fw6 and cbh2rv7

Positive clones were grown in expression medium (3.2.19) and samples were drawn after 24, 48 and 72 hours. 3 x 1 mL were drawn and centrifuged to separate the mycelium from the supernatant. Western blot analysis was done from the wild type and from clone 1/3, detected with *Cel16* antibody. The

sample showing the strongest signal was identified, samples drawn the day before the day that specific sample was taken were used for RNA isolation. For the day providing the strongest signal a Western blot for all clones was performed. The isolated RNA was subjected to RNA electrophoresis, transferred to a nylon membrane (cf. Roche DIG application manual) and analyzed by Northern Hybridization.

4.5.1 Western blot analysis

On the x-ray film one can see that the level of CBH II expression is the highest after 72 hours of cultivation. The bands are at ca. 75 kDa (instead of 55 kDa), which is due to glycosylation. Samples of the day before the highest signal on the x-ray film (second day, 48 hours) were taken for RNA isolation (Northern blot).

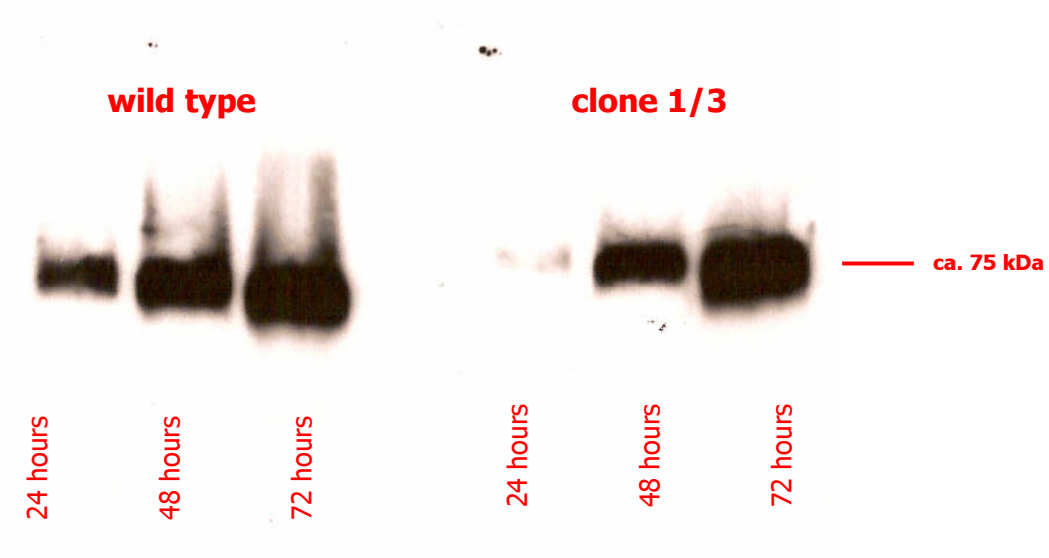

Fig. 12: Western blot of wild type and transformant 1/3 detected with *Cel16* antibody

4.5.2 Northern blot analysis

The x-ray film of the Northern blot shows that plenty of CBH II RNA is present in the wild type and all transformed clones, but no PDH RNA can be detected. Only the positive control (RNA from *A. bisporus*) can be observed on the film.

