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Pantothenic Acid Degradation in *Trichoderma reesei*

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Zusammenfassung

Pantothensäure bzw Vitamin B_5 ist ein wichtiger Precursor für die Biosynthese von Coenzym A (CoA), das als Acylgruppenträger fungiert und u.a. am Auf- und Abbau von Kohlenhydraten und Fetten beteiligt ist. Im Gegensatz zum Aufbau der Pantothensäure ist über deren Abbau wenig bekannt. Im *T. reesei* Genom sind die für den Pantothensäureabbau notwendigen Gene in einem Cluster zusammengefasst, der auch noch einen $Zn(II)_2Cys_6$Transkriptionsfaktor PAA1 und eine Permease PAP1 umfasst.

Um den Einfluss des Transkriptionsfaktors PAA1 auf die Expression des Pantothensäureclusters aufzuzeigen, wurden die Expression der anderen 5 Gene in einem Stamm, der im Regulator deletiert ist, untersucht. Es zeigte sich, dass die Induktion dieser Gene durch Pantothensäure in Abwesenheit des PAA1 Regulators verloren geht, aber in einem Stamm in dem der intakte Regulator wieder eingeführt wurde, wiederhergestellt werden konnte. Die Funktion der Permease PAP1 wurde mittels einer Komplementationsassays der *Saccharomyces cerevisiae* Pantothensäure-Permease-Mutante *fen2* untersucht. Die Einbringung des *T. reesei paa1* in die *fen2* Mutante, führte zur Aufhebung des Wachstumsdefektes des *fen2* Stammes und bestätigte somit die Funktion der PAP1 als Pantothensäurepermease. Weiters wurden die 2 Enzyme der ersten beiden Schritte des Pantothensäureabbaus in *E. coli* exprimiert und über einen His Tag gereinigt. Die Pantothenase PAN1, welche für den Abbau der Pantothensäure zu Pantoat und ß-Alanin verantwortlich ist, wurde in Inclusion Bodies produziert und konnte erst nach Optimierung der Wachtsumsbedingungen in löslichen Zustand produziert werden. Die Pantoat 4-dehydrogenase PAN2, welche die Oxidation von Pantoat zu R-4-dehydropantoat katalysiert, hat ein Molekulargewicht von 27 kDa, K_M(Pantoate) von 19,51 mM und eine v_{max} von 3,33 U/mg. In der vorliegenden Arbeit wurden somit weitere Bestandteile des Pantothensäureabbaus in *T. reesei* funktionell charakterisiert.

Abstract

Pantothenic acid (Vitamin B_5) is essential for life as a precursor for coenzyme A (CoA) biosynthesis. CoA functions as an acyl group carrier required for carbohydrate and fat metabolism. The biosynthesis pathway of pantothenate is already well documented in many bacteria, fungi and plants whereas the pantothenate catabolism is largely unknown.

Genes involved in pantothenate catabolism are clustered in the *Trichoderma reesei* genome and include beside four metabolic enzyme encoding genes, a putative pantothenate permease PAP1 and a $Zn(II)_2Cys_6$ transcription factor PAA1.

In this thesis the effect of the deletion of *paa1* on the expression of the other genes of the cluster was studied. It was shown that in the absence of *paa1* no induction could be observed. Induction of the genes was, however, restored in strains in which *paa1* was reintroduced. The function of the putative pantothenate permease PAP1 was tested in a complementation assay. Therefore, the *T. reesei pap1* was introduced into the *Saccharomyces cerevisiae* pantothenate permease mutant *fen2*. In these strains the growth defect of the *fen2* mutants was abolished which showed that *pap1* indeed encodes a pantothenate permease. The first two enzymes of pantothenate catabolism were expressed in *E. coli* and purified as His-Tag proteins. Pantothenase PAN1, which is responsible for the degradation of pantothenic acid to pantoate and ß-alanine, was mainly produced in inclusion bodies and could only be produced in soluble form after optimization of the growth conditions. Pantoat 4-dehydrogenase PAN2 which oxidizes pantoate to R-4-dehydropantoate, was produced in soluble form with a Mw of 27 kDa, K_M (pantoate) of 19,51 mM and a v_{max} of 3,33 U/mg. With the present work further components of the novel pathway of pantothenate catabolism could be identified.

Danksagung

An dieser Stelle möchte ich mich bei allen bedanken, die mich während meiner Diplomarbeit unterstützt haben.

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Parts of this thesis will be used for a manuscript entitled „Pantothenate catabolism is clustered in the *Trichoderma reesei* genome and positively regulated by a $Zn(II)_2Cys_6$ fungal transcription factor.“

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1. Introduction

1.1. *Trichoderma reesei*

Nowadays the imminent global climate change and the resulting natural disasters are prevalent. To decrease the harmful greenhouse effect, as well as to satisfy our future energy demands, targeted research towards cleaner and sustainable fuels is crucial. A possible alternative to fossil fuels are first generation biofuels, mainly produced from food crops and thus they also have several drawbacks - cue "food vs fuel". More promising alternatives are second generation biofuels ("lignocellulosic bioethanol") from agricultural and forest residues, like tree cuttings, corn cobs and straw. For an efficient lignocellulose hydrolysis a cocktail of hydrolytic enzymes (cellulases and hemicellulases) is required (Sims *et al*., 2008 & Mach-Aigner LAB project).

Trichoderma reesei is the most common (hemi)cellulase producer worldwide (Schuster *et al.*, 2010). In addition to its ability of degrading lignocellulose for bioethanol production these enzymes are also well established in the paper, pulp, food and textile industry. Based on these advantages *T. reesei* became a paradigm for commercial scale production of cellulolytic enzymes. In addition, the fermentation technology of this organism is also well established (Seiboth *et al*., 2011).

The cellulose degradation machinery of *T. reesei* faces some challenges, because lignocellulose biomass mainly consists of cellulose, hemicellulose, lignin and pectin and thus builds a complex structure (Seiboth *et al*., 2011). Therefore additional pretreatment procedures are necessary to improve cellulose digestibility (Himmel *et al*., 2007). For sufficient degradation four major types of enzymes - cellobiohydrolases (CBHs), endoglucanases (EGs), ß-glucosidases (BGLs) as well as LPMOs (lytic polysaccharide monooxygenases) - are necessary. LMPOs are metalloenzymes and act directly on the surface of crystalline cellulose by generating oxidized and non-oxidized chain ends. The increasing depolymerization results in a better access of the hydrolytic enzymes (Fig. 1) (Payne *et al*., 2015; Seiboth et al., 2011).

