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Pantothenic Acid Catabolism in *Trichoderma reesei*

ausgeführt am
Institut für Verfahrenstechnik, Umwelttechnik und technische Biowissenschaften
der Technischen Universität Wien

unter der Anleitung von
Priv. Doz. Dr. Bernhard Seiboth und
Dr. Robert Bischof

durch

Alexander Beinhauer, BSc.
Bandgasse 7/3/11
1070 Wien

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Datum Unterschrift

Zusammenfassung

Der Enzym produzierende Pilz *Trichoderma reesei* ist ein idealer Kandidat für die Verzuckerung von pflanzlicher lignozellulosischer Biomasse zu löslichen Zuckern, die zu Bioethanol oder anderen Bioraffinerie Produkten umgewandelt werden können. Für die Produktion von Lignozellulose abbauenden Enzymen sind regulierbare Expressionssysteme notwendig, die weder das Pilzwachstum noch die Enzymproduktion beeinflussen. Pantothensäure ist ein dafür geeigneter Induktor, der die Expression von einigen in einem Cluster angeordneten Genen in *T. reesei* auslöst. Die Gene dieses Clusters kodieren für eine Pantothenat Permease, einen $Zn(II)_2Cys_6$ Transkriptionsfaktor und vier Enzyme, die vermutlich in den Katabolismus von Pantothenat involviert sind. Die ersten beiden Enzyme des Stoffwechselweges, die Pantothenase (PAN1) und die Pantoat 4-Dehydrogenase (PAN2) wurden in *Escherichia coli* unter Verwendung des pET21a(+) Expressionssystems überexprimiert. Es wurden Enzymassays für diese beiden Enzyme entwickelt und die rekombinant produzierten Enzyme teilweise charakterisiert. Analysen der Gendeletionsstämme des Pantothensäurestoffwechsels zeigten, dass eine Deletion des *pan1* Gens zu einem höheren und stabileren Transkriptionslevel der anderen induzierbaren Gene des Clusters führt, was mit einer höheren Konzentration von Pantothenat in diesen Stämmen einhergeht. Zusammengefasst erbringt diese Arbeit einen biochemischen Beweis dafür, dass die Gene des Clusters für Enzyme kodieren, die in den Pantothensäureabbau involviert sind und die Manipulation des Abbauweges dazu benutzt werden kann, um den Transkriptlevel in dem Pantothensäure induzierbaren Expressionssystem zu erhöhen.

Abstract

The fungal enzyme producer *Trichoderma reesei* is an ideal candidate for the saccharification of lignocellulosic plant biomass to soluble sugars, which can be converted to e.g. bioethanol or other biorefinery products. For the production of lignocellulolytic enzymes tuneable promoter systems are needed which do not influence fungal growth and enzyme production. Pantothenic acid is such a candidate inducer that triggers expression of several clustered genes in *T. reesei*. The genes of this cluster encode a putative pantothenate permease, a fungal $Zn(II)_2Cys_6$ transcription factor and four enzymes putatively involved in the catabolism of pantothenate. The first two enzymes of the pathway, the pantothenase (PAN1) and the pantoate 4-dehydrogenase (PAN2) were overexpressed in *Escherichia coli* using the pET21a(+) expression system. Assays were developed for the two enzymes and the enzymes partially characterized. Analysis of gene deletion strains in the pantothenic acid pathway showed that deletion of *pan1* leads to higher and more stable transcript level of the other pantothenic acid cluster genes putatively due to a higher level of pantothenate in these strains. In summary these results provide biochemical evidence that the genes present in the pantothenic acid inducible gene cluster encode enzymes involved in the pantothenic acid degradation and that their manipulation can be used to enhance the transcript levels in the pantothenic inducible expression system.

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Einleitend möchte mich an dieser Stelle herzlichst bei all denen bedanken, die mich bei meiner Diplomarbeit unterstützt und motiviert haben.

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Table of Contents

Disclaimer

Parts of this thesis will be used in a research paper on pantothenic acid catabolism in *T. reesei*.

List of Figures

List of Tables

1. Introduction

1.1. *Trichoderma reesei*

Recent demands for new fuel sources, caused by environmental problems related to the usage of fossil fuels and its decreasing availability, called for targeted research towards cleaner and sustainable fuels. Second generation biofuels, derived from the abundant and renewable lignocellulosic biomass, are a promising alternative for the upcoming years to replace fossil fuels [1]. Filamentous fungi such as *Trichoderma reesei* have the ability to degrade plant cell wall polymers efficiently by the secretion of cellulases and hemicellulases and are therefore ideal candidates for saccharification of plant biomass to soluble sugars which are in the next step converted by other microorganisms such as *Saccharomyces cerevisiae* to ethanol. Furthermore other approaches to produce building block chemicals or specialty chemicals are at the moment developed in so called biorefinery concepts [2]. Today, *T. reesei* is the main industrial production organism for cellulases and the fermentation technology for this organism is already well established as it is used for homologous or heterologous production of proteins for about 25 years [3].

Lignocellulosic biomass consists mainly of cellulose, hemicellulose, lignin and pectin which are setup in a complex composition making its degradation difficult. Pretreatment steps are necessary to make the cellulose accessible for the enzymes [4, 5]. The presence of lignocellulosic biomass in the medium induces the expression and secretion of different cellulases and hemicellulases in *T.reesei*, including for example endoglucanases which hydrolyze internal bonds of cellulose, cellobiohydrolases which cleave cellobiose from both ends of the cellulose chain which leads to oligosaccharides of different sizes. Intra and extracellular ß-glucosidases further degrade these oligos to ß-D-glucose [6]. Apart from the degradation of lignocellulose for bioethanol production these enzymes are currently used for pulp, paper, food and textile industries [6, 7].

1.2. Tools for Recombinant Protein Expression

For genetic manipulation of *T. reesei* there is already a multifunctional genetic toolbox established [8], useful for transformation, high throughput gene deletions or gene expression. Important tools for functional genomics and biotechnology are promoters, enabling the controlled expression of a target gene. Beside a number of constitutive promoters, tuneable promoters including inducible and repressible promoters are available which are also able to decouple cell growth from protein production. The presently most efficient promoter of *T. reesei* is the *cel7a* promoter of the cellulase cellobiohydrolase 2 [6]. Currently expression systems in *T. reesei* are limited to promoters of different xylanase and cellulase genes. These promoters can influence cellulase production by titrating positive transcription factors necessary for cellulase gene transcription and their presence can

therefore also have an influence on growth on cellulose containing media. Therefore alternative promoters are necessary to drive recombinant protein production. Ideally an inducible promoter is induced by a cheap, easy available and non-toxic substance to a high transcription level. In addition it should not influence cellulase production and growth of the fungus. Such promoters were recently investigated and developed, with pantothenic acid being such a promising inducing substance that showed to trigger gene expression of a number of genes present in a novel fungal gene cluster in *T. reesei* (*vide infra*). Induction of the different genes of the cluster was found even at concentrations far below 1 mM (R.Bischof, A. Beinhauer, C.P. Kubicek and B. Seiboth, unpublished results).

1.3. Pantothenic Acid

Pantothenic acid was discovered by Dr. R. J. Williams in 1933 and is commonly known as vitamin B_5 [9]. It is soluble in water and an amide of pantoic acid and ß-alanine. Pantothenic acid is used for the biosynthesis of acyl carrier protein (ACP) and Coenzyme A (CoA) which are needed as an acyl group carrier in metabolism of lipids, carbohydrates and proteins. A deficiency in pantothenic acid is very uncommon for humans, as it is taken up throughout a wide range of foods. Pantothenic acid rich sources are liver, kidney, yeast, fish, avocado, yogurt and whole grains. There is no recommended daily allowance stated for pantothenic acid, but an adequate intake level of 5 mg/day is recommended [10]. Pantothenic acid deficiency in humans was induced by adding an antagonist and common symptoms observed were depression, cardiac instability, personality changes, fatigue, insomnia and headaches [11]. Pantethine, a derivative of pantothenic acid, proved to be effective in decreasing cholesterol. The use of pantethine as a nutritional supplement at 600 – 900 mg/day leads to a significant decrease of low density lipoprotein cholesterol (LDL-C) and total cholesterol which lowers the risk of cardiovascular disease [12]. Pantothenic acid itself is supplemented as either calcium pantothenate or pantothenol as they are more stable. It is used preventative as a supplement in food and in cosmetics and it showed to decrease facial acne [13]. Dexpanthenol is a precursor of pantothenic acid and improves wound healing. It is used as an anti-inflammatory drug when applied to the skin [14].

Concerning its high potential for increasing health there has always been interest in production of pantothenic acid. Williams and coworkers [15] first isolated pantothenic acid by concentration and purification from liver and achieved a first total synthesis of the pantothenic acid sodium salt by the condensation of the lactone with ß-alanine [16]. Pantothenic acid monophosphate was produced synthetically by condensation of pantolactone phosphate and ß-alanine [17]. Also several commercial companies filed a patent for preparation of pantothenic acid, e.g. Merck & Co. for the fusion of a ß-alanine salt with an α-keto or α-hydroxy-acid that lactonises or F. Hoffmann-La Roche & Co. for the condensation of D-pantolactone with ß-alanine [18]. As all the chemical procedures show

many disadvantages there have also been several attempts for a biotechnological production of pantothenic acid.

Pantothenic acid is an essential vitamin for animals whereas some bacteria, fungi and plants are known to be capable of pantothenic acid biosynthesis [19]. The *E. coli* pathway for pantothenate biosynthesis consists of 4 steps catalyzed by enzymes encoded by *panB (*ketopantoate hydroxymethyltransferase - EC 2.1.2.11*)*, *panC (*pantoate β-alanine ligase - EC 6.3.2.1*)*, *panD (*aspartate 1-decarboxylase - EC 4.1.1.11*)* and *panE (*2-dehydropantoate 2-reductase - EC 1.1.1.169*)* [20]. Bacterial biosynthesis finishes with a condensation of pantoate with ß-alanine both originating from 3-methyl-2-oxobutanoate and L-aspartate, respectively. There are several possibilities of pantothenic acid production in microorganisms as for example with *Azotobacter vinelandii* in a chemically defined medium [21], in *E. coli* using ATP regeneration [22] and there is also a patent for fermentative production in Enterobacteriaceae strains [23]. Sahm *et al.* [24] achieved D-pantothenate synthesis in *Corynebacterium glutamicum* with a production of up to 1g/l. Interestingly *S. cerevisiae* does not have a *panD* homologoue and uses a different way of synthesizing ß-alanine by oxidation of spermine with an amine oxidase encoded by *FSM1* [20]. As this step was rate limiting the overexpression of FSM1 led to secretion of pantothenic acid into the medium due to its overproduction.

1.4. Pantothenic Acid Transport and Catabolism

The *Schizosaccharomyces pombe liz1* gene is homologous to the *FEN2* and *VHT1* genes of *S. cerevisiae*. Those genes are responsible for the transport of pantothenate and biotin through the plasma membrane. Stolz *et al.* [25] were able to express *liz1* in a *S. cerevisiae FEN2* knockout mutant and showed retained cell growth on plates containing pantothenate, whereas the *ΔFEN2* strain did not grow on pantothenate which suggested *liz1* being a pantothenate transporter. Furthermore they proposed that higher extracellular concentrations of pantothenate lead to its uptake even in *Δliz1* knockout mutants and that the pantothenate transporter is inevitable only at lower concentrations than 1µM. The *S. pombe Δliz1* strain showed to be still viable through pantothenate synthesis from uracil but it showed defects in cell division most probably because of the excessive uptake of uracil itself.

Pantothenic acid, being either synthesized *in vivo* or taken up from the environment, is then converted into CoA throughout five enzymatic reactions [19]. The enzymes catalysing the reactions of this pathway are in the order of the reaction: pantothenate kinase (EC 2.7.1.33), phosphopantothenoylcysteine synthetase (EC 6.3.2.5), phosphopantothenylcysteine decarboxylase (EC 4.1.1.36), phosphopantetheine adenyltransferase (EC 2.7.7.3.) and dephospho-CoA kinase (E.C. 2.7.1.24).

1.5. Pantothenic Acid Metabolism in *T.reesei*

As already mentioned above, a novel gene cluster was discovered in *T. reesei* which contained different genes induced by the addition of pantothenic acid. This gene cluster contains 6 genes located within an area of around 12 kb. A fungal $Zn(II)_2Cys_6$ transcription factor present in this cluster was already characterized and showed to be the activator for the other genes of this cluster [26]. The cluster contains further a gene encoding a permease orthologue of the *liz1* gene of *S. pombe* which is therefore most likely responsible for the pantothenate transport and four genes encoding different enzymes. Pantothenic acid catabolism has till now only been described in more detail in the bacterium *Pseudomonas* P-2 including the purification and enzyme characteristics of the different enzymatic components of the pathway. However, the gene or protein sequences were until now not reported. Nurmikko *et al.* [27] characterized the pantothenate hydrolase as an enzyme showing activity in degrading pantothenate to ß-alanine and pantoate when the organism is grown on pantothenate. The second step of the degradation was described by Goodhue *et al.* [28] as an oxidation of pantoate into R-4-dehydropantoate via pantoate dehydrogenase. Finally R-4-dehydropantoate is further oxidized into R-3,3-dimethylmalate and then decarboxylated to 3-methyl-2-oxobutanoate [29]. Based on this information combined with an in silico analysis of the enzymes encoded in the pantothenic acid gene cluster a putative pathway was created for *T. reesei* (Figure 1).