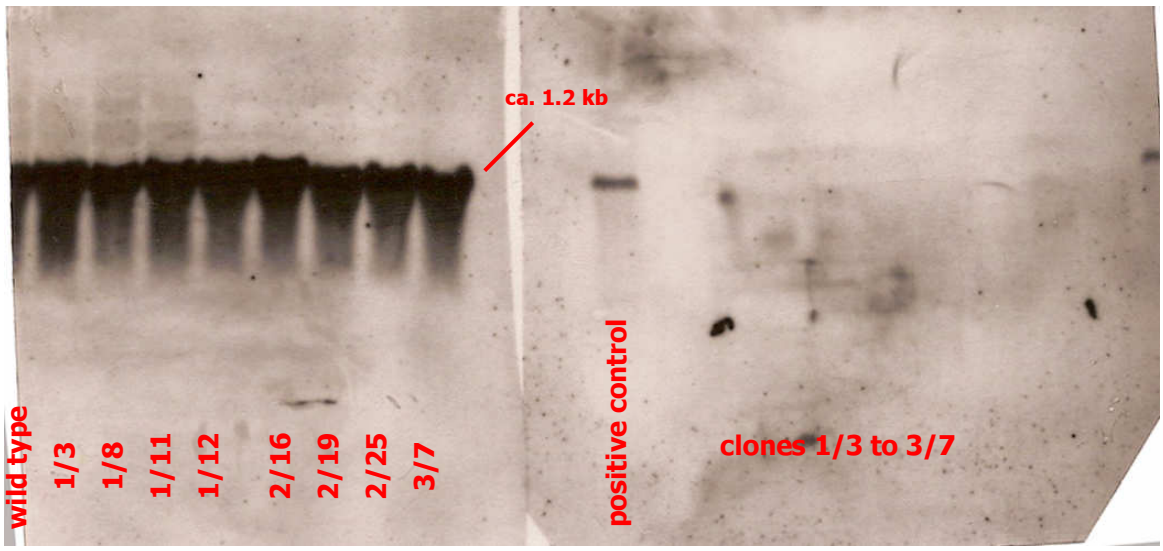

Fig. 13: Northern blot of a DIG labeled *cbh2* probe (left) and a DIG labeled *pdh* probe (right)

4.6 Deglycosylation of PDH from A. bisporus

Fig. 14 shows a PAA gel with samples of native and deglycosylated PDH as well as native and deglycosylated CDH as a reference. Sample PDH 'Roman' was a PDH sample previously deglycosylated by Roman Kittl, as well used as a reference. The glycosylation was done with Endo Hf and α–mannosidase (described in 3.2.23.2). The samples were mixed 1:1 with Laemmli buffer and boiled at 99 °C for 2 min. The amount of the samples applied on the gel are given in the illustration. 5 µL of the prestained dual color standard were applied on two lanes of the gel. The gel was stained using the Coomassie Blue method (3.2.12). The illustration shows a difference of the molecular mass of native and deglycosylated proteins that suggests a successful deglycosylation.

Fig. 14: PAA gel visualization of deglycosylation results (Endo Hf + α-mannosidase), colors modified for better visualization

With the aid of the known molecular mass of the standard bands in connection with the migration distance of the bands on the gel the molecular mass of native and deglycosylated PDH and CDH could be calculated (giving approximation values!). Fig. 15 shows the compensation curve for the standard, and Tab. 20 gives the calculations for estimation of the glycosylation rate. The calculations out of the PAA gel give results of ca. 7.3 % glycosylation of PDH and 10.8 % of CDH, which is about the expected value for both the proteins.