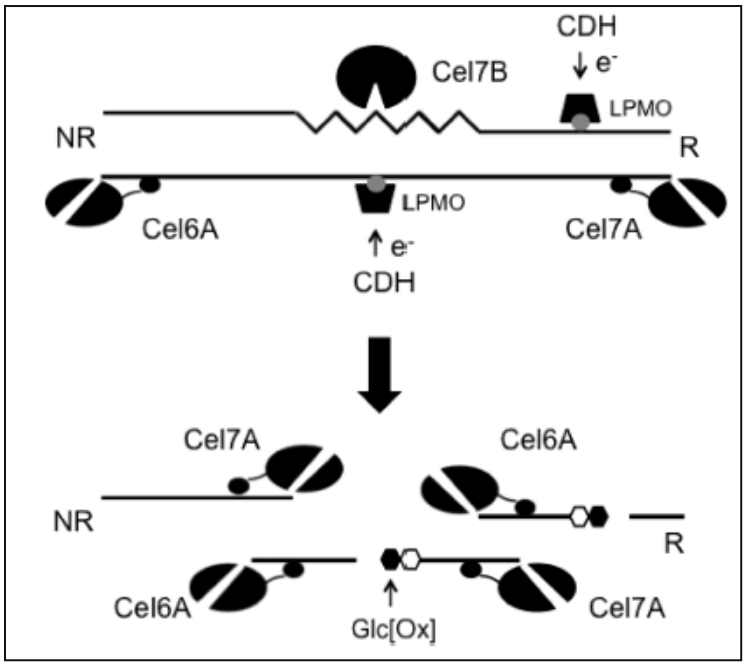

Figure 1: Cellulase degradation combining hydrolytic and oxidative fragmentation reactions. The cellobiohydrolases Cel6A and Cel7A function as exocellulases, which cleave two to four units from the cellulose ends. LMPOs generate oxidized and non oxidized chain ends and thus allow better access for the hydrolytic enzymes (Payne *et al*., 2015).

Filamentous fungi are versatile cell factories and thus they are utilized for heterologous protein expression (Schuster *et al*., 2010). As they are able to grow on various substrates and have the ability to degrade cellulosic biomass to glucose monomers, they are an attractive resource for new enzymes (Seiboth *et a*l., 2011). Tools for recombinant protein expression are already available in *T. reesei*. Different transformation systems, including the protoplast mediated transformation (PMT), electroporation, biolistic transformation and the *Agrobacterium* mediated transformation (AMT) are used for engineering virtually all *Trichoderma spp*.. For successful genetic transformation a number of selection markers are available. Promoters play a crucial role in protein production, as they control the expression of the target gene. In contrast to constitutive promoters, tunable (inducible or repressible) promoters have the huge advantage to decouple cell growth from protein production. This is an important aspect when the protein production displays a negative effect on cell growth. Under non induced conditions the ideal inducible promoter has no or only a very low basal expression level and a high expression level after induction (Bischof *et al*., 2014).

1.2. Pantothenic acid

Pantothenic acid (Fig. 2), also known as vitamin B_5, belongs to the water soluble B vitamins and was identified 1933 by Dr. Roger John Williams (Williams, 1939). The vitamin's name is derived from the Greek word "panthos" and can be translated as "from everywhere" (Rucker *et al.*, 2007 & Kelly, 2011). It reflects the fundamental importance for a successful biochemistry in almost every living cells (Higdon, 2003).

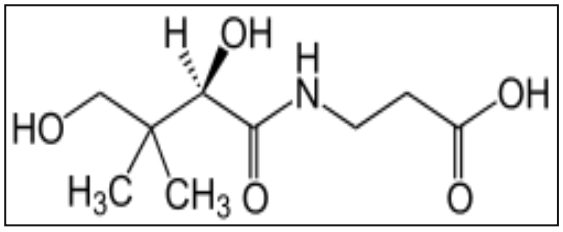

Figure 2: structural formula of pantothenic acid

The presence of vitamin B_5 in organisms plays an important part in different metabolic pathways, as it is required for the biosynthesis of coenzyme A (CoA). CoA is needed in the synthesis and oxidation of fatty acids and the oxidation of pyruvate in the citric acid cycle (Kelly, 2011 & EFSA, 2014). Pantothenic acid is also involved in the synthesis of various secondary metabolites such as steroid hormones, acetylated components, neurotransmitter and non ribosomal peptides and polyketides (Kelly, 2011). As pantothenic acid is taken up through a wide range of foods, a deficiency is normally rare, but it can be induced by the uptake of antagonists. Common symptoms are: fatigue, insomnia, depression, vomiting, stomach pain, cardiac instability and headaches (Kelly, 2011 & Smith, 1996).

Pantothenic acid can be synthesized by some microorganisms and plants de novo and is an amide of D-pantoic acid and ß-alanine. In contrast mammals and humans are not able to produce pantothenic acid and are thus dependant on the dietary intake. Rich sources are liver, kidney, chicken, duck, milk, yeast, broccoli, legumes, avocado, tomatoes, peanuts and whole grain products. Unlike other vitamins, Vitamin B_5 has no officially recommended daily allowance, but experts recommend a daily uptake of 5 mg for males and females over 14 years. During pregnancy and lactation the dietary reference intake is 7 mg/day (Kelly, 2011 & EFSA, 2014).

The derivates and precursors of vitamin B_5, used in dietary supplements and cosmetics, are produced by chemical synthesis. Because it is rather unstable and can be easily destroyed by heat, alkaline and acid conditions, the more stable calcium pantothenate is used in dietary supplements (Kelly, 2011).