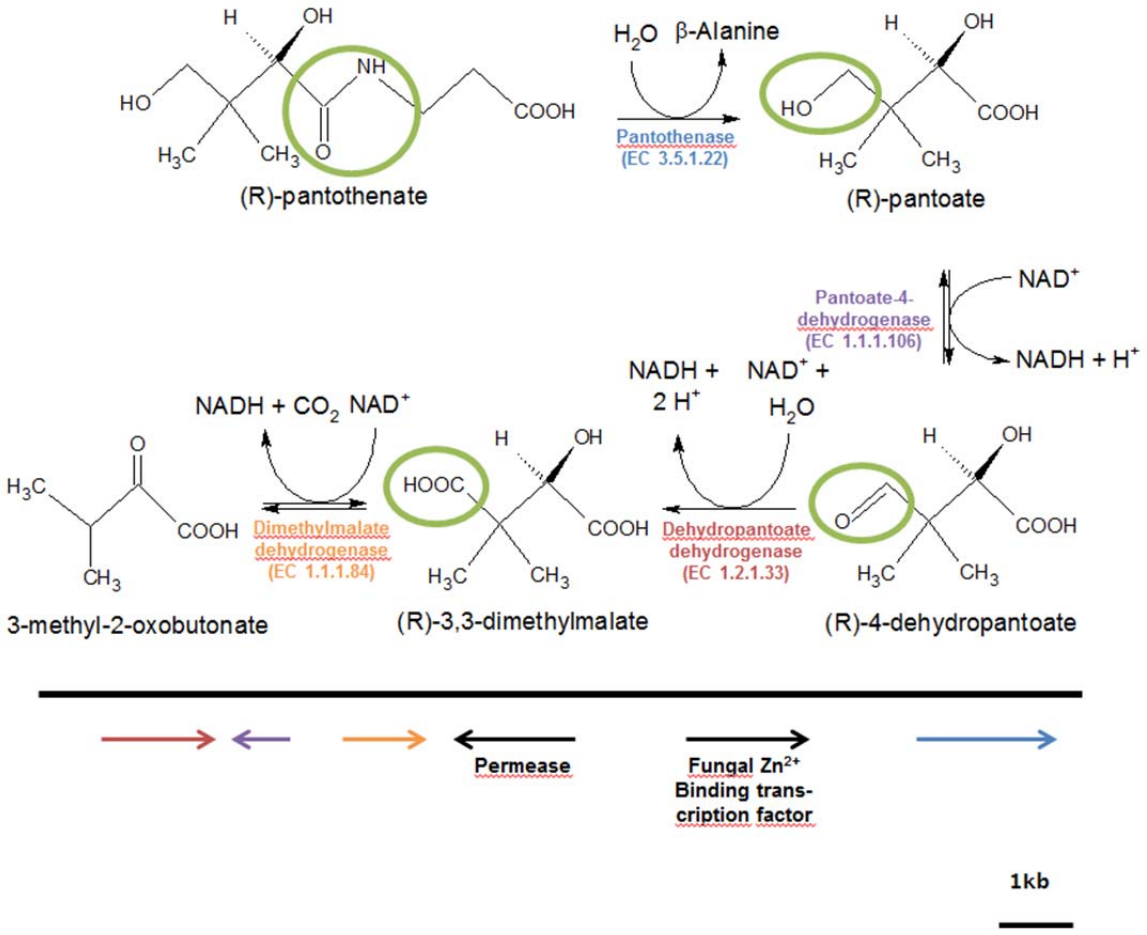

Figure 1: A putative pantothenate pathway in *T. reesei.* The pathway was created based on the in silico analysis of the enzymes encoded in the 12kb pantothenate inducible gene cluster and literature data on pantothenate catabolism in *Pseudomonas P-2*. The enzymes encoded in the cluster include besides a permease and a transcription factor also 4 enzymes responsible for the degradation of pantothenate.

Based on the pantothenate biosynthetic pathway in *S. cerevisiae*, homologous enzymes present in the *T. reesei* genome (http://genome.jgi-psf.org/Trire2/Trire2.home.html) were identified. An overview of the pantothenate metabolism and its putative corresponding genes in *T.reesei* are given in Figure 2.

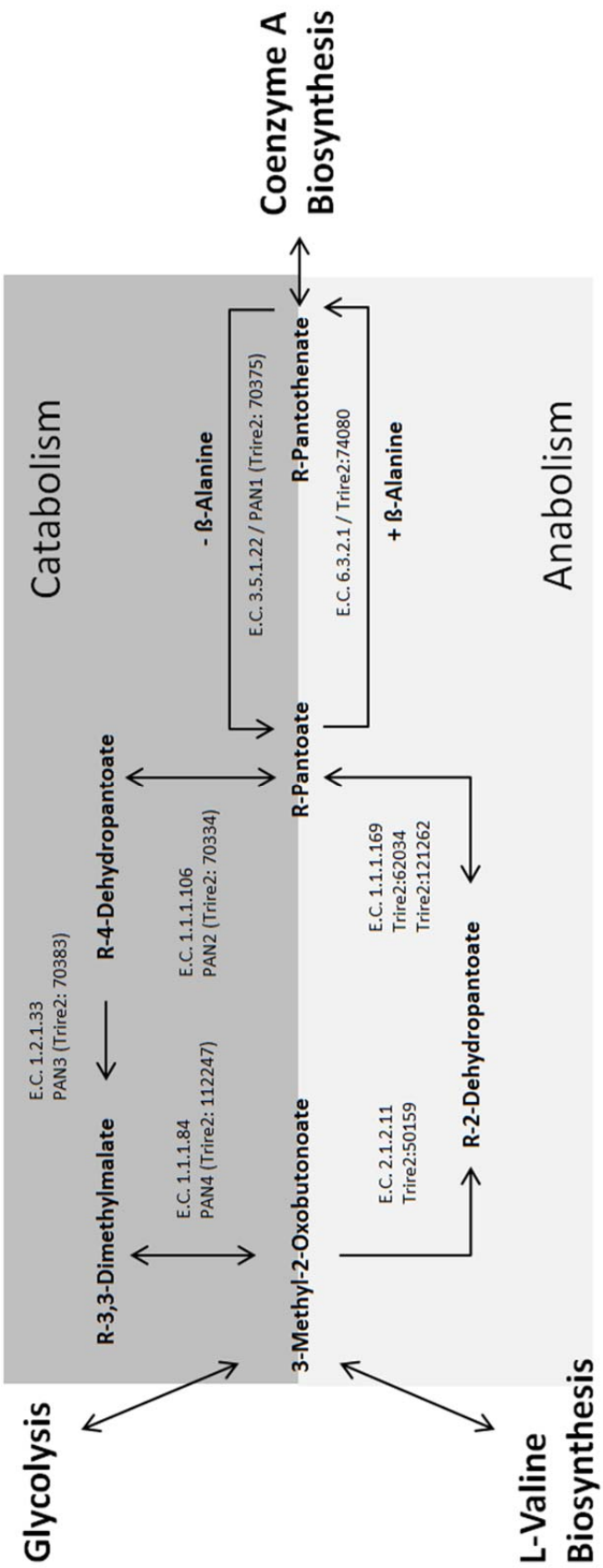

Figure 2: Overview of the pantothenate metabolism in *T.reesei.* The pathways are based on the data presented in Fig. 1 on the catabolic pathway for pantothenate and a search for homologous enzymes of the pantothenate anabolism of *S. cerevisiae* in the *T.reesei* genome database.

1.6. Aim of the Thesis

The primary goal of this thesis was to further investigate and characterize this novel gene cluster of *T. reesei*.

One aim was to establish that the enzymes encoded in this cluster are involved in pantothenic acid degradation. Therefore selected genes of the pathway were recombinantly expressed, the encoded enzymes purified and characterized.

The second aim was to investigate if the expression levels of the promoters can be modified by metabolic engineering of the pathway. The original motivation behind this topic was to find new promoters which can be induced by the cheap inducer pantothenic acid without interfering with cellulase production or growth of *T. reesei* as currently available expression systems in *T. reesei* are limited to promoters of xylanases and cellulases. Pantothenic acid was identified as a possible inducer which activates a number of genes present in a cluster. To find out if the expression strength of the promoters can be further manipulated by metabolic engineering of the pathway, strains deleted in the respective steps of the putative catabolic pathway for pantothenate were investigated with respect to the expression of the non-deleted genes of the pathway.

2. Materials and Methods

2.1. Strains and Cultivations

2.1.1. *Trichoderma reesei*

For this work the strains *T. reesei* QM9414 (ATCC 26921), a strain derived from it by gene deletion of the *T. reesei tku70* (QM9414*Δtku70,* C. Ivanova and B. Seiboth, unpublished results) and the deletion strains *Δpan1*, *Δpan2*, *Δpan3*, *Δpan4, Δpap1* and *Δpaa1* of the putative pantothenic acid catabolic pathway (T. Lindner, R. Bischof, C.P. Kubicek and B. Seiboth, unpublished results) were used and kept on potato dextrose agar (PDA 39 g/l) at 28°C. The strains were cultivated in 200 ml unbuffered Mandels Andreotti (MA) medium using 1L Erlenmeyer flasks. 10g/l each of glycerol, pantothenic acid, ß-Alanine or pretreated wheat straw were used as the carbon source as stated in the results section. The genes of the putative pantothenic acid cluster were renamed throughout this thesis as shown in Table 1.

Table 1: Overview of the putative pantothenic acid pathway genes.

Gene ID	Putative Function	Gene Name	Plasmid
trire2:70383	dehydropantoate DH	*pan3*	pAB-5, pAB-6
trire2:70334	pantoate 4-DH	*pan2*	pAB-3, pAB-4, pAB-10
trire2:112247	dimethylmalate DH	*pan4*	pAB-7, pAB-8
trire2:70349	pantoate permease	*pap1*	
trire2:70351	Zn^{2+} transcription factor	*paa1*	
trire2:70375	pantothenase	*pan1*	pAB-1, pAB-2, pAB-9

MA medium was prepared including wheat straw and autoclaved after pH adjustment to 4.8 with 1M KOH. Glycerol was autoclaved separately together with $CaCl_2$. ß-alanine and pantothenic acid as a carbon source or inducer were added as a sterile filtered stock solution. $CaCl_2$ was autoclaved separately at 20x concentration and 10 ml of it were added to 190 ml of MA medium in the Erlenmeyer flask. The medium was then inoculated with 2 x 10^6 conidiospores per ml (unless stated otherwise) and the cultures cultivated in a rotary shaker at 250 rpm and 28°C.

Suspensions of the conidiospores were obtained by mixing 10 ml NaCl/Tween solution to harvest spores of 10-14 days old PDA plates using a Drigalski spatula. Spore solution was filtered through glass wool tubes. 75 µl of the spore suspension was then diluted with 1 ml of NaCl/Tween and absorption was measured at 600 nm. Spore concentration (c) was calculated using equation (1).

$$A = 3.41 * 10^{-6}\,\mathrm{c} + 4.63^{-2} \qquad (1)$$

A absorbance at 600 nm wavelength

Biomass determination was performed using preweighted glass microfiber filters and 25 ml of each culture. Samples were washed with desalted H_2O, vacuum dried and further dried at 80°C for 3 days before being weighed again.
Biomass samples for qPCR analysis were gathered by filtering the culture broth through Miracloth and the retained mycelium was immediately frozen in liquid nitrogen after washing with cold dH_2O. Samples were stored at -80°C until extraction.

Media

NaCl/Tween Solution: 0.9 % NaCl, 0.05 % Tween® 80

Mandels-Andreotti unbuffered (1 L): 1.4 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 0.3 g $MgSO_4 \times 7H_2O$, 0.3 g $CaCl_2 \times 2H_2O$, 0.3 g urea, 1 g peptone (casein), 20 ml trace elements

Trace elements (1 L): 250 mg $FeSO_4 \times 7H_2O$, 80 mg $MnSO_4 \times H_2O$, 70 mg $ZnSO_4 \times 7H_2O$ and 100 mg $CoCl_2 \times 2H_2O$ (pH 2).

Chemicals

$(NH_4)_2SO_4$	Roth
$CaCl_2 \times 2H_2O$	Sigma
$CoCl_2 \times 2H_2O$	Merck
D-Pantothenic acid hemicalciumsalt	Sigma
ß-alanine	Serva
$FeSO_4 \times 7H_2O$	Merck
Glycerol	Roth
KH_2PO_4	Merck
KOH	Merck
$MgSO_4 \times 7H_2O$	Roth
$MnSO_4 \times H_2O$	Merck
Peptone from casein	Merck
Potato dextrose agar	Difco
NaCl	Roth
Tween® 80	Merck
Urea	Merck
Pretreated Wheat Straw	Clariant Produkte Deutschland GmbH
$ZnSO_4 \times 7H_2O$	Merck

Instruments

Infors HT Multitron Standard 230 V	Infors HT, Switzerland
IKA® Vortex	IKA, Germany
Magnetic stirrer Hei Standard	Heidolph Instruments, Germany
Miracloth 475855	Calbiochem, Austria

Whatman Glass Microfibre filters, 47mm	Whatman, England
Vacuum pump	ILMVAC
PHM 82 pH Meter	Radiometer Copenhagen
CERTOclav Type CVII/1600	Kelomat, Traun, Austria
Pipetman® L-series	Gilson, Wisconsin, USA

2.1.3. *Escherichia coli*

Stellar™ Competent Cells (Clontech) were used for construction of the expression vectors and amplification of the expression vectors. The pET21a+ plasmid was provided already transformed into *E.coli* TOP10F´. For enzyme production *E.coli* BL21(DE3) was used with the bacteriophage T7 promoter-based expression system and pET21a+ vector.

For cultivation LB medium was used and Ampicillin was used at a concentration of 100 µg/ml. For protein production Super-Broth (SB) was used.

Media

LB: 1% peptone, 0.5% yeast extract, 1% (0.5%) NaCl, 1.5% agar for plates, pH 7.0

SB: 3.2% peptone, 2% yeast extract, 0.5% NaCl, NaOH to pH 7

Chemicals

Ampicillin sodium salt ≥99%	ROTH
Zeocin	Genaxxon Bioscience

2.2. Construction of Expression Vectors

The pET21a(+) vector system uses the T7 promoter and contains the DNA for a C-terminal his-tag. For the construction of expression vectors, pET21a(+) containing *E. coli* were propagated in LB medium and the plasmid subsequently purified using the DNA GeneJET™ Plasmid Miniprep (Thermo Fisher Scientific, Bremen, Germany) Kit or PureYield™ Plasmid Midiprep System (Promega). pET21a(+) was then digested using the restriction enzymes NdeI and XhoI and purified using agarose gel electrophoresis. After excision of the plasmid-containing band from the gel, the DNA contained therein was extracted with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

The inserts were amplified from cDNA of the pantothenic acid induced strain *T. reesei* RB-VIII-3 with Phusion High-Fidelity DNA Polymerase (Thermo Scientific) using the primers listed in Table 2 and purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The cDNA of *T. reesei* RB-VIII-3 was generated as described below. RNA was isolated from a 48h old wheat straw culture, 2.5h after induction with 1mM pantothenic acid. Primers 70375_Infus_fw and 70375_Infus_nostop_rv were used to amplify the gene *pan1* and

primers 70334_Infus_fw and 70334_Infus_nostop_rv (Table 2) to amplify the gene *pan2*. In both cases, terminal 15 bp long overlaps for the recombination cloning were introduced.
Vectors were constructed using In-Fusion® HD Cloning Kit (Clontech® Laboratories, Inc.) and transformed into Stellar™ Competent Cells according to the manufacturer's instructions. Successfully transformed cells were selected through growth on LB-amp plates and picked for isolation of the plasmid.
Nucleotide sequences were obtained from JGI platform version 2 of the *T. reesei* genome database (http://genome.jgi-psf.org/Trire2/Trire2.home.html). Primers were designed using the "Convert PCR Primers Into In-Fusion® Primers" design tool of Clontech® (http://bioinfo.clontech.com/infusion/convertPcrPrimersInit.do).
Plasmids were named pAB-9 (pET21a+; *pan1*) and pAB-10 (pET21a+; *pan2*) and digested for control with 10x FastDigest® Green Buffer at 37°C using XbaI and EcoRV, respectively.
DNA concentrations were determined using the NanoDrop spectrophotometer.