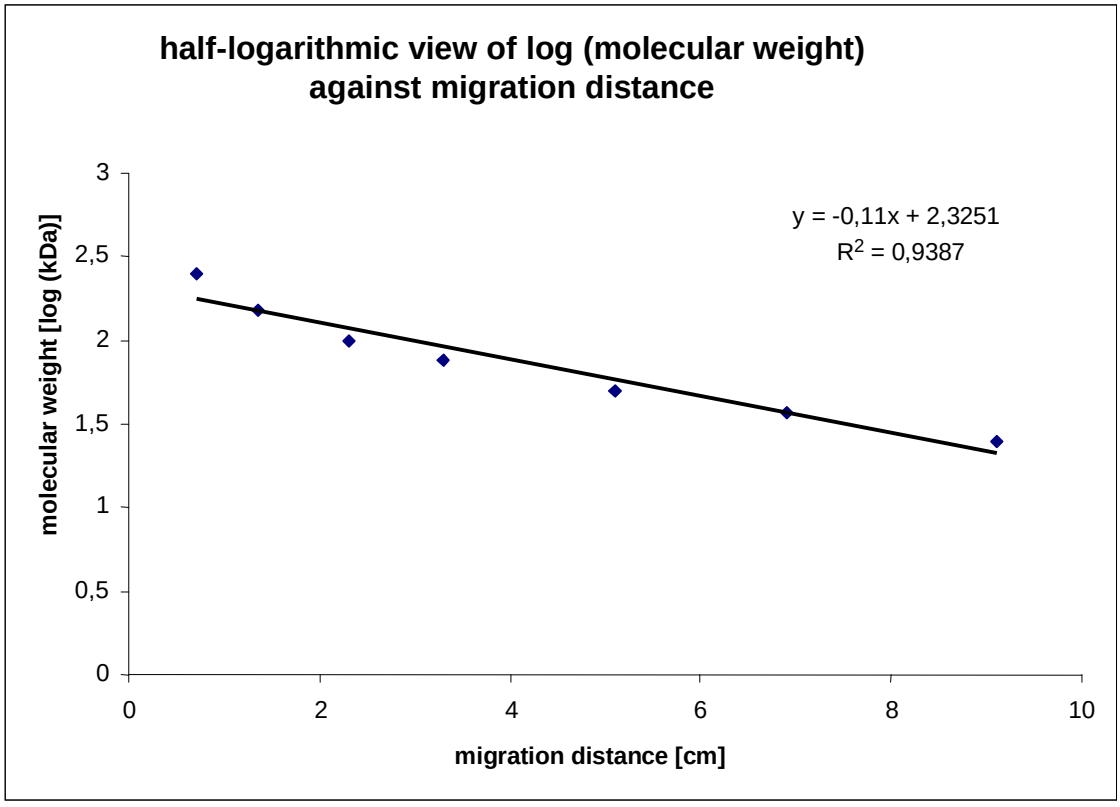

Fig. 15: Compensation curve for calculating molecular masses in a PAA gel

Tab. 20: Table for calculating mass differences of native and deglycosylated proteins

molecular standard dual color prestained	M [kDa]	log M	migration distance [cm]
	250	2,39794	0,7
	150	2,176091	1,35
	100	2	2,3
	75	1,875061	3,3
	50	1,69897	5,1
	37	1,568202	6,9
	25	1,39794	9,1

				difference [%] = glycosylation rate
PDH native a	80,74209244	1,9071	3,8	
PDH deglyc. 4h	74,83417927	1,8741	4,1	**7,3**
PDH deglyc. ON	74,83417927	1,8741	4,1	**7,3**
PDH native b	80,74209244	1,9071	3,8	
PDH deglyc. ‚ROMAN'	74,83417927	1,8741	4,1	**7,3**
				7,3
CDH native	124,1938242	2,0941	2,1	
CDH deglyc.	110,8153698	2,0446	2,55	**10,8**

Deglycosylation with Endo Hf and α-mannosidase was compared with deglycosylation with PNGase F. Fig. 16 shows a Coomassie Blue stained Phast gel with samples of native and deglycosylated PDH and ovalbumin and native P2Ox. Samples were mixed 1:1 with Laemmli buffer and boiled at 99 °C for 2 min. Samples were applied as described in (3.2.11). A difference between native and deglycosylated PDH is recognizable, but not too well, explained by the small size of the gel and the thereby weak separation performance. The illustration just served for a quick examination of the reaction result. It seems as if the two different deglycosylation methods are equally effective.