The significant health effect of pantothenic acid and its derivates are confirmed in several studies. The treatment with pantethine, a disulfide of pantothenic acid, as a nutritional supplement at 600 - 900 mg/day leads to a significant decrease in total cholesterol and thus reduces the risk of cardiovascular disease (Kelly, 2011 & Evans *et al.*, 2014). Dexpanthenol

is applied as topical or in injected form for cosmetic purposes or as anti-inflammatory drug for wound healing (Ebner *et al.*, 2002). Positive results were reported when treating rheumatoid arthritis with pantothenic acid (1x500 mg to 4x500 mg per day) thus yielding a significant reduction of pain and morning stiffness (Kelly, 2011).

1.3. Rising commercial interest in biotechnological production of pantothenic acid

Currently about 5000 tonnes per year of pantothenate, used as dietary supplements and for addition to cosmetic products, are produced by bulk chemical synthesis. The additional separation of the racemic mixture - only the D-isomer possesses biological activity - significantly increases the costs and thus results in an expensive process (Coxon *et al.*, 2005).

As some bacteria, fungi and plants are capable of producing pantothenate de novo, this pathway plays an interesting role in the biotechnological production of pantothenic acid. Biotransformation does not require the necessary purification step and thus guarantees a more economical production (Coxon *et al.*, 2005).

The pantothenate biosynthetic pathway is already well characterized in *E. coli* and includes 4 enzymatic reactions (Fig. 3). First α-ketoisovalerat is metabolized into ketopantoate by ketopantoate hydroxymethyltrasferase (EC 2.1.2.11), using the cofactor 5,10-methylene tetrahydrofolate. In the following step ketopantoate is reduced to pantoate, catalyzed by ketopantoate reductase (EC 1.1.1.169). Simultaneously, L-aspartate is converted to ß-alanine, catalyzed by L-aspartate-α-decarboxylase (EC 4.1.1.11). The final step results in a condensation between ß-alanine and pantoate - catalyzed by the ATP dependent pantothenate synthetase (EC 6.3.2.1) - forming pantothenate (Chakauya *et al.*, 2006).

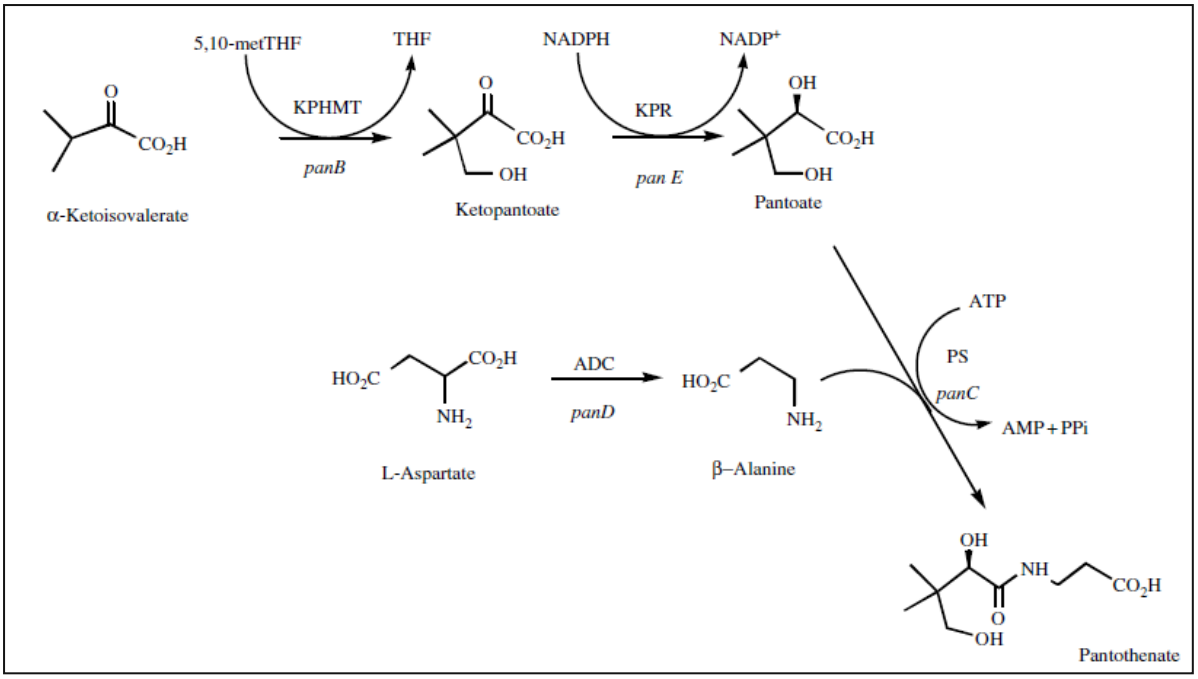

Figure 3: Pantothenate biosynthesis pathway in *E.coli* : α-ketoisovalerat is converted into ketopantoate by the ketopantoate hydroxymethyltransferase (KPHMT, encoded by *panB*), using the cofactor 5, 10-methylene tetrahydrofolate (5, 10 metTHF). Ketopantoate is reduced to pantoate by the ketopantoate reductase (KPR, encoded by *panE),* using NADPH as a hydrogen donor. L-aspartate-α-decarboxylase (ADC, encoded by *panD*) converts L-aspartate into ß-alanine, which is finally condensed with pantoate to form pantothenate by the ATP dependent pantothenate synthetase (PS, encoded by *panC*) (Chakauya *et al*., 2006).

In the past a variety of possibilities were discovered for pantothenic acid production in microorganisms. For example the production of pantothenate by *Azotobacter vinelandii* (Martinez-Toledo *et al*., 1996) and by *E. coli* cells using ATP regeneration (Kawabata *et al*., 1980). White *et al*. (2001) reported that *S. cerevisi*ae is capable of de novo pantothenate biosynthesis involving ß-alanine production from spermine.

As pantothenate biosynthesis does not occur in animals, the involved enzymes could also be optimal targets for the discovery of non-toxic antibiotics, herbicides and fungicides (Chakauya *et al*., 2006).

1.4. Coenzyme A

As already mentioned above vitamin B_5 is one of the precursors of CoA, which is an essential factor in all biological systems and thus plays a central role in different metabolic pathways. These include the tricarboxylic acid cycle and ß-oxidation, fatty acid and isoprenoid biosynthesis and the biosynthesis of different secondary metabolites. CoA functions as acyl group carrier and carbonyl activating groups (Coxon *et al*., 2005 & Chakauya *et al*., 2006).