Table 2: Primers for construction and sequencing of expression vectors

Primer	Sequence
70375_Infus_fw	AAGGAGATATACATATGATGACTGTCTCTGGAGATTCCA
70375_Infus_nostop_rv	GGTGGTGGTGCTCGAGCGCAGCCAGAGCTTCTAATACT
70334_Infus_fw	AAGGAGATATACATATGATGCCATCAAACCGCCTC
70334_Infus_nostop_rv	GGTGGTGGTGCTCGAGCACGCATCTTCCACCATCC
T7	TAATACGACTCACTATAGG
T7term	TGCTAGTTATTGCTCAGCGG

Chemicals and others:

GeneJET™ Plasmid Miniprep Kit	Fermentas
PureYield™ Plasmid Midiprep System	Promega
QIAquick Gel Extraction Kit	Qiagen, Germany
Phusion High-Fidelity DNA Polymerase	Thermo Scientific
QIAquick PCR Purification Kit	Qiagen, Germany
In-Fusion® HD Cloning Kit	Clontech®
Restriction Enzymes	Thermo Scientific
Isopropanol	Merck
Ethanol 96%	Merck
Oligonucleotides	Fermentas
Water for analysis EMSURE®	Merck

Instruments

Thermocycler T3000	Biometra GmbH, Germany

Centrifuge 5414R	Eppendorf AG, Hamburg, Germany
Nanodrop ND-1000 spectrophotometer	Thermo Scientific
Electrophoresis materials	Biorad

2.3. Transformation of *Escherichia coli*

E.coli BL21(DE3) electrocompetent cell aliquots of 50 µl were thawed on ice. Cuvettes were cooled on ice for 5 minutes. 500 ng of purified plasmid DNA diluted in 10 µl were added to the cells and kept on ice for 5 minutes. Then the cells were quickly pipetted into the cuvette and electroporated at 1.8 kV for 5.2 ms using a MicroPulser™ Electroporator (Biorad, Austria). Cells were quickly added to 1 ml of SOC medium and regenerated for 1h in a Thermomixer at 37°C / 800 rpm. Cells were then spread on LB-amp plates and incubated at 37°C overnight. Isolated colonies were picked onto fresh plates for longer storage.

2.4. Analysis of Transformants

BL21(DE3) transformants containing the expression vectors were analyzed by sequencing of the vectors. Therefore three colonies were picked for every transformation and plasmids were isolated using the GeneJET™ Plasmid Miniprep Kit. Samples were sequenced by Microsynth Austria (Vienna) using the T7 primer pair (T7 and T7term, Table 2) and aligned using CLC Main Workbench. Cryo-stocks of positive transformants were made in duplicate by mixing 300 µl of sterile 50% glycerol with 700 µl of a dense overnight culture to obtain a 15% glycerol stock at -80°C.

2.5. Overproduction and Isolation of the Target Enzyme

Enzyme production was started by picking a single *E. coli* colony into 10 ml of SB-amp in a 100ml Erlenmeyer flask as shown in Figure 3. The main culture was prepared by diluting the preculture to an OD_{600} of 0.5 in a 1 L flask containing fresh medium. Exact cell density was measured again using the photometer and 1 mL of a sterile filtered 100x IPTG stock solution was added at a temperature of 25°C for induction.

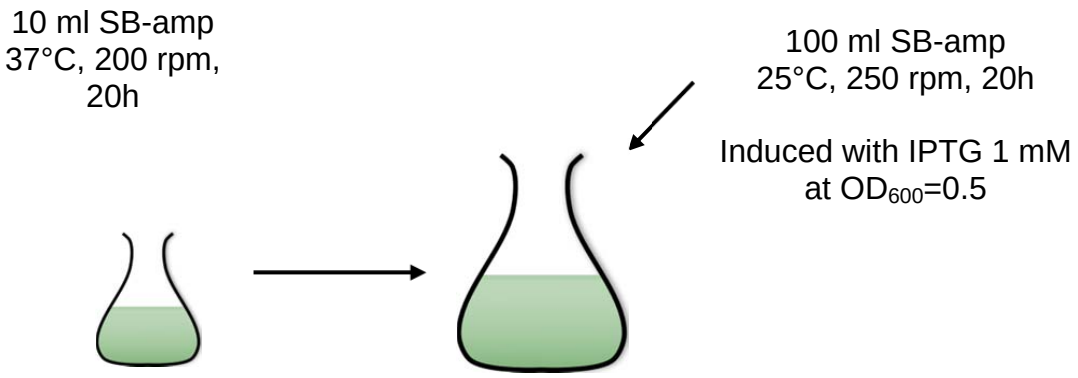

Figure 3: Enzyme production scheme for *E.coli* BL21(DE3) containing the pET21a(+) expression vectors.

Cells were harvested by centrifugation at 10.000 g for 10 min at 4°C. Cells were once washed in Buffer A (50 mM KH_2PO_4, 500 mM NaCl, 20 mM Imidazole) and then resuspended in 25 ml of Buffer A for further reprocessing. Cells were always kept at 4°C and then lysed by using a French press system Emulsiflex©C-3 Homogenizer. Pressure was adjusted to 6 bar primary pressure and a pressure of 1000-1500 bar main pressure. Cell suspension of 25 ml was recirculated for 5 min 15 sec. The cells were then immediately put back on ice. The French press system was flushed with 70% Ethanol and washed with dH_2O before and after use. Finally the homogenizer was stored under 70% ethanol.
Cell extract was then centrifuged 2x at 21.000g, 4°C before sterile filtration through 0.2 µm syringe filters.

2.5.1. ÄKTA – FPLC

Protein was purified using the ÄKTA Fast Protein Purification Liquid Chromatography (FPLC) System (GE Healthcare Life Sciences) operated by UNICORN 5 control software. The purification principle was Immobilized Metal Ion Affinity Chromatography (IMAC) using a Ni^{2+} Sepharose High Performance 1 ml column (GE Healthcare Life Sciences, HisTrap HP).
The FPLC system was always flushed with 100 ml of 20% ethanol before use by starting the program "Pumpwashpurifier" in UNICORN 5. Ethanol and both buffers A and B were sterile filtered (0.2 µm) prior to use. Then the tubings for Pump A and B were placed in the respective buffer solution and a gradient of 50% B was started to equilibrate both tubings. After flushing about 20 ml at 3 ml/min the column was placed right before the UV detector by adjusting a low flow rate of 47 cm/h. Column was first opened at the top and the tubing was adjusted, then the bottom of the column was quickly removed to prevent a high pressure in the column. Then the column was connected right before the UV-detector. The column always has to be connected under a positive flow rate to prevent getting oxygen into the sepharose bed.

The column was equilibrated at 312 cm/h by flushing at least 5 column volumina (CV) of 50% B gradient followed by 5 CV of buffer A. Buffer A was flushed until the UV signal at 280 nm was stable.

Then the sample was loaded as follows: The crude extract was poured into a measuring cylinder and the flow paused. Tubing A was changed from buffer A to the cylinder containing the sample and the flow was started again. The sample was then loaded at 312 cm/h and the total flowthrough was collected in a 50 ml falcon tube. All samples were kept on ice whenever possible. After loading the sample, the tubing was changed back to buffer A and column was flushed until the primary UV signal was reached again. Column was flushed again with at least 5 CV of buffer A until a linear gradient to 100% buffer B was started. Gradient was set for 10 min at a flowrate 312 cm/h. Sampling was started right at the start of the gradient by collecting 1.4 ml each in a 1.5 ml Eppendorf tube. As soon as the UV signal was stable again after elution, sampling was stopped and the column was flushed again with buffer A until the basal UV level was reached.

Samples were pooled according to the UV signals and several measurements were performed for determination of the purification factor and yields. Therefore the exact volume, protein content and enzyme activity was determined for all of the crude extracts, flowthroughs and eluted fractions, respectively.

Calculations:

Protein concentration was determined using the Protein Assay Kit (Biorad, Hercules, USA) as per the manufacturer's instruction and multiplied with the volume of each fraction to obtain the amount of total protein.

Specific activity was calculated after measuring the activity of each fraction. Activity was divided by the protein content of each fraction to obtain its specific activity (see "2.6. Enzyme Characterization").

Protein recovery rate was determined using equation (2).

$$\text{protein recovery } [\%] = \frac{p_{FT}+p_{F1}+\ldots..+p_{Fx}}{tp_{CE}} * 100 \qquad (2)$$

p_{FT}, $p_{F1, \ldots}$, p_{Fx} = protein content of flowthrough, fraction 1, …, fraction x. [mg]

tp_{CE} = total protein/protein content of crude extract [mg]

Protein yields were determined by measuring the activity in all fractions. Ideally, there is only activity found in one pooled fraction after elution and no activity in the flowthrough.

$$\text{protein yield } [\%] = \frac{specific\ activity_{F1} * \text{p}_{\text{F1}}}{specific\ activity_{CE} * \text{tp}_{\text{CE}}} * 100 \qquad (3)$$

F1 = Fraction containing the target enzyme

Purification factor was determined by relating the specific activity of the purified enzyme fraction to the specific activity of the crude extract.

Media

Buffer A (binding buffer): 50 mM KH_2PO_4, 500 mM NaCl, 20 mM imidazole
Buffer B (elution buffer): 50 mM KH_2PO_4, 500 mM NaCl, 500 mM imidazole

Chemicals

Imidazole APS grade	Sigma

Instruments

Emulsiflex©C-3 Homogenizer	Avestin
ÄKTAmicro	GE Healthcare Life Sciences
HisTrap HP, 5 x 1 ml	GE Healthcare Life Sciences

2.6. Enzyme Characterization

2.6.1. Pantothenase PAN1

Pantothenase assay was performed as described by Nurmikko *et al.* [27] and the formed ß-alanine was measured in a modified version of the ninhydrin colorimetric analysis for amino acids based on the method of Yemm and Cocking [30].

The assay was performed using different substrate concentrations and a reaction time of 1h. Reaction was stopped by heating to 100°C for 3 min in a water bath. As a blank the assay was first heated to 100°C and enzyme was added at that temperature.

Then the amount of ß-alanine formed was determined by heating 1 ml of the assay solution with 1 ml ninhydrin reagent and 3 ml of 0.2M acetate buffer for 15 min to 100°C in test tubes together with boiling chips. The reaction was stopped by cooling in a water bath and adding EtOH (50% v/v) up to a volume of 5ml. Absorption was determined at 570 nm.

Media

Potassium phosphate buffer: 2 mM KH_2PO_4, pH 7.4

Acetate buffer: 0.2 mM sodium acetate trihydrate, pH 5.5 with NaOH

Ninhydrin: 8% (w/v) ninhydrin in acetone

Chemicals

Boiling chips	Roth
Ninhydrin	Loba
Sodium acetate trihydrate	Merck
Acetone	Merck

2.6.2. Pantoate 4-dehydrogenae PAN2

Short-chain dehydrogenase or pantoate 4-dehydrogenase (PAN2) assay was performed as described by Goodhue *et al.* [28]. The assay was pipetted into 1.5 ml semimicrocuvettes and reaction was followed at 340 nm. The assay was always filled up to 1 ml with dH_2O leading to the following final concentrations:

Table 3: Components of the standard enzyme assay for pantoate dehydrogenase

Chemical	Concentration (1ml reactions)
Glycin – NaOH pH 10 270 mM stock	90 mM
NAD^+ 100 mM stock	1 mM
Enzyme dilution	100 µl
(Pantoate 50 mM stock)	(2.5 mM)

The reaction was followed 2 times for each enzyme solution. First the background reaction was followed by excluding pantoate as a substrate in the reaction. Second the reaction was measured exactly the same way but with addition of the substrate R-pantoate. Mastermixes contained all components except the enzyme solution. Once the enzyme was added, reaction was mixed quickly by pipetting up and down for about 3-4 times and absorption was measured in the first 30 seconds for the overexpressed enzyme.

Measurements in *T. reesei* cell extracts had to be measured much longer to get significant results, as the absorption changes were lower. Those reactions were followed for 20 minutes to calculate the ΔA/min.

ΔA/min was calculated for the enzyme reaction by the photometer for the short measurements and manually when reaction had to be followed 20 minutes. Background reaction was always subtracted for each measurement. Specific activity [U/mg] was calculated after equation (4).

$$Units/mg = \frac{\Delta A/\min}{6.22\ x\ \frac{mg\ enzyme}{ml\ reaction\ mixture}} \quad (4)$$

Chemicals

Glycin	Roth
Pantoate	synthesized as described in 3.6.1.
NAD^+	Sigma
$NADP^+$	Sigma

For every strain 3 independent measurements were made. To determine the statistical significance of the differences, an unequal variance two-sided t-test was performed using the online calculator of GraphPad Quickcalcs (http://graphpad.com/quickcalcs/).

2.6.1. Preparation of R-Pantoate

R-Pantoate was prepared as follows: 10 g of D-pantothenic acid hemicalcium salt were completely hydrolysed by reflux heating in fuming hydrochloric acid for 16 hours. ß-alanin was removed from that mixture with a cation exchange resin (CM Sephadex C-50, SIGMA). The residual water was then removed by vacuum evaporation. The purity of the R-pantoate fraction was verified by NMR (1H NMR (D_2O, 200 MHz): δ = 0.84 (s, 3 H, CH_3), 1.01 (s, 3H, CH_3), 3.93 (s, 2H, CH_2), 4.20 (s, 1H, CH)).

2.6.2. Generation of *T. reesei* Cell Extracts

Cell extracts for pantoate dehydrogenase (PAN*2*) enzyme assays of *T.reesei* were prepared by first growing *T. reesei* for 24 hours on MA-medium with glycerol. The cultures were then induced by addition of 0.5% pantothenic acid. Biomass was harvested by filtrating the culture broth through Miracloth or Whatman filters, washing once with dH_2O and freezing the samples in liquid N_2. Samples were stored at -80°C until use.

Cell extracts were generated by an adapted method originally described by Mustalathi [31]. Approximately 100 mg of mycelium were mixed with 900 µl of extraction buffer and 1 g of 0.5 mm acid washed glass beads using 2 ml reaction tubes. The tubes were cooled on ice at 4°C and homogenized using a ball mill (Retsch MM301, Düsseldorf, Germany) for 3 x 1min. at 30 Hz. and cooling breaks of 2 min. Supernatant was separated from the cell debris and glass beads by centrifugation at 14.000 g 2x 10min.

2.7. Gel Electrophoresis

2.7.1. Agarose Gel Electrophoresis

For gel electrophoresis 0.9 % (w/v) of Starpure Agarose were suspended in TAE buffer and melted in the microwave. After cooling down to about 50°C in a water bath, SYBR^R Safe DNA

gel stain was added (2-5 µl in 60–100 mL). After mixing, the solution was poured into a gel tray with an appropriate comb and solidified. The gel was transferred into the gel chamber containing TAE buffer. For analysis always 5 µl of a DNA ladder (GeneRuler 1kb) were added. Gels were run at 110 V.