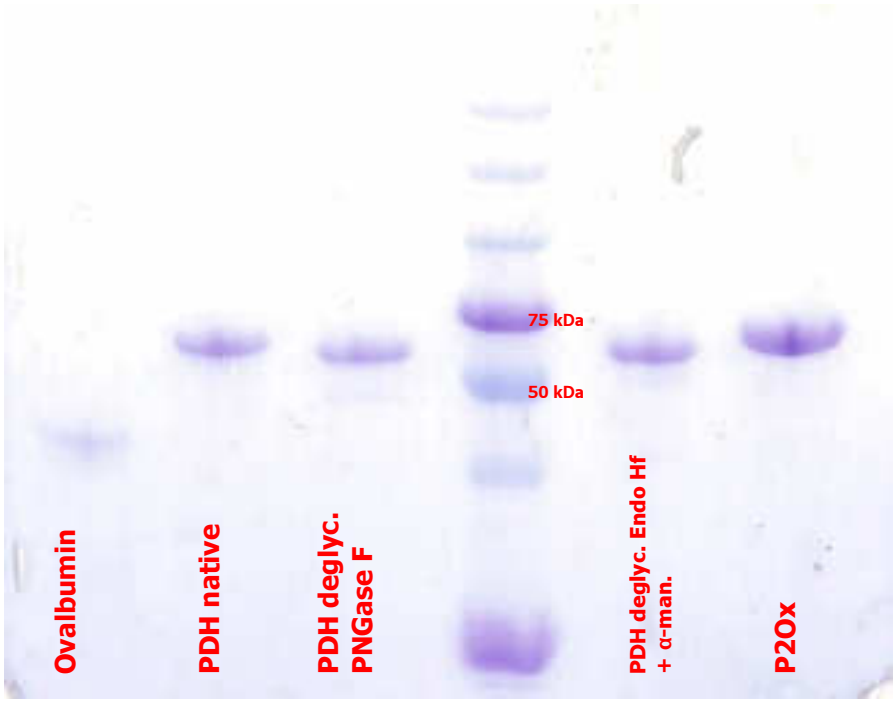

Fig. 16: Phast gel for comparison of deglycosylation strategies

4.7 Dansyl hydrazine staining of PDH-bound carbohydrates

The procedure of the staining method is described in 3.2.24. In the first experiment for dansyl hydrazine staining of PDH-bound carbohydrates on a PAA gel the visualization was not successful. The second time, using a small Phast gel to get results faster and more convenient, visualization was successful, but quite weakly, as it can be seen in Fig. 17. There are slight bands for the native PDH as well as for ovalbumin and no band for the negative sample P2Ox (not glycosylated). This suggests a successful carbohydrate staining, though visualization on the small Phast gel is quite unsuitable. It seems that the samples of deglycosylated PDH are somehow not totally deglycosylated or even not at all, because faint bands are visible for those samples (indicated in Fig. 17).