CoA is biosynthesized in five enzymatic steps, requiring pantothenate, cysteine and 3 equivalents of ATP (Fig. 4). Pantothenate kinase (EC 2.7.1.33) phosphorylates pantothenate to 4'-phosphopantothenate, followed by a condensation of 4'-phosphopantothenate with cysteine, catalyzed by 4'-phosphopantothenoyl cysteine synthase (EC 6.3.2.5). The subsequent decarboxylation, catalyzed by 4'-phosphopantothenyl cysteine decarboxylase (EC 4.1.1.36), results in 4'-phosphopantetheine, which is adenylated by the enzyme phosphopantetheine adenylyl transferase (EC 2.7.7.3). Finally, dephospho-CoA is phosphorylated by dephospho-CoA kinase (EC 2.7.1.24) to CoA (Leonardi *et al*., 2005 & De Villiers *et al*, 2015).

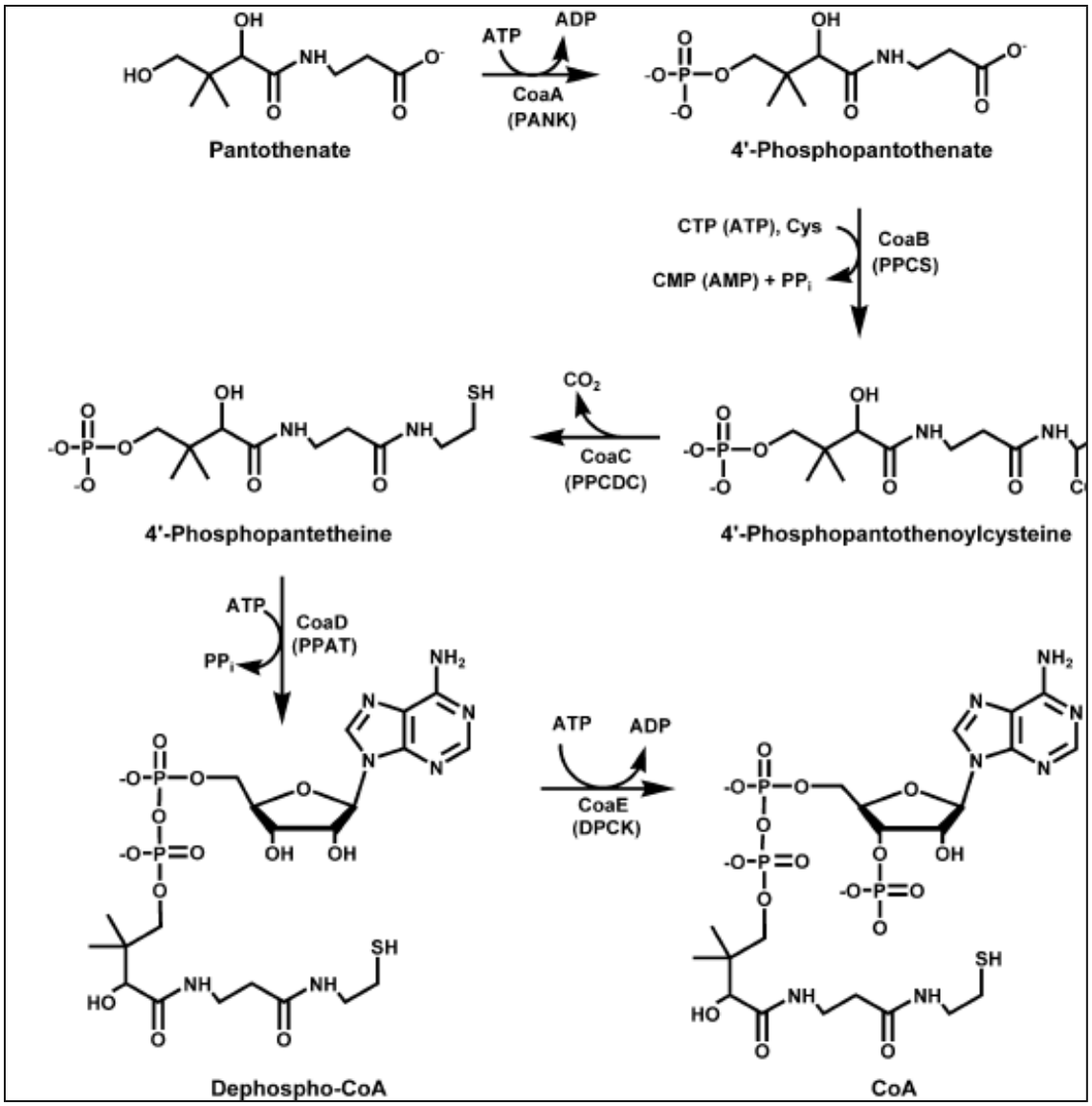

Figure 4: CoA biosynthesis pathway involves five enzymatic steps: Pantothenate is phosphorylated by the pantothenate kinase (PANK) to 4'-phosphopantothenate, which is condensed with cysteine (Cys) to form 4'-phosphopantothenylcysteine, catalyzed by the 4'-phosphopantothenyl cysteine synthetase (PPCS). The 4'-phosphopantothenyl cysteine decarboxylase (PPCDC) catalyzes the subsequent decarboxylation to 4'-phosphopantetheine, which is adenylated to dephospho-CoA by the phosphopantetheine adenylyl transferase (PPAT). The dephospho-CoA kinase (DPCK) finally phosphorylates Dephospho-CoA to Coenzyme A (CoA) (Leonardi *et al.*, 2005).

As an inhibition of CoA biosynthesis is lethal, all involved enzymes are possible targets for selective antimicrobial drug development. This strategy is possible because the specific enzymes catalyzing the CoA biosynthesis show considerably diversity in structure, sequence and mechanism. PanK, PPCS and PPAT are the most promising enzymes for drug targets. Developed antimicrobial agents include target specific inhibitors (triazole, biaryl acetic acid, cycloalkyl pyrimidines, CJ-15,801) and also CoA antimetabolite precursors which are able to reduce CoA levels and also interfere with reactions based on this cofactor. Fig. 5 shows the pathway of normal CoA biosynthesis compared to the "hijacked" anti-CoA pathway. The

antimetabolite anti-Pan is similar to the vitamin B_5 precursor of CoA, but it prevents the incorporation of the catalytically essential thiol, which results in a reduction of the CoA level (De Villiers *et al.*, 2015 & Moolman *et al.*, 2014).