Chemicals

Rotiphorese® 50x TAE Puffer	Roth
Starpure Agarose	Starlab

2.7.2. SDS – PAGE

SDS gels were always made fresh in a composition as listed in Table 4. Small gels were used with a 4.5% stacking gel and a separating gel of either 12% or 15% depending on the separation. As a reference PageRuler Prestained Protein Ladder 10-170kDA (ThermoScientific) was used. About 15 µg of total protein was loaded when separating cell extracts and 3 µg were loaded of purified proteins. After separation the gels were stained overnight using colloidal Coomassie blue staining and again destained overnight using dH_2O. Samples were prepared before use by mixing them with the 5x loading dye so as to reach the desired concentrations. Samples were heated to 97°C for 7 min and immediately loaded. Gels were started at 80 V for 15 min and run at 120 V until completion.

Table 4: Composition of the SDS-PAGE gels

Chemical	Stacking gel	Separating Gel 12 %	Separating Gel 15 %
dH_2O	1.5 ml	2.1 ml	1.5 ml
Gel buffer	0.625 ml	1.5 ml	1.5 ml
AA 30%	0.375 ml	2.4ml	3 ml
10% APS	12.5 µl	30 µl	30 µl
TEMED	2.5 µl	5 µl	5 µl

Media

Acrylamid-Stock, 30% (500 ml): 150g acrylamid, 2g bisacrylamid,

Separating Gel Buffer (500 ml): 90.75 g Tris, 2 g SDS, pH 8.8 with HCl

Stacking Gel Buffer (500 ml): 30.25 g Tris, 2 g SDS, pH 6.8 with HCl

10x Running Buffer (500 ml): 15g Tris, 72g Glycin, 5g SDS

Ammoniumpersulfate: 0.1g per 1 ml dH_2O

Colloidal Coomassie (100 ml): 8 g $(NH_4)_2SO_4$ + 78.1 ml dH_2O + 1.9 ml 85% H_3PO_4, 1.6 ml 5% Coomassie Blue G250, 20 ml MeOH

5x Loading Dye: Bromphenol blue 0.25%, DTT 0.5 M, Glycerol 50%, SDS 10%, Tris HCl 0.25M, pH 6.8, ß-mercaptoethanol 5% (added before use)

Chemicals

PageRuler Prestained Protein Ladder 10-170kDA	ThermoScientific
Acrylamide	Merck
Bisacrylamid 4K ultrapure	APPLICHEM
Sodiumdodecylsulfate	Merck
Coomassie Blue G250	Merck
Bromphenolblue	Roth
Glycerol	Roth

All other chemicals were supplied by SIGMA Aldrich

2.8. RNA Isolation and cDNA Synthesis

Extraction of RNA for qPCR analysis of the *T. reesei* samples from wheat straw cultures was performed using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) as described in the manufacturer's instructions.

After determination of the concentration (Nanodrop spectrophotometer) RNAs were stored at -80°C or immediately transcribed into cDNA. Therefore RNAs were first treated with DNAse I (5 µg RNA, 1 µl DNAse I, 1 µl 10x buffer and dH_2O to 10 µl) by incubation for 30 min. at 37°C. After addition of 1 µl EDTA + 1 µl dH_2O the samples were further incubated for 10min. at 65°C. Finally cDNAs were synthesized following the instructions of the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Bremen, Germany) using both oligo(dT) and random hexamer primers and stored at -20°C.

2.9. Quantitative Real Time PCR

All qPCR measurements were conducted on Mastercycler® ep realplex 2 using the method previously described by Bischof [32]. Several measurements to determine transcription of the pantothenic acid permease were made using the primer pair for *pap1*. This gene was chosen as it is present in all gene deletion strains of the pantothenate catabolic pathway. The housekeeping gene *tef1* was used as a reference to compensate for differences in the total amount of cDNA in the assay. Relative expression levels were calculated using the REST© Software tool (http://www.gene-quantification.de/rest.html). The primers used for the qRT-PCR measurements are listed in Table 5.

Table 5: Primers used in qRT-PCR measurements

Primer	Sequence	target mRNA	Efficiency
70383_1_fw	CCTTGTTGTTCGTGCCACATCG	*pan3*	0.89
70383_1_rv	CCCTTTGCGAGAGCATCCTTG		
70375_1_fw	CATTGGCAAGGGAACTCAGCAC	*pan1*	0.83
70375_1_rv	ACGGTGTTGAACGGCATGGTAG		
70349_1_fw	GCCTGTCGTTGAGGAACACTCG	*pap1*	0.91
70349_1_rv	CAGCGATGACCCAGAGCATGAC		
70334_2_fw	ACTCCAACAAGGCGACAGAGGG	*pan2*	0.9
70334_2_rv	AAGCTGCCAGGGCGATTCTC		
112247_2_fw	CAACGGGATCTACGAGCCAGTG	*pan4*	0.81
112247_2_rv	GCAGCAGCATCGCCAGACTTAG		
qPCR_tef1_for	CCACATTGCCTGCAAGTTCGC	*tef1* (reference gene)	0.87
qPCR_tef1_rev	GTCGGTGAAAGCCTCAACGCAC		

2.10. HPLC Measurements

For HPLC measurements the strains QM9414*Δtku70*, the deletion strains QM9414*Δtku70Δpan1*-#51 and QM9414*Δtku70Δpan2*-#11 were cultured on wheat straw and induced with 0.1 mM pantothenic acid after 48h. The *Δpan1* strain was also cultured without induction as a control. Samples were taken before induction and 2, 4, 8, 12, 16, 20 and 24h after induction.

Sampling was performed by filtration of 5 ml of each culture on Miracloth. After washing with dH_2O the mycelium was placed in a 1.5 ml Eppendorf reaction tube and stored at -80°C after quickly freezing in liquid N_2.

Sample preparation for HPLC measurement was started by thawing the mycelium in the reaction tube. 1 ml of a 10 mM K_2HPO_4 buffer was added and the samples were cooked at 99°C for 10 min. After 10 min of centrifugation at 14k rpm, 4°C, the supernatant was removed into another reaction tube and centrifuged again. This supernatant was now ready for HPLC measurement and transferred into the HPLC vial. Pellets were washed with 1 ml of buffer and centrifuged again. Supernatant was removed carefully, discarded and the samples were dried for 3 days at 80°C until determination of dry biomass.

Standards were made for pantothenic acid, pantoate and ß-alanine in concentrations of 0.016, 0.08, 0.4, 2, 10 and 50 mM. An ion exclusion column was used with a constant flow rate of 0.5 ml/min. and the mobile phase was run isocratically.

Media

K_2HPO_4 buffer: 10 mM K_2HPO_4, pH to 7.4 using H_3PO_4

HPLC running buffer: 0.1% H_3PO_4

Chemicals

K_2HPO_4		Merck
Ortho-phosphoric acid	85%	Merck

Instruments

HP Agilent Series 1100	Agilent Technologies, USA
SUPELCOGEL C-610H	Sigma-Aldrich, USA
RI Detector	Agilent Technologies, USA

2.11. Plate Growth Tests and Sporulation Assay

Plate growth tests were performed as follows: Equal pieces of mycelium from freshly sporulated plates were cut and transferred to freshly poured plates (Ø 94mm) containing 20ml of minimal medium with 1% w/v glucose. Tests were run as biological triplicates.

Sporulation was measured as follows: Equal pieces of mycelium from freshly sporulated plates were cut and transferred to freshly poured plates (Ø 60mm) containing 5ml of minimal medium. After 66 hours, spores were harvested in 4ml NaCl Tween solution and the spore concentration measured photometrically.

Media

Minimal Medium (1L): 6 g $(NH_4)_2SO_4$, 10 g KH2PO4, 1 g $MgSO_4$ x $7H_2O$, 3 g sodium citrate, 10 g glucose, 15g gar noble, 20 ml trace elements

3. Results

3.1. Effect of the Deletion of different Genes of the Pantothenate Catabolism on Biomass Formation.

Biomass formation on pantothenate as a sole carbon source was examined for deletion strains of the putative pantothenate catabolic pathway compared to the reference strain QM9414*Δtku70* in biological triplicates. In addition we also cultivated QM9414*Δtku70* on 1 % ß-alanine. The first step in degradation of pantothenate is hydrolysis into pantoate and ß-alanine and the ß-alanine control was made to examine if growth is possible on just one part of pantothenate. The pH value of both media was adjusted to pH 5.5-6.0. Biomass samples were taken after 28h and 56h of growth in shake flasks.

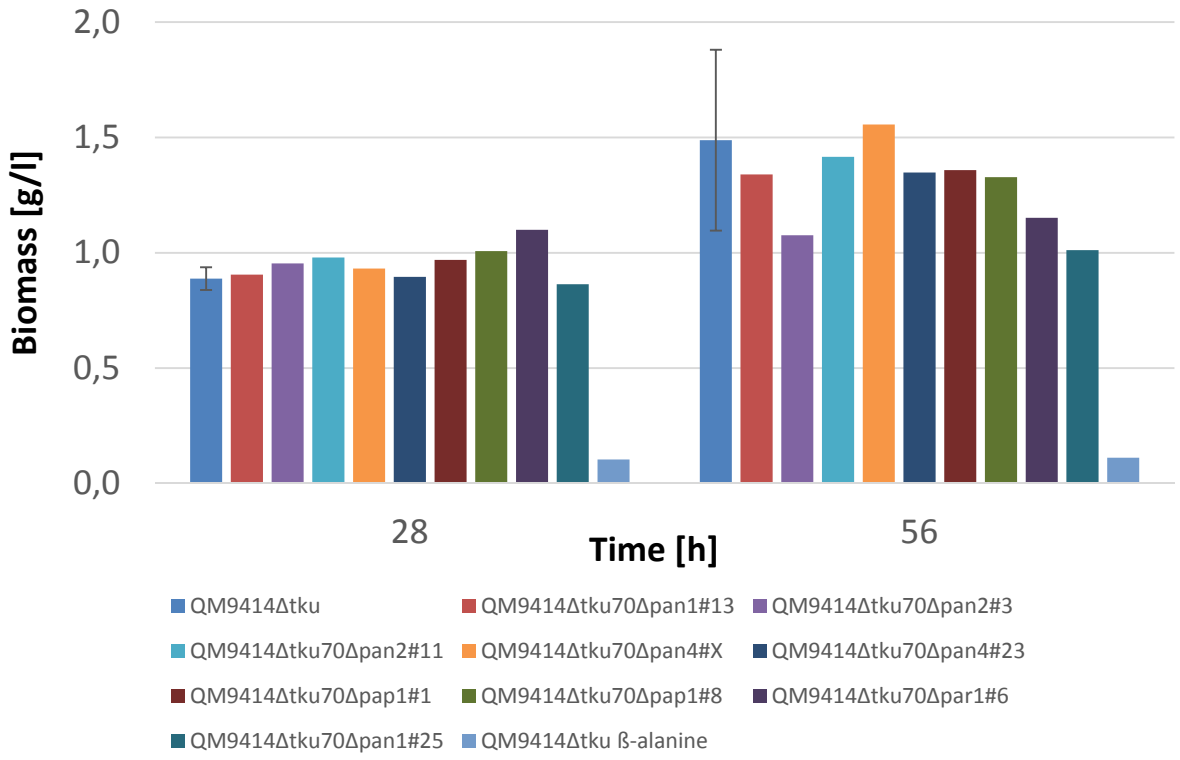

Figure 4: Production of biomass in different strains deleted in different steps of the putative pantothenate catabolism: Strains were cultivated in unbuffered MA-medium containing 1 % w/v pantothenic acid or ß-alanine as a carbon source and samples were taken after 28 and 56 h.

As shown in Figure 4 all deletion strains growing on pantothenate show similar biomass formation within the first 28 hours. Only the reference strain grown on ß-alanine shows a reduced accumulation of biomass. The residual growth is most probably due to the utilization of peptone (0.1%) present as starter in the media. So ß-alanine itself cannot be used as a carbon source by QM9414. Further experiments will be necessary to investigate if ß-alanine cannot be taken up by the fungus under these conditions or if it cannot be

metabolized at all. We can however also not rule out, that the high concentration of ß-alanine is growth inhibiting.

After 56 hours of cultivation all strains showed an increase in biomass formation. Also the standard deviation of the reference strain is quite high, presumably due to the problem that during further growth, a ring of sporulating mycelium was formed on the interior of the flasks which complicated the biomass measurement and led to a high standard deviation.

3.2. Plate Growth Tests and Sporulation Assay

Plate growth and sporulation of QM9414*Δtku70* reference strain was compared to 3 different *Δpan2* strains. Therefore the strains were cultivated in triplicates on PDA plates and also MM + Glucose plates and growth was measured several times as shown in Figure 5 and Figure 6.

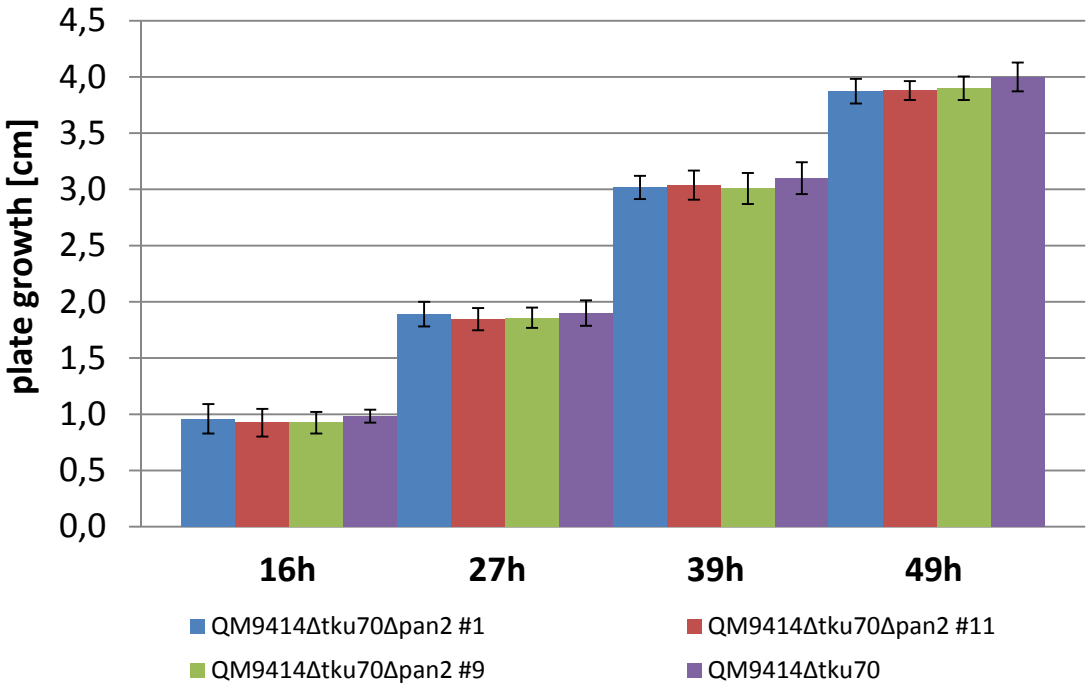

Figure 5: Radial growth of 3 different *Δpan2* strains and QM9414 on PDA plates.