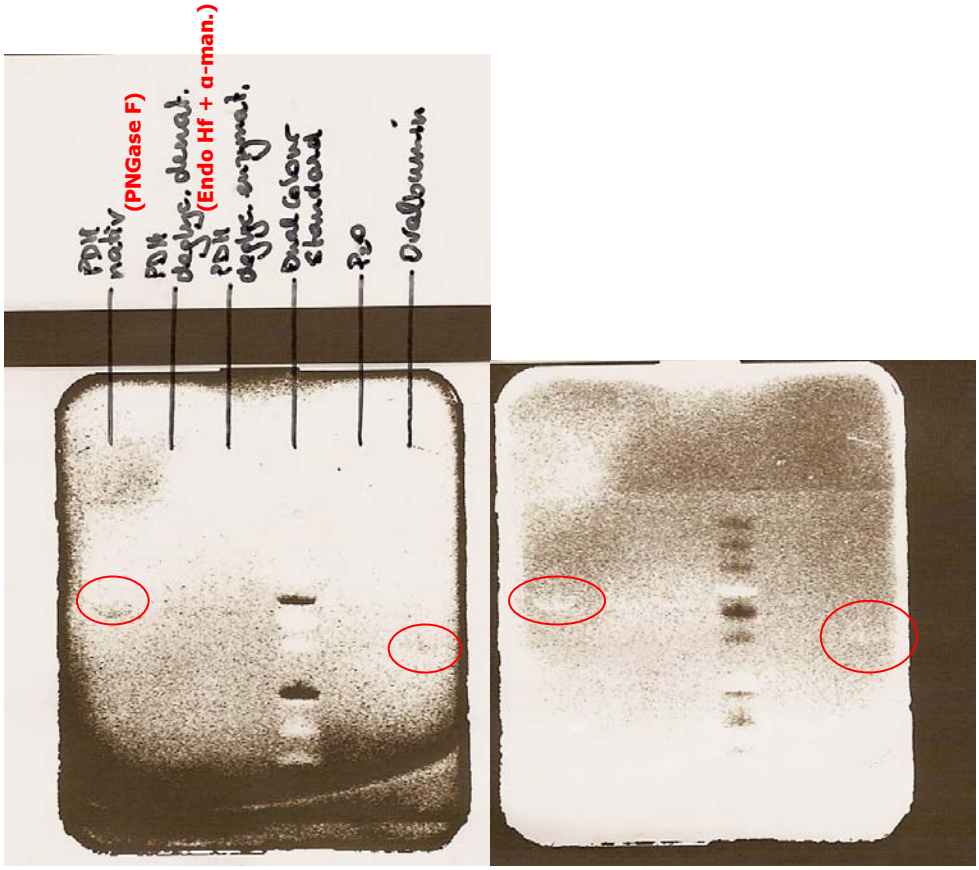

Fig. 17: SDS Phast gel dansyl hydrazine staining; positive (left) and negative (right)

Another attempt on dansyl hydrazine staining was made with a PAA gel containing native and deglycosylated PDH samples, P2Ox as a negative control and albumin as a positive control (Fig. 18). Native PDH shows a band for stained carbohydrates, as expected, but also the Endo Hf / α-mannosidase deglycosylated gives a positive signal. The band of albumin as a positive control is very weak and therefore can hardly be interpreted as a positive signal. The negative sample P2Ox expectedly shows no band. PNGase F deglycosylated PDH shows an extremely weak signal, which may be caused by residual sugars through incomplete deglycosylation.

Fig. 18: PAA gel dansyl hydrazine staining

5 Discussion

5.1 Vector construction

Construction of expression vectors either linked to the *cbh1* promoter or to the *cbh2* promoter turned out to be rather time-consuming, since it contained several crucial steps that required severalfold repeats of work steps.

Firstly, the PCR conditions for amplification of particular DNA fragments using iproof polymerase had to be adapted in each case in order to get proper products free of undesired side-products. Therefore annealing temperatures were increased, leading to the wanted amplicons. *Pfu* polymerase was used alternatively in the beginning, but PCRs using iproof polymerase proved to be more efficient in the present case. Since some DNA fragments were quite large (e.g. the *cbh2* coding region with about 4 kb or the CBH II / PDH fusion protein with ca. 5.6 kb) PCR amplification as well as blunt end cloning into the TOPO vector required repeats of the procedure (possibly using altered reaction conditions).

Blunt end ligations represented the limiting step in some of the experiments, offering the idea of considering different strategies for future construction design if possible. In addition to basic problems concerning blunt end ligations another limiting factor was the size of resulting plasmids (up to 9 kb in case of the CBH II fusion protein in the TOPO blunt vector). Another issue was the fact that blunt-ended fragments could be ligated to themselves, leading to AA or BB fragment patterns instead of correct AB ligations, including the possibility of two different orientations of each fragment. Plasmid transformation into and multiplication in *E. coli* of the different plasmids (DNA fragment in the pCR II TOPO blunt vector) was elaborate, since usually several attempts were needed to obtain clones that contained the desired plasmid. *Taq* polymerase PCRs with volumes of 25 µL (instead of 50 µL in other cases), running 30 cycles (instead of usually more) showed to be suitable for screening of transformants.

Dissolving of gel pieces containing DNA fragments or purification of PCR products respectively using the PROMEGA kit sometimes resulted in loss of DNA in dimensions of up to 50 %. There seemed to be residues that clung to the purification column. This problem could not be handled satisfactorily by compliance of the working instructions.

5.2 Transformation results

Growing of mycelium for protoplast production was successful with both the media MEA and PDA, but since the cultivation on PDA was much faster the experiments were done on this medium. Production of protoplasts did not pose a problem, since in every attempt sufficient protoplasts could be observed. At the beginning difficulties of controlling the working conditions for ten or more transformations simultaneously (for preliminary tests) affected the results immensely. Since speed and handling of the transformation procedure improved with every attempt, successful transformations could be performed at last. For the *cbh2*-linked construct co-transformed with pLH1 a transformation rate of 5.5×10^4 colonies per mg plasmid could be scored. The regeneration rate could not be determined since the plates containing the positive control of the transformation reaction (without plasmids, only with buffer) showed colonies that grew together so that counting of the colonies was not possible. 20 out of initially 66 colonies growing on hygromycin were left after antibiotic screening, resulting in eight clones including both the antibiotic resistance and the construct. This gives a resistance rate of 1.7×10^4 colonies per mg plasmid pLH1 and a rate of 6.7×10^3 colonies per mg plasmid pLH1 that included the construct.