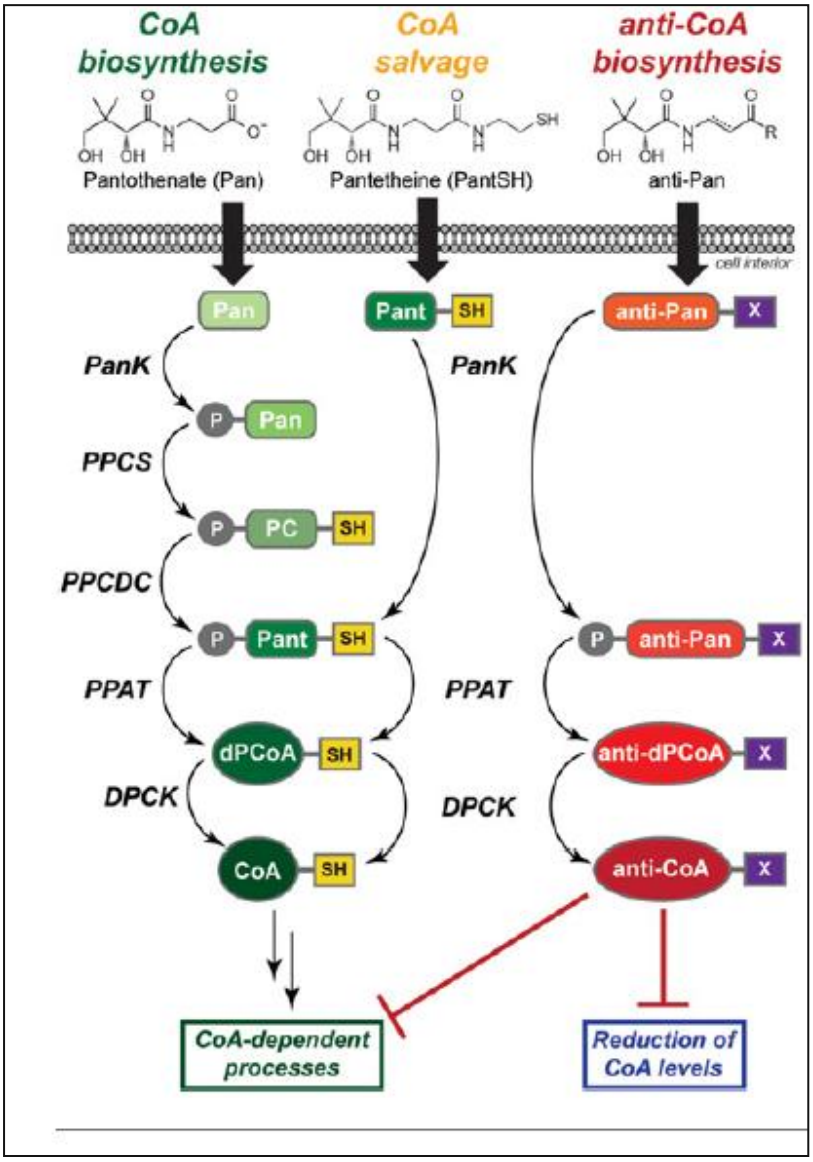

Figure 5: CoA biosynthesis and utilization as an antimicrobial drug target: Five enzymes (PanK, PPCS, PPCDC, PPAT, DPCK) are involved in CoA biosynthesis from pantothenate (Pan). However, CoA can also be metabolized from pantetheine (PantSH) due to a "shortened salvage pathway"which involves the enzymes PanK, PPAT and DPCK. The pantothenate antimetabolite anti-Pan is able to "hijack" the "salvage pathway" by prohibiting the incorporation of the catalytically important thiol (SH) and thus reduces the CoA level (Moolman *et al.*, 2014).

Altogether the discovered inhibitors of CoA biosynthesis emphasize the great potential of this kind of antimicrobial drug development. However, the drugs analysed so far do not provide sufficient inhibitory potency to achieve a commercial viability (De Villiers *et al.*, 2015 & Moolman *et al.*, 2014).

1.5. Pantothenate transport systems in different organisms

1.5.1. Pantotenate transport in *E. coli*

The pantothenate uptake and transport in *E. coli* is mediated by PanF, a pantothenate permease that imports pantothenic acid from the medium due to a Na^+-cotransport mechanism. The K_M of 0.4 µM indicates the transport system to be highly specific for pantothenic acid (Jackowski *et al*., 1990 & Leonardi *et al*., 2005).

Jackowski *et al.* (1990) further demonstrated that over expression of the *panF* encoded permease results in a significant increase in the pantothenate uptake rates. The concomitant elevation of the intracellular pantothenate concentration, however, did not result in an increased CoA biosynthesis (Jackowski *et al*., 1990 & Leonardi *et al*., 2005).

1.5.2. Pantothenate transport in *Saccharomyces cerevisiae*

In *S. cerevisiae* the *FEN2* gene product is responsible for the uptake and transport of pantothenate. It was characterized as an ATP dependant symporter, localized in the plasma membrane (Stolz *et al*., 1999). Growth analysis of a wild type strain and a *fen2* mutant on media, also containing D-glucose with none and various pantothenate concentrations were performed and the different pantothenate uptake rates were analyzed. In the absence of pantothenate both strains were not able to grow. However, only 10µM pantothenate were sufficient to detect growth of the wild type strain. The *fen2* mutant required a 2000 times higher concentration, which is evidence for a pantothenate transport defect. The relatively low K_M of pantothenate (K_M = 3.5 µM) indicates that *FEN2* encodes a high affinity transporter and thus is able to transport physiological pantothenate concentrations at high rates. The expression of the *FEN2* gene encoding the pantothenate transporter is also increased with decreasing extracellular pantothenate concentrations (Stolz *et al*., 1999).