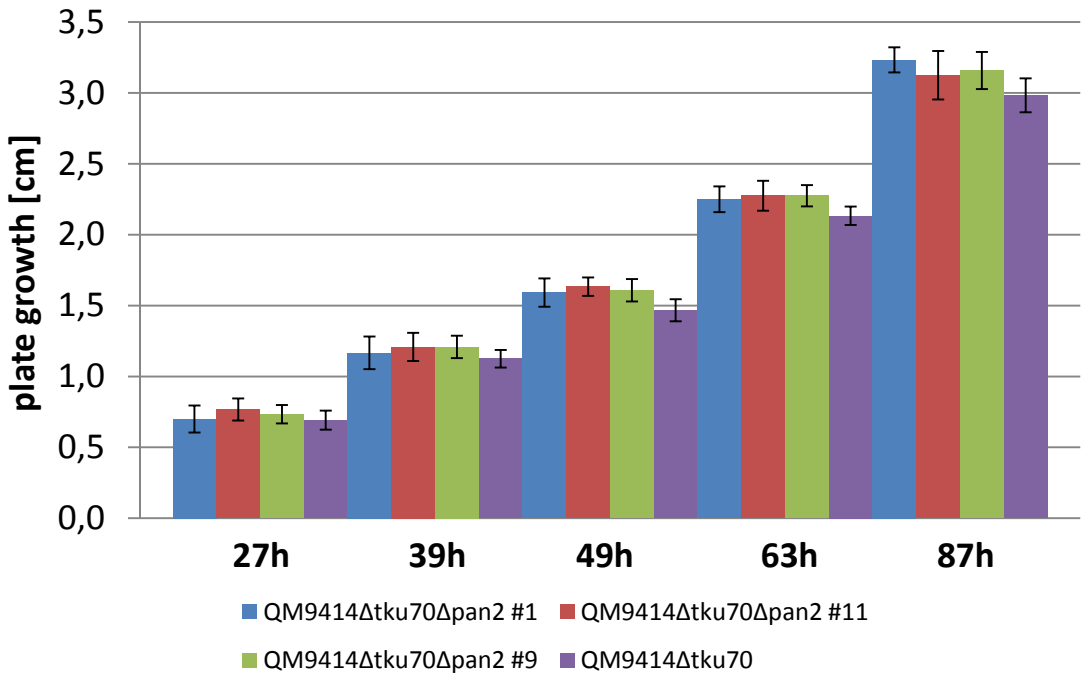

Figure 6: Radial growth of 3 different *Δpan2* strains and QM9414 on MM + Glucose plates.

The growth experiment showed that there is no significant difference in radial growth between the knockout mutants and the reference strain.

Sporulation was measured after growth for 66 hours on medium PDA plates for the same strains in duplicates. As it can be seen in Figure 7, the *Δpan2* strains show significantly higher amount of spores than the reference strain.

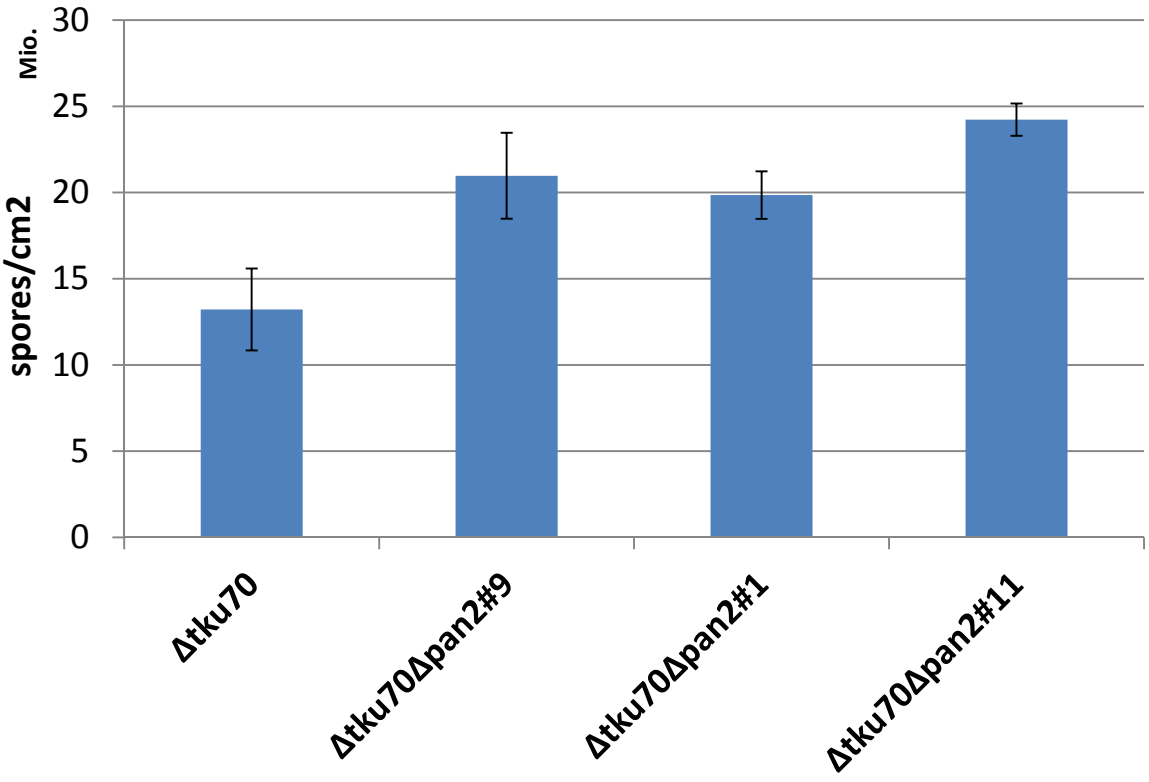

Figure 7: Amount of spores after growth on PDA plates for 66 hours.

3.3. Effect of the Deletion of the Genes of the putative Pantothenate Catabolism on Gene Expression

The strains deleted in the different steps of the putative pantothenate catabolic pathway were used to study which step(s) are essential for inducer formation of the pathway. In the experiment we used a cellulase production medium with wheat straw as inducing carbon source to study pantothenate induction under enzyme production relevant conditions.

Therefore the different knockout strains were grown for 48h on wheat straw as carbon source on this cellulase production medium and were then induced with 0.1 mM of pantothenic acid. Samples were taken before and 2, 4 and 8h after addition of pantothenic acid. The effect of the different gene deletions on gene expression was analyzed by assaying the transcript levels of the putative pantothenate permease gene *pap1* by qRT-PCR. This gene was chosen as it is present in all gene deletion strains of the pantothenate catabolic pathway.

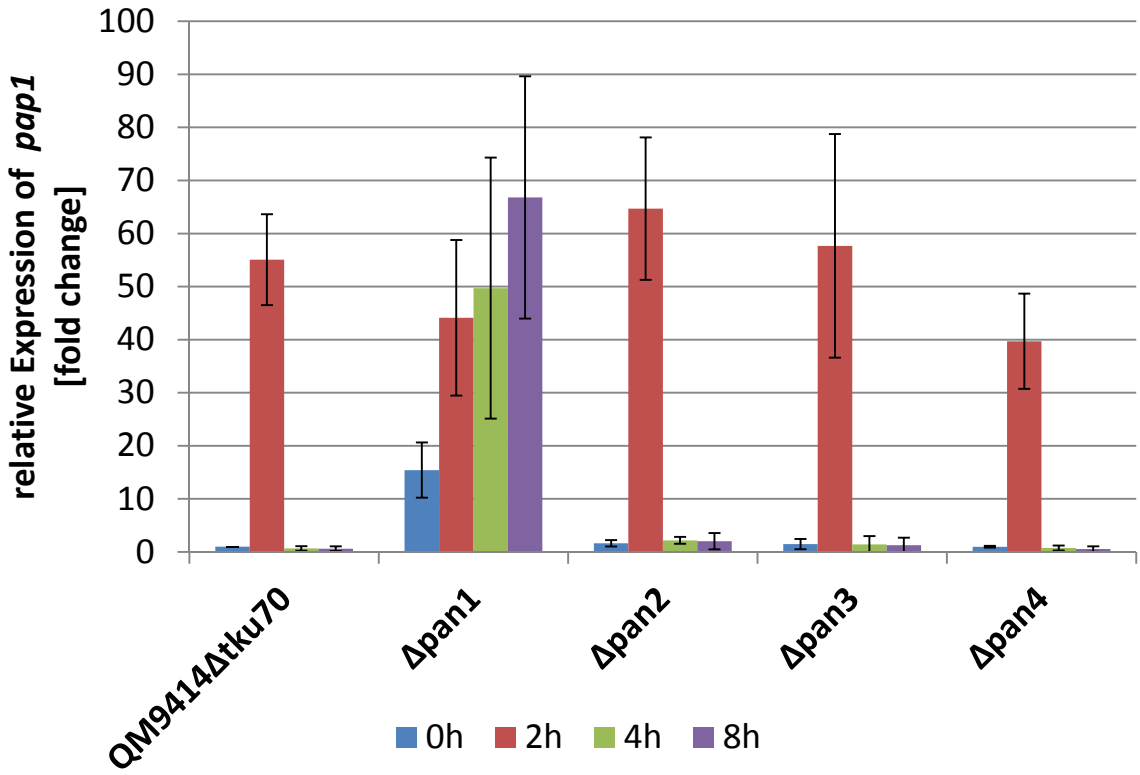

Figure 8: Relative gene expression of *pap1*: The expression of the pantothenic acid permease *pap1* is normalized to *tef1* and related to the QM9414Δ*tku70* sample before induction (time point 0 h) with pantothenic acid. The samples were grown 48h on wheat straw and induced by 0.1 mM pantothenic acid. All the samples were analyzed in biological duplicates, *Δpan1* in triplicates.

As shown in Figure 8, all gene deletion strains and the reference strain show a highly upregulated gene expression of *pap1* after 2 hours following addition of pantothenic acid. Only the three tested *Δpan1* strains show a prolonged presence of the transcript of *pap1* even after 4 and 8 hours following addition of pantothenic acid, while the knock out strains of the other three genes (*pan2-4*) behave essentially as the control strain. Analysis of a sample after 26h of induction for the *Δpan1* #51 strain still showed an about 50 fold elevated transcript level of *pap1* compared to the reference strain (data not shown). Beside that the induced level of the *pap1* gene is higher in *Δpan1* stains, the results also show that the basal transcript level before pantothenic acid addition is already significantly increased in the *Δpan1* strains as compared to the reference strain and to all other knockout strains.

Of all the tested knockout strains, only the strain missing the putative pantothenate hydrolase PAN1 remained induced which strongly indicates that pantothenate itself can serve as the inducer of *pap1* and in conclusion of the whole pantothenate gene cluster. Beside this increased transcript presence the basal level of gene expression before induction is also increased significantly in the *Δpan1* strains, which could be explained by an increased steady state concentration of pantothenate in the cells due to the absence or a reduction in pantothenate catabolism.

3.4. Intracellular Accumulation of Pantothenate in *T. reesei Δpan1* Strains

To investigate if pantothenate is accumulating in *Δpan1* strains, the concentration of pantothenate was measured in the cells by HPLC. Therefore the strains were grown in unbuffered Mandels-Andreotti medium with wheat straw as carbon source and induced with 0.1 mM pantothenic acid after 48h of growth. The *Δpan1* strain was also cultured without induction as a control. Samples were taken before induction and 2, 4, 8, 12, 16, 20 and 24h after induction.

Figure 9 shows the accumulation of pantothenate in the *Δpan1* knockout strain following addition of pantothenic acid compared to the QM9414*Δtku70* control. After 16 hours about 30 µM/mg cell dry biomass were found compared to the reference strain which accumulated only 5 µM/mg.

The uninduced *Δpan1* strain, however, did apparently not accumulate pantothenate intracellularly, and the levels of pantothenate were similar to the controls which included the reference strain QM9414*Δtku70* and a *Δpan2* strain. Therefore, the data of the prolonged expression of *pap1* in the *Δpan1* strain following pantothenate addition can now be explained by accumulation of pantothenate but don't provide an explanation for the significantly increased basal expression level in the *Δpan1* strains before pantothenate addition. One explanation could be that the absence of PAN1 itself has an effect on *pap1* gene expression or that the deletion of *pan1* in the gene cluster would lead to this effect. But further experiments would be necessary to support any of these possibilities.

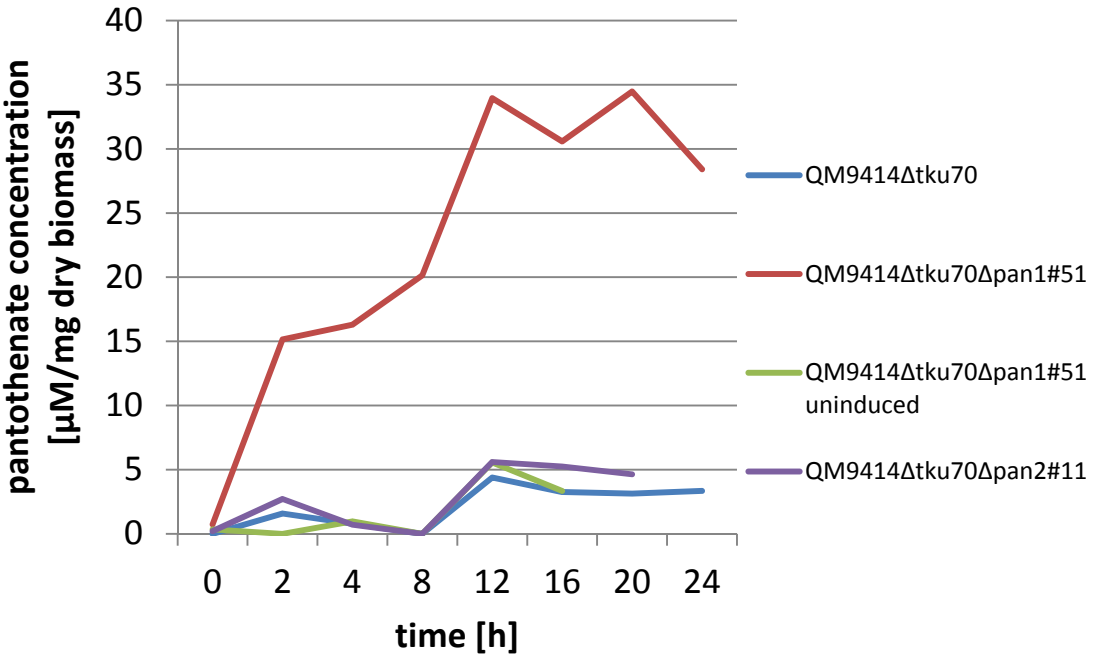

Figure 9: Concentration of pantothenate in *T. reesei* cell extracts: Pantothenate was measured in QM9414Δ*tku70,* a *Δpan2* strain and in a *Δpan1* strain (both induced and uninduced) after induction with pantothenic acid (time point = 0) after a 48h cultivation on unbuffered MA with wheat straw. Accumulation of pantothenate is seen only for the induced strain lacking the putative pantothenase.