Due to the performance of the transformations as co-transformations together with hygromycin-resistance carrying plasmids conclusions about whether the *pdh* containing construct was taken up into the genome could be drawn just after screening for PDH.

5.3 cbh1-linked vector analysis

As mentioned before, two out of three strategies had to be dropped by virtue of problems with final blunt end ligations. The successful strategy for vector construction provided a construct of the *pdh* gene under control of the *cbh1* promoter, nevertheless it was not possible to express PDH when cultivating the transformants that had taken up the construct into the genome. Since CBH II is expressed (proved by Western blot) the problem has to be associated to the construct and not to protein expression as such.

One reason could be that integration into the genome led to damage of the construct. Another possible explanation could be that the RNA was unstable (because of the construct size or some other reason). *cbh1* RNA could be detected for all the clones except for two (T5, T6) which may support the assumption of unstable RNA. It is also possible that the promoter was damaged so that in the end no enzyme expression could be achieved, or the vector was integrated in an area where no expression takes place. RNA of *cbh1* could be detected, whereas no *pdh* RNA could be found in any of the six clones.

5.4 cbh2-linked vector analysis

The results of the Western blot analysis show that CBH II is expressed, and Northern blot analysis shows that *cbh2* RNA is present in all the eight analyzed clones, whereas no *pdh* RNA could be detected.

As for the *cbh1*-linked construct the *cbh2*-linked construct may potentially got damaged during integration. The size of the construct RNA could also be a reason for the unsuccessful experiment, as well as unstable RNA, like mentioned before. It was not checked if the construct may have replaced native *cbh2*, which may have caused damage to the RNA. This damage may appear as a modified secondary structure of the RNA.

As well as for the *cbh1*-construct integration of the *cbh2*-construct could have taken place in an area of no expression, the construct or only the promoter might be damaged.

5.5 Deglycosylation

Deglycosylation of PDH was done using Endo Hf and α-mannosidase on the one hand and PNGase F on the other hand. The results seem to be about the same, proved by several attempts. Visualization was not too significant on small Phast gels and even not on larger PAA gels. For quantification of small glycosylation rates like in the case of PDH the use of larger PAA gels is recommendable, even though the Phast system is unbeatable concerning speed and convenience. If significant conclusions are required small gels are only suitable for preliminary tests.

The calculated value of 7.3 % of deglycosylation of the 10 x 15 cm polyacrylamide gel proved to be close to determinations provided by literature. The glycosylation rate of CDH as a reference with 10.8 % is close to the literature value.

Therefore deglycosylation with Endo Hf and α-mannosidase seem to be adequate for PDH and CDH deglycosylation, PNGase F also seems suitable for PDH deglycosylation.

5.6 Dansyl hydrazine staining

The dansyl hydrazine staining method should represent an effective method for visualization of carbohydrate residues bound to proteins. In this case it was successfully used to detect carbohydrates, but not to the expected degree. The results were rather undetermined for some samples, giving unspecific bands, while the performance of the method was quite time consuming. In order to get acceptable results the method needs to be optimized in performance, which

could not be done during this thesis because of lack of time. Repeated practice of the method and consequently better handling will certainly lead to more satisfying results.

5.7 Future prospects

In order to be able to exploit the strong *T. reesei (H. jecorina)* promoters for heterologous PDH production the problems concerning expression have to get solved and a closer look at where and why protein synthesis gets stuck has to be taken. Additionally, it will be necessary to know where in the genome the constructed vectors integrate. The challenge will be to establish a stable expression system for heterologous protein production in satisfactory amounts, with adequate protein folding and glycosylation.

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