The resistance of *fen2* mutants against the anti-fungal agent fenpropimorph was also revealed in previous studies. Stolz *et al*. (1999) demonstrated that fenpropimorph inhibits the transport of different substrates (pantothenate 76,1%; D-glucose 76.8%; L-lysine 85,5%; biotin 92,9%) in the presence of 3 mM fenpropimorph and thus the sensitivity of wild-type cells to this fungicide cannot only be explained by a specific interaction with Fen2. Furthermore, inhibition was also detected in *fen2* mutants which expressed Gap1, the yeast general amino acid permease. Stolz *et al.* (1999) concluded therefore that the inhibitory effect of fenpropimorph depends on the existence of intracellular pantothenate - essential for ergosterol biosynthesis (acetyl-CoA) and for the fatty acid biosynthesis and elongation (malonyl-CoA). Furthermore, it is assumed that the antifungal agent interacts directly with

different membrane components, in particular with very long chain fatty acids and sphingolipids (Stolz *et al*., 1999). It is demonstrated that β-alanine can suppress the growth defect of *FEN2* wild-type and *fen2* mutant cells on pantothenate-free medium (Stolz *et al*., 1999).

1.5.3. Pantothenate transport in *Schizosaccharomyces pombe*

Phylogenetic studies and direct protein comparisons of proteins belonging to the allantoate transporter families of *S. pombe* and *S. cerevisiae* showed that Liz1 is most related to the Fen2 pantothenate transporter of *S. cerevisiae* (Stolz *et al*., 2004).

Stolz *et al.* (2004) identified the pantothenate transporter LIZ1 in *S. pombe*, by expressing *liz1* in a *S. cerevisiae FEN2* knockout mutant. Compared to *fen2* mutants only the *S. cerevisiae liz1* complemented strains grew on plates containing pantothenate. This result proved that *liz1* is the functional homologue of *FEN2* of *S. cerevisiae* and thus it can be stated to be a pantothenate transporter.

Furthermore, the studies showed that *Δliz1* mutants are viable, because they are capable of synthesizing some pantothenate from uracil. However, they exhibit slow growth and mitotic defects (Stolz *et al*., 2004).

1.6. Degradation pathway of pantothenic acid in *Pseudomonas p-2*

The biochemistry of pantothenic acid degradation has only been analyzed in detail in the bacterium *Pseudomonas p-2.* A series of papers led by Esmond E. Snell, then a leading expert of vitamin B biochemistry, describes the purification and enzyme characteristics of the involved enzymes. However, gene and protein sequences are not yet reported (Goodhue *et al.*, 1966).

Nurmikko *et al.* (1966) described the first step of pantothenate degradation and identified pantothenate hydrolase as a pantothenate inducible enzyme, that is responsible for the hydrolysis of pantothenic acid to pantoic acid and ß-alanine. The enzyme activity was characterized by ß-alanine determination by means of a ninhydrin colorimetric analysis, based on the method of Yemm and Cocking (1955). The result of the study showed that the enzyme functions optimally at 28°C and a pH of 7.4. The K_M value for pantothenate is 5 mM (Nurmikko *et al*., 1966).

The following steps in the degradative metabolism were characterized by Goodhue *et al*. (1966). The enzyme pantoate dehydrogenase catalyzes the NAD^+ dependent oxidation of pantoate to D-aldopantoate. Its activity was determined by measuring NADH formation at 340nM. The analyzed K_M value for pantoate is 0.033 mM at a pH of 10 (Goodhue *et al*., 1966).

Subsequently D-aldopantoate was oxidized to ß, ß-dimethyl-D-malate via D-aldopantoate dehydrogenase. The final oxidative decarboxylation to α-ketoisovalerate was catalyzed by dimethylmalate dehydrogenase (Magee *et al*., 1966).

1.7. The catabolism of pantothenic acid in *T. reesei*

The genes encoding putative pantothenic acid catabolic enzymes were identified by their induction in response to the addition of pantothenic acid to a wheat straw medium (Bischof *et al.*, data not published yet).

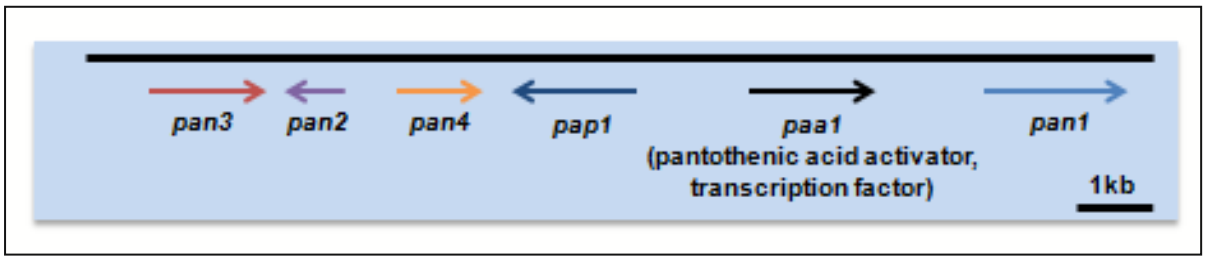

Figure 6: Genes involved in the pantothenate catabolism in *T. reesei*: *paa1* encoding the fungal $Zn(II)_2Cys_6$ transcription factor; *pap1* encoding the permease; *pan1* encoding the pantothenase PAN1; *pan2* encoding the pantoate 4-dehydrogenase PAN2; *pan3* and *pan4* encoding two further dehydrogenases.

The genes are found in a cluster in the *T. reesei* genome, which spans a region of around 12 kb (Fig. 6).The fungal $Zn(II)_2Cys_6$ transcription factor PAA1 was proposed to be the activator of the other 5 genes - *pap1*, *pan1*, *pan2*, *pan3* and *pan4* - located in this cluster (Bischof *et al.*, data not published yet). The permease PAP1 is an orthologue to the *FEN2* gene in *S. cerevisiae* (Stolz *et al.*, 1999) and is most probably responsible for the pantothenate import in *T. reesei* (Bischof *et al.*, data not published yet).