3.5. Effect of the Deletion of *pan2* on Pantoate 4-dehydrogenase Activity in *T. reesei*

To measure if the deletion of *pan2* has any effect on the intracellular pantoate 4-dehydrogenase activity, we compared this activity in cell free extracts of *T. reesei* and a *pan2* deletion strain. Enzymatic activity of the short-chain dehydrogenase pantoate 4-dehydrogenase was first measured in *T. reesei* cell free extracts by following NADH formation at 340nm. As the cell extracts showed a rather low pantoate 4-dehydrogenase activity, the changes in absorption were recorded usually between 5 and 20 minutes after starting the reaction by pantoate addition, as there was a linear increase of the absorption during that period.

To obtain cell free extracts, three strains were cultured for 24 hours on MA-medium with glycerol and then induced by addition of 0.5% pantothenic acid. Biomass was harvested after 3 hours and cell free extracts were prepared.

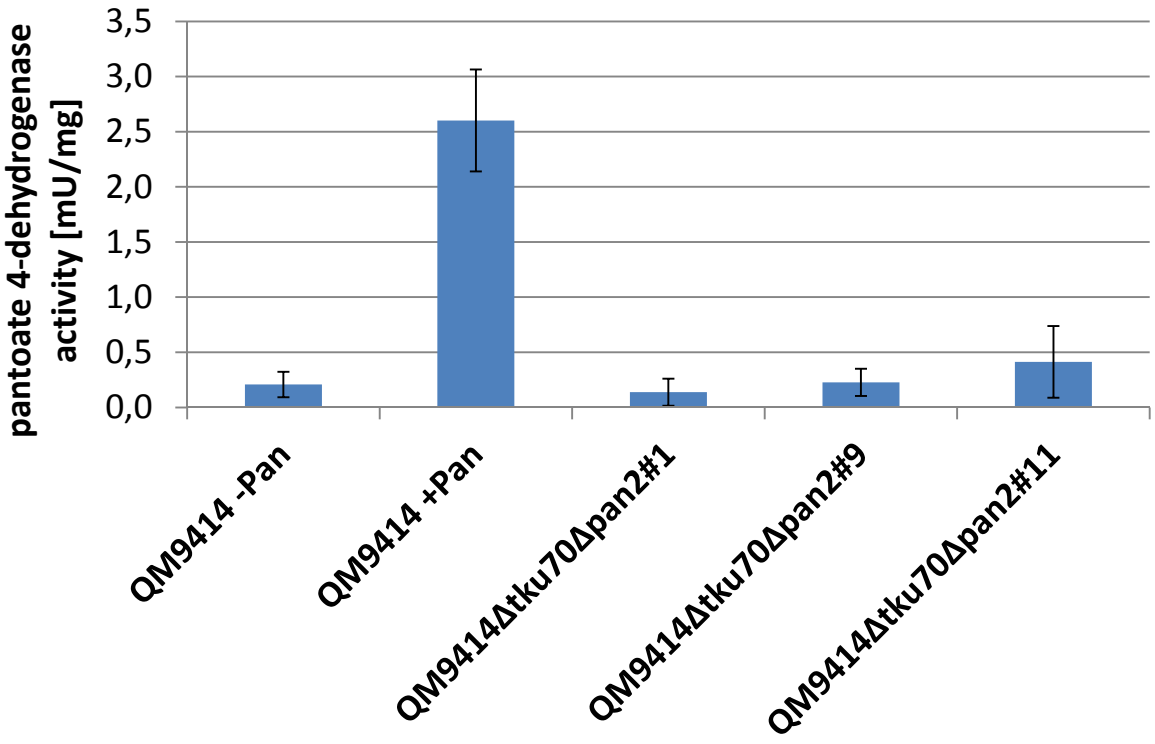

Figure 10: Pantoate 4-dehydrogenase activity in *T. reesei* cell free extracts: The reference strain and three pan2 deleted stains were induced or mock treated with pantothenic acid following preparation of cell free extracts. Activity was measured in technical triplicates in 3 isogenic *pan2* deletion strains compared to uninduced and induced samples of the reference strain QM9414.

Clearly, pantothenic acid addition to the medium induced an enzyme in QM9414 with pantoate dehydrogenase activity. The reduced activity in the three *Δpan2* strains adds to the notion that PAN2 is the enzyme responsible for the pantoate 4-dehydrogenase.

The enzyme assay was also repeated using $NADP^+$ instead of NAD^+ but no $NADP^+$ pantoate 4-dehydrogenase dependent activity was found (results not shown). Therefore the enzyme exhibiting pantoate dehydrogenase activity appears to be strictly NAD^+ dependent.

3.6. Heterologous Production of Pantothenase PAN1 and Pantoate 4-dehydrogenase PAN2 in *E.coli*

To characterize the enzymatic properties of the different enzymes of the putative pantothenic catabolic pathway, the enzymes responsible for the first two steps were recombinantly expressed in *E. coli*. Therefore the pET21a(+) plasmid was digested using the restriction enzymes NdeI and XhoI; the inserts were amplified from *T. reesei* cDNA (pantothenic acid induced culture) using the primers listed in Table 2. Thereby 15 bp overlaps were introduced for recombinational cloning of the cDNA insert into the plasmid.

The presence and orientation of the cDNA inserts (*pan1*: 1692 bp; *pan2*: 768 bp) was verified by restriction digests using XbaI (pAB-9: pET21a(+); *pan1*) and EcoRV (pAB-10: pET21a(+); *pan2*). The digest of pAB-9 was only partially successful due to the methylation protection of

the XbaI site in the *pan1* cDNA , but it can be seen that the plasmid has the expected size of 7 kb. The digest of pAB-10 led to two fragments of 1878 bp and 4233 bp as shown in Figure 11 which is in accordance to the expected sizes of the construct.

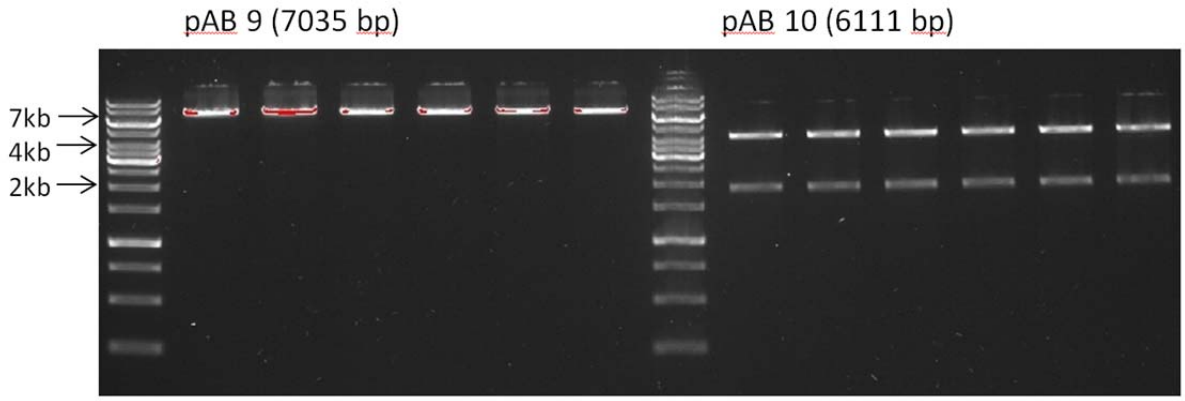

Figure 11: Control digest for pAB-9 (XbaI) and pAB-10 (EcoRV). The digest with XbaI led to a plasmid of 7kb while the EcoRV digest led to two fragments with 1.9kb and 4.2kb.

Successful integration of the coding region of the two genes into the pET21a(+) vector and absence of mutations was verified by sequencing of the isolated plasmids. Analysis of the cDNA sequence showed that an intron was not correctly assigned in the *pan1* sequence present at the JGI genome homepage (http://genome.jgi-psf.org/Trire2/Trire2.home.html). The complete corrected *pan1* and *pan2* sequence and the corresponding protein sequences are shown in the supplementary material.

Initially three *E. coli* strains for each plasmid were grown together with a control strain. The strains containing the plasmid pAB-9 or pAB-10 were named AB-I-x or AB-II-x, where x is the number of the transformant. Precultures in 10 ml SB-amp were grown at 37°C and 200 rpm for 19 hours and 1 ml of these precultures were then used to inoculate the main culture medium (100 ml SB-amp, 37°C, 250rpm). After 2 hours of further growth at 37°C the temperature was decreased to 25°C and OD_{600} was measured. Then the cultures were induced with 1mM IPTG. Cultures were harvested 21 hours after IPTG induction and the pellets were frozen at -20°C until use.

Table 6: OD600 values of IPTG induced production strains

Strain	Induction t=0	Harvest t=21h
AB-I-1	0.528	10
AB-I-2	0.655	9.4
AB-I-3	0.513	9.58
AB-II-1	0.416	9.68
AB-II-2	0.44	9.82
AB-II-3	0.46	9.4

3.7. Characterization of the recombinantly produced PAN1

PAN1 was isolated from the cell extract of the *E. coli* strain AB-I-1. Additionally one control strain was prepared which did not contain the plasmid pET21a(+). Protein was purified by use of the ÄKTA Fast Protein Purification Liquid Chromatography System. During elution of the protein extracts the control strain showed no peak suggesting that no protein bound to the Ni^{2+} Sepharose High Performance column. In contrast affinity purification of the cell free extract of the expression strain resulted in two peaks during elution similar to the PAN2 purification. Several fractions assigned to a peak were pooled and analyzed together with the crude extract and flowthrough by SDS-PAGE.

As shown in Figure 12 a protein band corresponding to the 62 kDa putative pantothenase PAN1 proves that enzyme was successfully overexpressed and purified. The size of PAN1 is slightly above 60 kDa which is in accordance with the theoretical size (62 kDa) and it could be purified very well being separated from the other proteins without any protein in the flowthrough. The control does not show any peak at the height of the target protein.

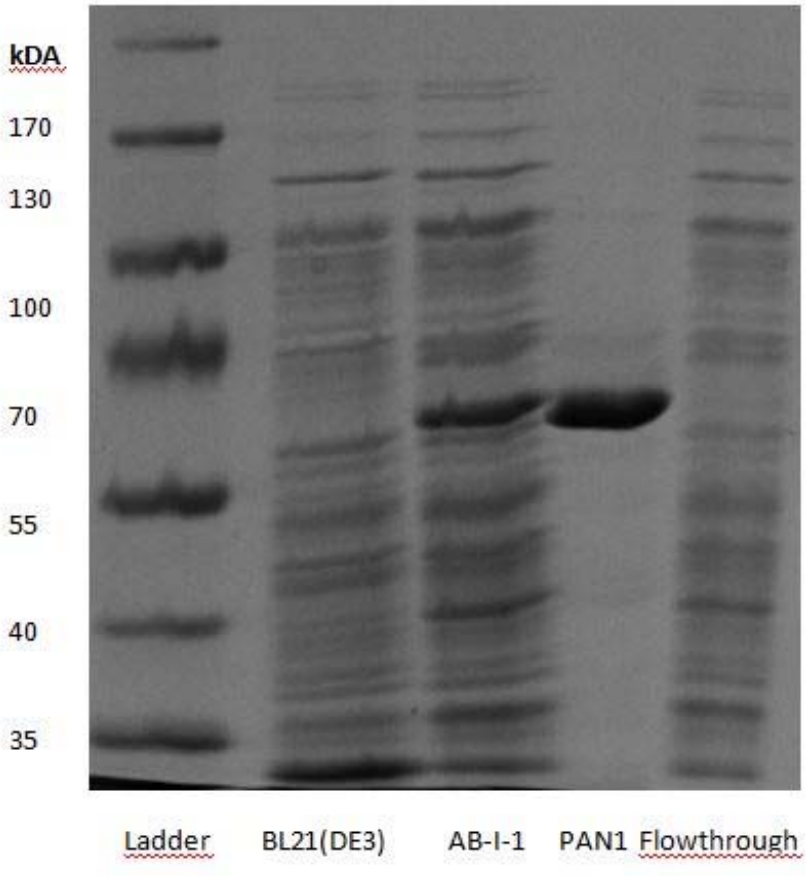

Figure 12: 12.5 % SDS-PAGE of control strain, crude extract of PAN1 producing *E. coli*, the affinity purified PAN1 and the flowthrough. The crude extract of the AB-I-1 strain is shown compared to that of a control strain without pET21a(+). The target enzyme PAN1 is found at the expected size of 62 kDA.

3.7.1. Enzymatic Properties of the *T. reesei* PAN1

The pantothenase PAN1 catalyzes the degradation of (R)-pantothenate into (R)-pantoate and ß-alanine. Enzyme assays were made as described in the methods section using the purified enzyme fraction shown in Figure 12. To follow the reaction the formation of Figure 15ß-alanine was measured by a ninhydrin assay. To determine the Michaelis constant K_m the enzyme test was performed with varying concentrations of the substrate pantothenic acid. The reaction was started by addition of the enzyme and stopped by heating to 100 °C for 3 min. Then ß-alanine formation was measured using the ninhydrin assay. The concentration of pantothenic acid was varied between 2.5 and 20 mM.