A blueprint of the putative pathway for pantothenic acid degradation in *T. reesei* was created, based on the information on the pantothenic acid catabolism in *Pseudomonas p-2* (see 1.6.) and on an *in silico* analysis of the enzymes encoded in the pantothenic acid inducible gene cluster (Bischof *et al.*, data not published yet). This putative pantothenate pathway is shown in Fig. 7 (Beinhauer, 2014). The pantothenase PAN1 (EC 3.5.1.22) catalyzes the metabolization of (R)-pantothenate to pantoate and ß-alanine. Pantoate is then oxidized to (R)-4-dehydropantoate by the pantoate-4-dehydrogenase PAN2 (EC 1.1.1.106). Subsequently the dehydropantoate dehydrogenase PAN3 (EC. 1.2.1.33) oxidizes (R)-4-dehydropantoate to (R)-3,3-dimethylmalate which is decarboxylated to 3-methyl-2-oxobutonate by the dimethylmalate dehydrogenase PAN4 (EC. 1.1.1.84) (Beinhauer, 2014).

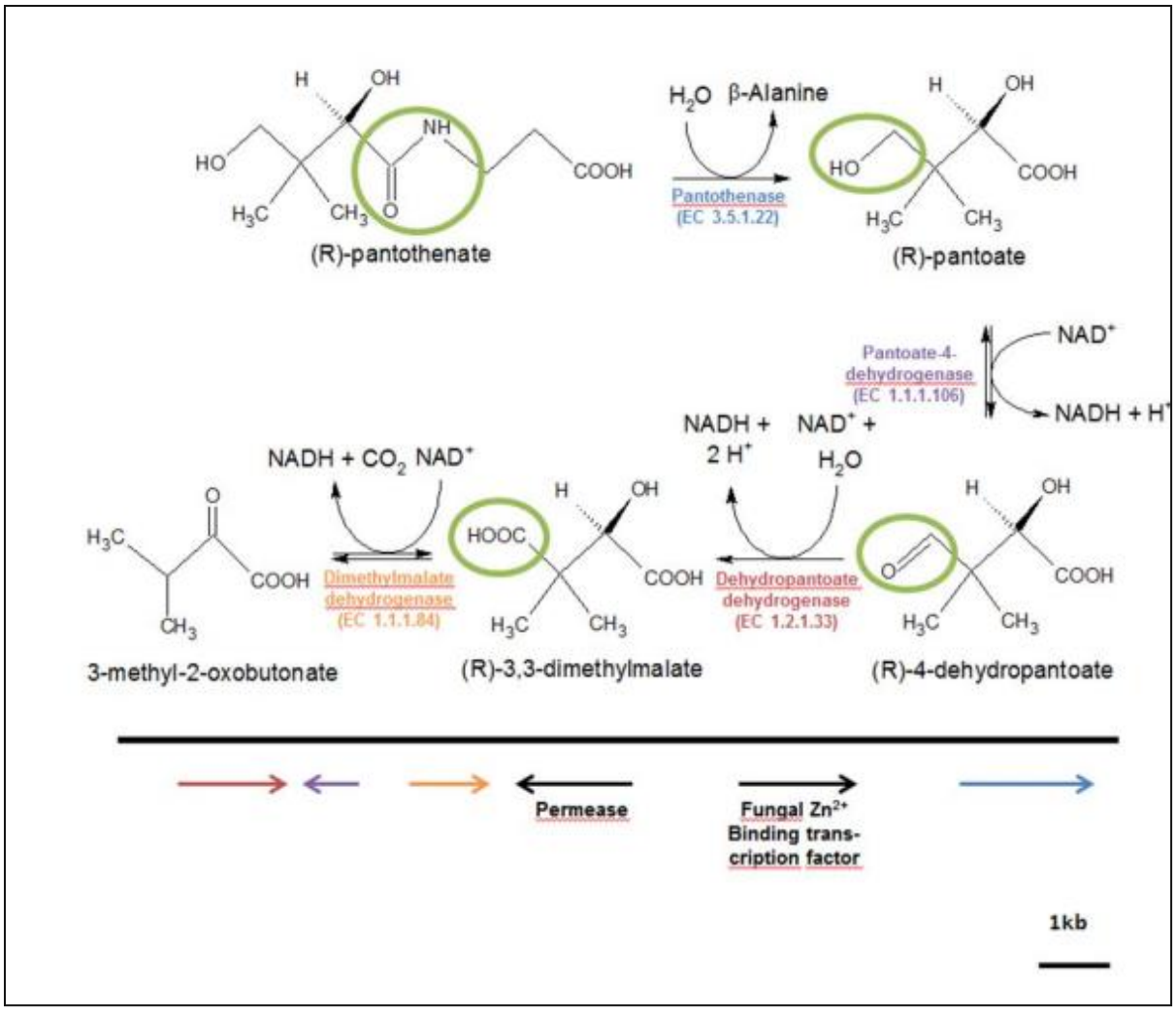

Figure 7: Catabolic pathway of pantothenate in *T. reesei* : Pantothenate is degraded to pantoate and ß-alanine by the pantothenase PAN1. Pantoate is converted to (R)-4-dehydropantoate, catalyzed by the pantoate 4-dehydrogenase PAN2. Finally, (R)-4-dehydropantoate is oxidized to (R)-3,3-dimethylmalate and subsequently decarboxylated to 3-methyl-2-oxobutonate (Beinhauer, 2014).

2. Aim of the thesis

Pantothenic acid is an indispensable nutrient which is required for the survival of all organisms (Higdon, 2003). In contrast to its biosynthesis pathway, which is already well characterized in many bacteria, fungi and plants, the pantothenate catabolic pathway and its respective genes are still not documented. Previously Bischof *et al.* (data not published) identified 6 clustered genes in the *T. reesei* genome which are involved in pantothenate degradation.

The objective of this research work was to characterize the novel pantothenate inducible gene cluster in *T. reesei*, including (I) the regulatory effect of the fungal transcription factor PAA1, (II) the pantothenate transport and (III) the characterization of the first two enzymes involved in the pantothenate catabolic pathway.

3. Materials & methods

3.1. *T. reesei* strains & cultivation conditions

3.1.1. Strains

The *Δtku70* knockout strain was prepared by Christa Ivanova (unpublished data) similar as described by Guangtao *et al*. (2009). Therefore the *tku70* open reading frame was replaced with a pyrithiamine resistance cassette and the *hphB* resistance cassette was used to knockout the transcription factor *paa1* (Lindner, 2013*)*. The *paa1* restored strains were produced by sexual reproduction as described by Schuster *et al*. (2012).