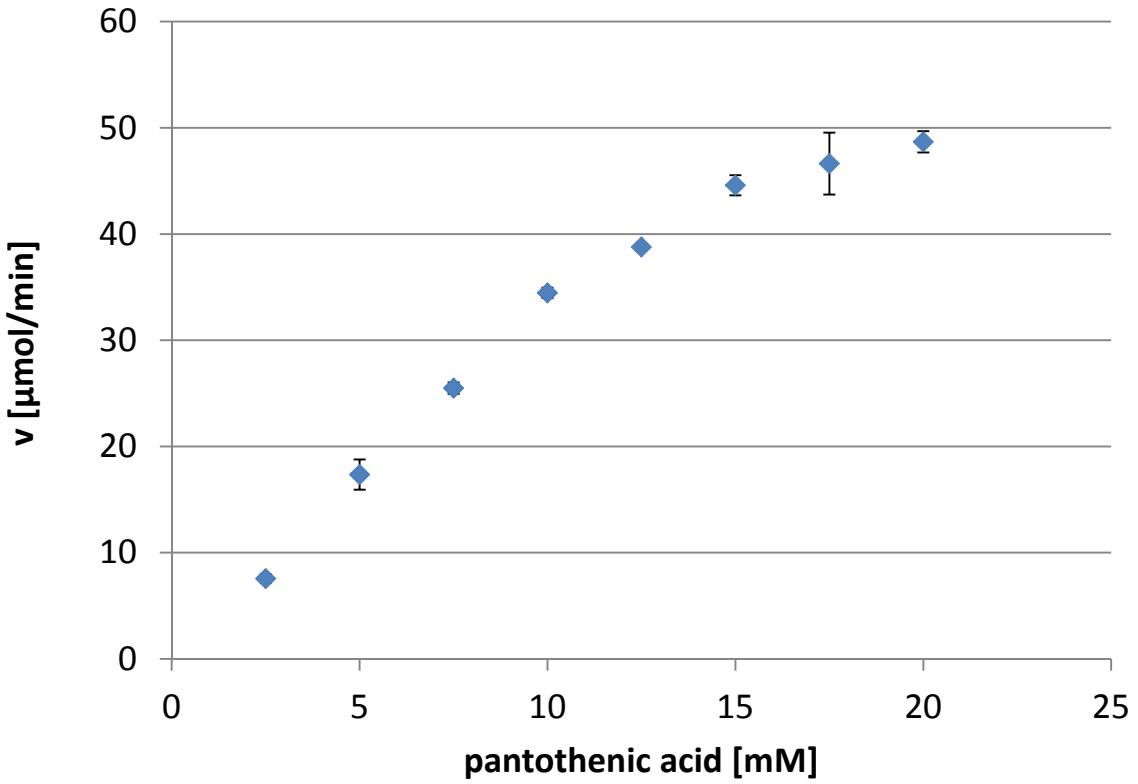

Figure 13: Michaelis Menten plot showing the measurements of the reaction velocity at different substrate concentrations. Pantothenase PAN1 catalyzes the reaction of pantothenic acid to ß-alanine and pantoate and its activity was measured over one hour; ß-alanine formed was measured by a ninhydrin assay.

The corresponding Lineweaver-Burk plot did lead to a linear trendline cutting the negative axis of abscissa to determine the Michaelis constant (Figure 14). The intersection with the abscissa was used to calculate a K_m constant of 15 mM.

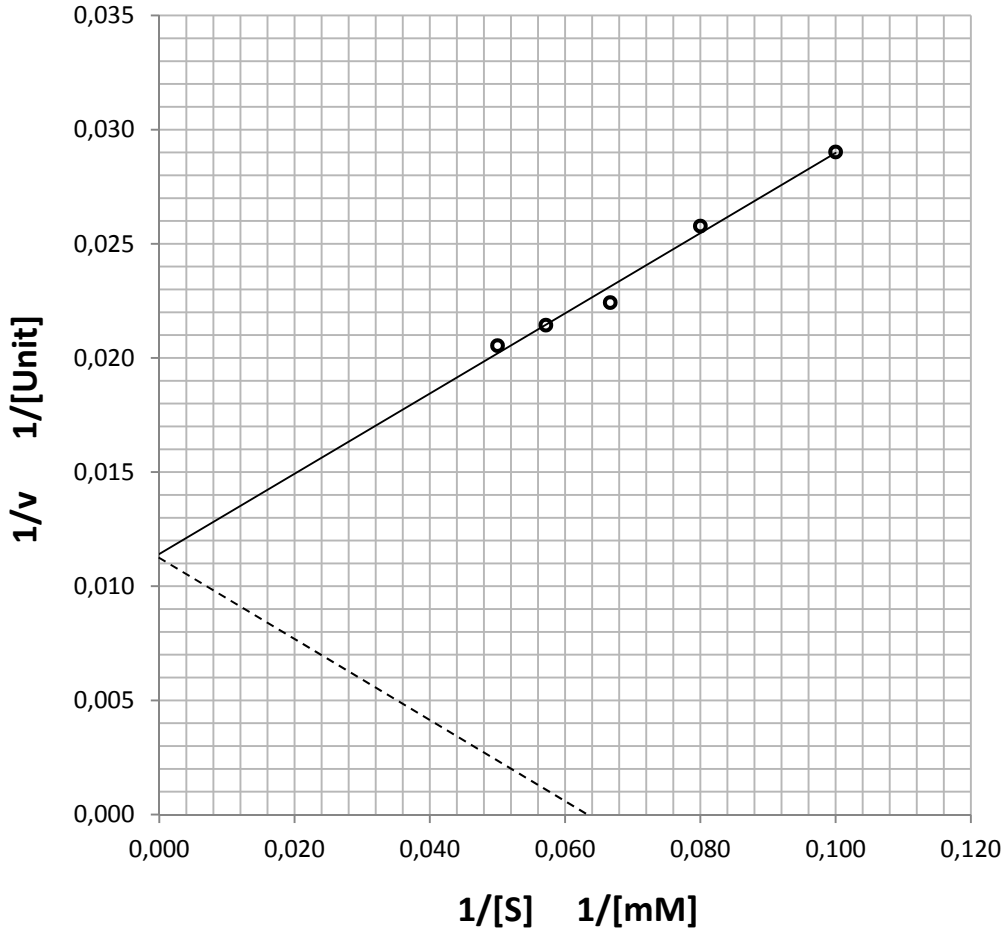

Figure 14: Lineweaver-Burk plot of the pantothenase assay. The intersection with the abscissa leads to a K_m constant of 15 mM.

3.8. Characterization of the recombinantly produced PAN2

PAN2 was first isolated from the cell extracts of strains AB-II-1 and AB-II-2. Additionally one control strain was prepared which did not express PAN2. PAN2 was purified through affinity of the fused C-terminal His-Tag to a Ni^{2+} sepharose column by use of the ÄKTA Fast Protein Purification Liquid Chromatography System. During elution the protein extracts of the control strain did not yield a peak suggesting that no protein bound to the Ni^{2+} Sepharose High Performance column. In contrast affinity purification of the cell free extracts of the two expression strains resulted in two peaks during elution. One very small peak was observed after just a few minutes of increasing percentage of the elution buffer and then a larger peak starting at a 70% of the elution buffer. Several fractions assigned to a peak were pooled and analyzed together with the crude extract by SDS-PAGE.

As shown in Figure 15 a band around 27 kDa is found in the SDS PAGE which suggests that the putative pantoate 4-dehydrogenase PAN2 was successfully overexpressed and purified. The size of PAN2 is in accordance with the theoretical molecular mass (27 kDa) and it could be purified very well being separated from the other proteins. The fraction 1 (F-1) shows the first smaller peak after starting elution from the Ni^{2+} column. This fraction comprises all proteins that weakly bind to the column. The second fraction shows a band corresponding to the purified short-chain dehydrogenase PAN2. The control strain without the enzyme encoding gene does not show that protein band at around 27 kDa.

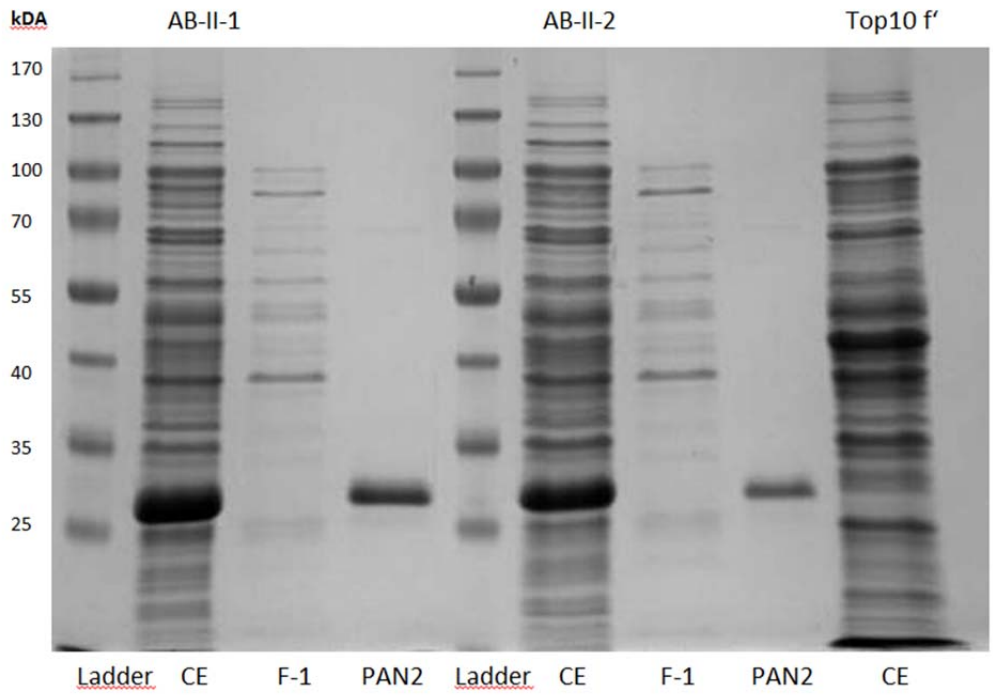

Figure 15: SDS-PAGE of crude extracts of PAN2 producing *E. coli* and the affinity purified PAN2. Crude extracts and different pooled fractions following affinity purification were separated in a 15% SDS PAGE. The crude extracts of two different strains (AB-II-1 and AB-II-2) are shown compared to a control strain (Top10 f`) with the empty pET21a(+). The target enzyme PAN2 is found slightly above 25 kDa.

3.8.1. Enzymatic Properties of the *T. reesei* PAN2

Enzyme assays were made as described in the methods section using the purified enzyme fractions shown in Figure 15 and activity was measured by following the NADH formation at 340 nm on the photometer. The results of the purification and the enzyme assay are listed in Table 7. In the crude extract of the reference sample no activity could be measured.

Table 7: Summary of the PAN2 affinity purification and pantoate 4-dehydrogenase enzyme assays

		Specific Activity [mU/mg]	Protein Amount [mg]	Yield	Purification Factor	Recovery
AB-II-1	**Crude Extract**	3.2	98.2			90 %
	Purified Enzyme	19.4	14.4	89 %	6.1	
AB-II-2	**Crude Extract**	3.0	107.8			87 %
	Purified Enzyme	29.0	9.9	89 %	9.7	

To determine the Michaelis constant K_m, the enzyme test was performed with varying concentrations of the substrate pantoate. Therefore the reaction was started by addition of the pantoate and the absorption at 340 nm was measured every 10 seconds for a minute. The concentration was varied between 1.5 and 10 mM pantoate. Results are shown in Figure 16 revealing that the reaction does not show significant absorption changes until a concentration of at least 3.5 mM. Lower concentrations did not lead to reliable results due to very slow changes of absorption.

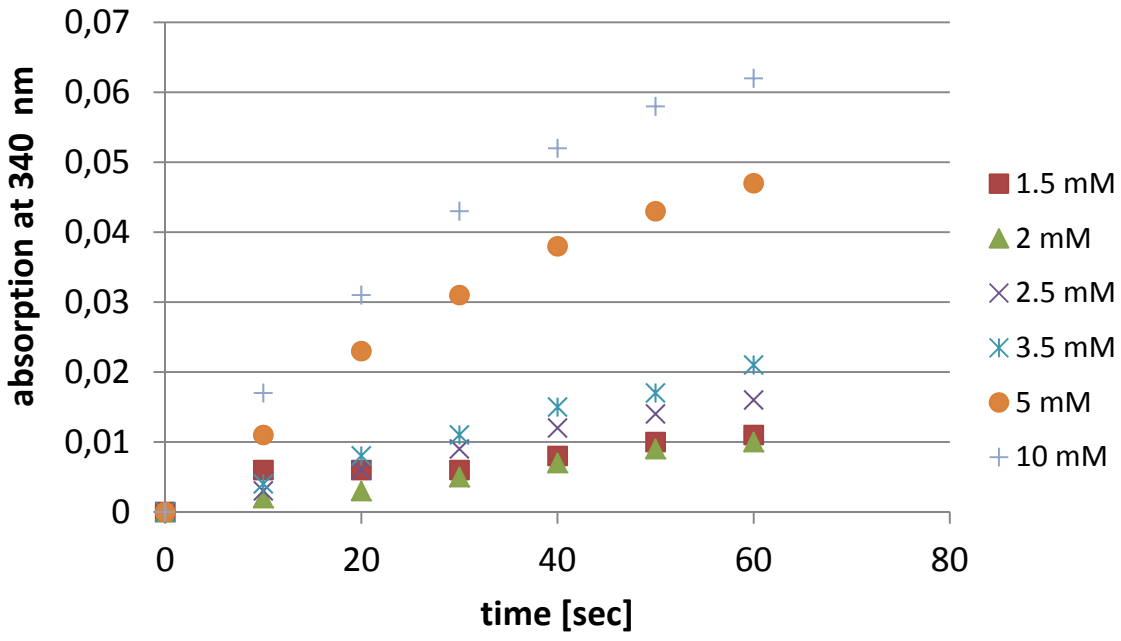

Figure 16: Pantoate 4-dehydrogenase assay: Concentration of pantoate was varied between 1.5 and 10 mM and the reaction was followed for a minute.

The corresponding Lineweaver-Burk plot did not lead to a linear trendline cutting the negative axis of abscissa to determine the Michaelis constant. The reaction is strongly product inhibited and the substrate was synthesized without exact determination of the purity which both complicates the enzyme assay. For further measurements more substrate will be needed first and the optimal temperature and pH range of the enzyme should be determined to reach higher absorption changes. Another interesting point will be to check whether or not the dehydrogenase contains zinc as indicated by an analysis of Pfam domains (see Table 8).

Table 8: PFAM domains for PAP2

Start	End	Protein Family	Accession	Score	E-value	Domain
9	173	Short chain dehydrogenase	PF00106	78.40	6.2E-23	Adh_short
11	39	NAD dependent epimerase/dehydratase family	PF01370	-0.30	0.39	Epimerase
19	63	Methyltransferase domain	PF08241	0.1	0.52	Methyltransf_11
9	173	Short chain dehydrogenase	PF00106	80.30	1.3E-22	Adh_short
7	141	Zinc-binding dehydrogenase	PF00107	-16.70	4.1E-4	ADH_zinc_N
11	242	NAD dependent epimerase/dehydratase family	PF01370	-69.40	1.1E-3	Epimerase
9	224	Pyridine nucleotide-disulphide oxidoreductase	PF07992	-82.30	0.53	Pyr_redox_2

Nevertheless the enzyme definitely shows activity in degrading pantoate, but the measurements still have to be repeated until reliable results are received and the K_m can be determined.

4. Discussion

Pantothenic acid was recently identified to be a neutral inducer for *T. reesei* with respect to growth and cellulase gene expression during growth on a number of carbon sources (Bischof *et al.*, unpublished data). By microarray analysis a number of different genes were identified which were upregulated upon addition of pantothenic acid. The most strongly induced genes were located in a cluster which contained a putative pantothenate permease, a fungal $Zn(II)_2Cys_6$ transcription factor and four enzymes putatively involved in the catabolism of pantothenate.

In silico analysis of the different enzymes and their putative activity in combination with published literature on the *Pseudomonas* pantothenic acid catabolism [33] led to a blueprint of a putative pantothenic acid catabolic pathway which consists of the following steps: Pantothenate is degraded by the pantothenate hydrolase (PAN1) to ß-alanine and pantoate followed by an oxidation of pantoate into R-4-dehydropantoate via pantoate dehydrogenase (PAN2). Finally R-4-dehydropantoate is further oxidized into R-3,3-dimethylmalate by the dehydropantoate dehydrogenase (PAN3) and then decarboxylated to 3-methyl-2-oxobutanoate by dimethylmalate dehydrogenase (PAN4).

The aim of the thesis was to investigate the genes of this cluster in more detail to provide evidence that the genes are involved in pantothenic acid degradation. Therefore the effect on the elimination of the different enzymatic steps was studied with respect to growth on pantothenic acid as sole carbon source. These investigations showed no significant difference between the reference strain and the four knockout strains. Apart from that the enzymes are not involved in pantothenic acid degradation, one possible explanation for this result is that the loss of the activity in these strains is compensated by other enzymes having the same activity. In this respect it is interesting to note, that for PAN3 and PAN4 enzymes with a high similarity are encoded in the *T. reesei* genome as shown in Table 9. Another possibility could be that an alternative pathway can compensate for the growth on pantothenate.

Table 9: Enzymes encoded in the *T. reesei* genome with a high similarity to PAN3 and PAN4.

Gene Name	Putative Function	Closest *T. reesei* Homologue	Putative Function	Blastp Score	E-value
pan3	Dehydropantoate DH	*trire2:22741*	Succinate semialdehyde DH	1037	9.95E-125
pan4	Dimethylmalate DH	*trire2:122233*	3-Isopropymalate DH	964	2.01E-98

Two possible candidate enzymes having such an overlapping activity based on their similarity to PAN3 and PAN4 are the succinate semialdehyde dehydrogenase and the 3-

isopropylmalate dehydrogenase. The succinate semialdehyde dehydrogenase (EC.1.2.1.16) is involved in alanine, aspartate and glutamate metabolism (http://www.genome.jp/dbget-bin/www_bget?tre:TRIREDRAFT_22741) and could potentially take over the function of 4-Dehydropantoate dehydrogenase. The other candidate is the 3-isopropylmalate dehydrogenase (EC.1.1.1.85) involved in leucine biosynthesis (http://www.genome.jp/dbget-bin/www_bget?tre:TRIREDRAFT_112247+tre:TRIREDRAFT_122233) which could take over the function of ß,ß-dimethyl malate dehydrogenase. The substrate for succiniate semialdehyde DH lacks the substituents on C2 (OH) and C3 [$(CH_3)_2$] compared to the PAN3 substrate (R)-4-dehydropantoate. The substrate for 3-Isopropylmalate DH differs from the respective pantothenate catabolic intermediate only by an isopropyl side chain instead of a ß,ß-dimethyl substituent. In accordance with our hypothesis, that residual activities are still present in deleted strains, we found that there is still a residual pantoate 4-dehydrogenase activity in the non-induced QM9414Δ*tku70* and in the pantothenic acid induced Δ*pan2* strains, which is around 10% of pantoate 4-dehydrogenase activity found under inducing conditions. For the *pap1* deletion strains, the lack of reduced growth is explained by the fact that undissociated pantothenic acid can be passively transported through the cell membrane by diffusion [25]. Since its pKS is about 4.40 and the unbuffered Mandels Andreotti medium has a pH of about 5.5, pantothenic acid was still partly undissociated in all our experiments according to the Henderson-Hasselbach equation. At the cytosolic pH, which typically is considerably higher (above 7.0), pantothenic acid would be present exclusively as pantothenate. Pantothenic acid is therefore imported into the cell until the cytosol is acidified to a point that allows a diffusion equilibrium to be established between the intra- and extracellular space. This would in turn lead to very high intracellular levels of pantothenate and subsequently its metabolites, meaning that even enzymes with a very low affinity towards pantothenate and its intermediates could take over the function of the pathway enzymes. Interestingly, the control with ß-alanine as the sole carbon source did not grow well at all. One obvious explanation for this would be that ß-alanine is not transported into the cells. In *E. coli*, the secondary H^+-symporter CycA is responsible for the transport of ß-alanine [34] as well as for that of L-alanine and D-alanine, D-serine and D-cycloserine and glycine [35, 36]. Schneider *et al.* [34] found that even if *cycA* was deleted from the *E. coli* genome, ß-alanine was still transported into the cell at a lower rate, and reasoned that it is likely transported by another transporter or via diffusion, indicating that there might be more than one amino acid transporter with an overlapping ability to transport ß-alanine in the absence of its preferred substrate. One such fungal permease that has a broad substrate specificity is the *S. cerevisiae* permease Agp1p that transports, amongst other amino acids, also those that are transported by the permease responsible for ß-alanine uptake in *E. coli* [37]. The *T. reesei* genome also encodes two proteins with high sequence similarity to this permease namely Trire2:5787 (E-value $2*10^{-117}$) and Trire2:75430 (E-value $5*10^{-108}$). We thus

consider it unlikely that lack of transport is the sole reason for *T. reesei*'s inability to grow on ß-alanine.
There is, however, another explanation: half of the known metabolic reactions where ß-alanine is a substrate are ATP-dependent condensation reactions. Consequently, growth in the presence of ß-alanine as the sole carbon source leads to a rapid depletion of the cellular ATP pool. In contrast, when pantoic acid is coproduced by the hydrolysis of pantothenate, its degradation generates NADH which can in turn be reoxidized in the respiratory chain to replenish the cellular ATP. Three of the other reactions are transamination reactions of E.C. 2.6.1.18 (3-oxopropanoate as aminoacceptor), E.C. 2.6.1.55 and 2.6.1.19 (in both cases 2-oxoglutarate as aminoacceptor). The last remaining reaction of E.C. 3.5.1.6 produces N-carbamoyl-ß-alanine with CO2 and NH3 and subsequently leads to uracil via two additional steps, E.C. 3.5.2.2 and E.C. 1.3.1.1 (NAD+ dependent) / EC 1.3.1.2 (NADP+ dependent).

Further experiments with the *pan1* deletion strain will be necessary to see if there is also a residual pantothenase reaction found in this strain. The accumulation of pantothenate in this strain points at least to the fact that the PAN1 enzyme is directly involved in pantothenic acid degradation.

The corresponding pantothenase PAN1 was partly characterized leading to a K_m of 15 mM which is in the expected range as compared to the *Pseudomonas* P2 pantothenate hydrolase, which has a K_m of 5 mM [27].

The second enzyme of the pathway pantoate dehydrogenase PAN2 could not yet be fully characterized but was shown to be strictly NAD^+ dependent as activity assays using $NADP^+$ as the cofactor did not lead to any activity. Further experiments will be needed to determine the K_m value of PAN2. It was assumed that its K_m would be around 33 µM [28]. The authors also mentioned that the reaction equilibrium lies on the left side of pantoate and the reaction is strongly product inhibited. In *Pseudomonas* P-2 the third reaction of the pantothenate pathway degrading aldopantoate is irreversible and therefore promotes the pantoate dehydrogenase reaction. It is also possible that the enzyme is regulated allosterically so as to prevent reaching a high aldehyde level, as they are reactive metabolites that influence biological function and cause toxic side effects [38, 39]. The fungal enzyme isolated within this thesis, however, seemed to have a higher K_m. In comparison several other fungal dehydrogenases and their K_m are shown in Table 10. As can be seen, it is not unusual for fungal dehydrogenases to have K_m values above 1 mM.

Table 10: List of fungal dehydrogenases and corresponding K_m values

Enzyme	Organism	K_m [mM]	Reference
L-rhamnose 1-Dehydrogenase	*Pichia stipitis*	1.5	[40]
D-xylose Dehydrogenase	*Hypocrea jecorina*	25	[41]
Glycerol Dehydrogenase	*Hypocrea jecorina*	0.9	[42]
Mannitol Dehydrogenase	*Hypocrea jecorina*	16	[43]
Glyceraldehyde 3-Phosphate Dehydrogenase	*Kluyveromyces lactis*	0.75	[44]
Sorbitol Dehydrogenase	*Aspergillus niger*	50	[45]
Galactitol dehydrogenase	*Aspergillus niger*	200mM for Galactitol 55mM for Arabinose	[46]

The different strains deleted in the four steps of the pantothenate pathway were also used to assess the effect of a metabolic engineering of the pathway. Therefore the expression of the pantothenic acid permease *pap1* was followed in the deletion strains as it is pantothenic acid inducible and present in all strains.

Transcript levels of *pap1* were higher and more stable in strains where the pantothenate hydrolase PAN1 was absent while the other deletion strains did not show an effect. This strongly indicates that pantothenic acid itself serves as the inducer of *pap1* and in conclusion of the whole gene cluster. This hypothesis is supported by the finding that pantothenate accumulates intracellularily in *Δpan1* strains after its addition to the medium. However, also the basal *pap1* transcript level before induction is increased significantly in the Δ*pan1* strain compared to the reference and all other knockout strains. This could indicate an increased steady state level of intracellular pantothenate leading to a pseudoconstitutive expression of *pap1* in the absence of PAN1. While this hypothesis is supported by the fact that most of the genes for the *de novo* synthesis of pantothenate appear to be present in the genome of *T. reesei* (vide supra), efforts to show the accumulation of pantothenate in an uninduced *Δpan1* strain did not lead to the expected result. Another possibility is that the replacement of the *pan1* open reading frame with the hygromycin resistance cassette *per se* causes a deregulation of the gene cluster or that the absence of PAN1 leads to this higher transcript

levels. Previous results have shown that all the cluster genes are positively regulated by the $Zn(II)_2Cys_6$ transcription factor PAA1 [26]. Likewise, the biosynthesis of the vitamin thiamin is regulated by such a $Zn(II)_2Cys_6$ transcription factor i*n S. cerevisiae* [47] as are the catabolism of the amino acids proline, serine and threonine [48-50]. The proline regulator Put3p of *S. cerevisiae* is constitutively bound to the promoters of prolin utilization genes but activated only through direct interaction with proline [51, 52]. Another possible *modus operandi* for $Zn(II)_2Cys_6$ transcription factors is that they interact with chromatin remodeling factors as has been shown, e.g. for Gal4p, whose activation domain recruits the SAGA complex [53] that by virtue of Gcn5p can loosen chromatin structure [54]. Since chromatin remodeling is known to contribute to the regulation of fungal gene clusters [55, 56], it is tempting to speculate that *paa1* might function in this way.

As previously described the promoters of *pan1* and *pan3* were used to drive the expression of heterologous genes in *T. reesei* (R. Bischof, A. Beinhauer, C.P. Kubicek and B. Seiboth, manuscript in preparation). A possible disadvantage of this expression system is that the induction of those genes is only transient depending on the presence and concentration of the inducer. The results presented here clearly underline the hypothesis that this is due to metabolism of the inducer by a pathway that resembles the bacterial pathway described by Goodhue *et al.* [33]. We reasoned that by disruption of the respective catabolic pathway we could identify the steps important for inducer formation respectively inducer degradation and thereby increase the inducer concentration and extend the expression of the pantothenic acid inducible genes. The accumulation of pantothenate in the *Δpan1* strain and the accompanying higher and more stable transcript levels are promising in this respect but come at the cost of lost tightness. To avoid this effect, a conditional knockdown strategy might be better suited for this purpose. One way to do this would be to replace one of the pathway genes with an antisense construct for *pan1*. Doing this would ensure that there still is a basal level of the PAN1 protein, but that the PAN1 activity is decreased when pantothenic acid induction takes place. Evaluation of the growth of *Δpan2* strains on agar plates with a complex (PDA) or defined (MA glucose) medium showed, that growth of *T. reesei* was unaffected in both cases, while sporulation was increased slightly for all gene deletion strains when grown on a complex medium (PDA). These results indicate that the *pan2* locus could be used to harbor the antisense construct, since deletion of *pan2* does not appear to have a serious impact on the fitness of *T. reesei*.

Beside the application of the *pan* system for gene expression, this pathway and its enzymes can provide further information for engineered production of pantothenic acid. The intermediates of the pantothenate catabolism have potentially interesting chemical structures for further synthesis and might be well suited as platform chemicals probably for the production of bioplastics or other biopolymers if produced by *T. reesei* on the cheap C-

source wheat straw. R-3,3-dimethylmalate for example shows high similarity to succinic acid which is a building block for commodity and specialty chemicals as for example fibers like lycra. C4 building block diacids in general have a significant market opportunity as precursor for biobased products [57].

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6. Supplementary Material

6.1. Sequences

6.1.1. pAB-9 – *pan1* in pET21a(+)

70375_Infus_fw
70375_Infus_nostop_rv
pan1

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6.1.2. Pantothenase Protein Sequence

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6.1.1. pAB-10 – *pan2* in pET21a(+)

70334_Infus_fw
70334_Infus_nostop_rv
pan2

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GCGGGATATAACATGAGCTGTCTTCGGTATCGTCGTATCCCACTACCGAGATATCCGCACCAACGCGCAGCCCGGACTCGG
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GCATGGTTTGTTGAAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATTTGATTGCGAGTGAGAT
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AATGCGACCAGATGCTCCACGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTTGATGGGTGTCTGGTCAGA
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TGATCAGCCCACTGACGCGTTGCGCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCG
ACACCACCACGCTGGCACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGCAGGGCCAGA
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CGCCATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAAACGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTGATA
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AGGAAGCAGCCCAGTAGTAGGTTGAGGCCGTTGAGCACCGCCGCCGCAAGGAATGGTGCATGCAAGGAGATGGCGCCC
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6.2.2. Pantoate 4-dehydrogenase Protein Sequence

MPSNRLEGKVAVITGAASGFGKGIATKFAQEGAKVIVADLSQEAGQQAASELNGIFIRTDVTKRSDWEAILALALKEYGQLDIVV
NNAGASYSNKATEGVIEQEFDMVMNVNVKSIYFSTNILVPYFLKENRPGSFIQIASTAGIRPRGGLAWYSASKGAAITATKALASE
YGPKQIRFNAVSPVVGITGMTNLFLGSTDISTFVSTVPLGRPSTPADIANACCYLASDEASFITGVNLEVDGGRCVLEHHHHHH