3.1.2. Cultivation

After sterilizing the unbuffered MA medium containing glycerol, peptone and urea the pre-cultures were prepared by addition of conidiospores to a concentration of 10^6 per ml. The cultures were then cultivated in a rotary shaker at 250 rpm and 28°C for about 24 hours. Biomass samples for qPCR were taken by filtering the culture broth. The retained mycelium was washed with cold dH_2O and was then immediately frozen in liquid nitrogen. The pre culture samples were stored at -80°C until RNA isolation. The main cultures were prepared by culture replacement. After filtering and washing the pre-culture broth the collected mycelium was transferred into 100 ml of unbuffered MA medium without urea, peptone and glycerol. Then 25 ml of the mycelium in the unbuffered MA medium were added to 165 ml of MA medium without urea, peptone and glycerol. The cultures were induced by addition of different pantothenic acid concentrations (0, 1, 50 mM) and were again cultivated in a rotary shaker for one hour at 28°C and 250 rpm. Thereafter biomass samples were taken as described above and stored at - 80°C.

3.1.3. Preparation of a spore solution

Ten ml NaCl/Tween solution are added to the spores of one PDA plate. The spore solution was filtered through Eppendorf tubes filled with glass wool. Subsequently 75 µl of this solution were diluted with one ml of NaCl/Tween solution. Absorption was measured at 600 nm.

The spore concentration was calculated by using the following equations:

linear function (OD_{600} ≤ .0.6):

$$y = 3.41 * 10^{-6} * x + 4.63 * 10^{-2} \quad (1)$$

quadratic function (OD_{600} = 0.6 - 0.9):

$$y = -4.37 * 10^{-12} * x^2 + 4.20 * 10^{-6} * x + 1.88 * 10^{-2} \quad (2)$$

3.1.4. Media

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

Mandels - Andreotti media (1 L) unbuffered: 1.4 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 0.3 g $MgSO_4*7H_2O$, 0.3 g $CaCl_2*2H_2O$, 0.3 g urea (5 mM), 20 ml trace elements, 15 g glycerol, 1.0 g peptone

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

Mandels - Andreotti media (1 L) unbuffered without urea, peptone and glycerol: 1.4 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 0.3 g $MgSO_4*7H_2O$, 0.3 g $CaCl_2*2H_2O$, 20 ml trace elements

3.1.5. Chemicals and instruments

Chemicals:

$(NH_4)_2SO_4$	Carl Roth GmbH, Germany
KH_2PO	Merck GmbH, Germany
$MgSO_4*7H_2O$	Roth GmbH, Germany
$CaCl_2*2H_2O$	Sigma, Germany
Urea	Merck GmbH, Germany
Glycerol	Carl Roth GmbH, Germany
Peptone from casein	Merck GmbH, Germany

$FeSO_4$ x $7H_2O$	Merck GmbH, Germany
$MnSO_4$ x H_2O	Merck GmbH, Germany
$ZnSO_4$ x $7H_2O$	Merck GmbH, Germany
$CoCl_2$ x $2H_2O$	Merck GmbH, Germany
NaCl	Carl Roth GmbH, Germany
Potato dextrose agar (PDA)	Difco, USA
Tween® 80	Merck GmbH, Germany
D-Pantothenic acid hemicalciumsalt	Sigma, Germany

Instruments:

Rotary shaker, Infors HT Multitron Standard 230 V	Infors HT, Switzerland
Spectrophotometer	Biolog, Germany
Miracloth 475855	Calbiochem, Austria
IKA® Vortex	IKA, Germany
Magnetic stirrer Hei Standard	Heidolph Instruments, Germany
CERTOclav Type CVII/1600	KELOMAT, Austria

3.2. Nucleic acid Techniques

3.2.1. RNA Isolation

RNA extraction was performed as described by Chirgwin *et al.* (1979). The mycelium was frozen with liquid nitrogen and pulverized by using a mortar. The pulverized mycelium was then added to 700 µl Chirgwin solution and 6 µl ß-mercaptoethanol. After homogenization 70 µl 2M sodium acetate, 700 µl phenol and 200 µl chloroform isoamylalcohol (49:1) were added, followed by an incubation of the mixture on ice for 15 minutes. Thereafter the samples were centrifuged for 10 minutes at 14 000 rpm and 4°C. The aqueous phase was removed and transferred into an Eppendorf tube containing 750 µl isopropanol. Precipitation took place at -20°C for 20 minutes. After centrifugation of the samples at 14 000 rpm and 4°C for ten minutes the supernatant was discarded and the RNA pellet was washed twice with 75% ethanol. The pellet was dissolved in 30 µl RNAse free water at 50°C for 15 minutes.

Subsequently the concentration was determined by Nanodrop spectrophotometer and the samples were either stored at -80°C or immediately reverse transcribed into cDNA.

3.2.2. cDNA Synthesis

The RNAs were treated with DNAse (5 µg RNA, 1 µl reaction buffer, 1 µl DNAse 1 RNAse free and dH_2O to 10 µl) by incubation at 37°C for 30 minutes. After adding one µl EDTA, the samples were incubated again for ten minutes at 65°C. For cDNA synthesis the instructions of the RevertAid H Minus First Strand cDNA Synthesis Kit were followed. Therefore 0.5 µl random primer, 0.5 µl oligo(dT) and 1 µl DEPC H_2O were added to the sample. After five minutes incubation at 65°C the samples were placed back on ice again. 4 µl 5x reaction buffer, 1 µl Ribolock™RNAse Inhibitor (20u/ µl), 2 µl 10mM dNTP mix and 1 µl RevertAid™H Minus M-MulV Reverse Transcriptase were added, gently mixed and incubated for five minutes at 25°C and then for 60 minutes at 42°C. The reaction was then terminated by heating the samples for 5 minutes at 70°C. Subsequently they were diluted with 80 µl qPCR water and stored at -20°C.

3.2.2.1. Solutions

Chirgwin solution: 250 g guanidinium thiocyanate, 293 ml dH_2O, 17.6 ml sodium citrate, 26.4 ml sodium laurylsarcosine (10%)

Chloroform-isoamylalkohol solution (50 ml): 49 ml chloroform and 1 ml isoamylalkohol

3.2.2.2. Chemicals and instruments

Chemicals:

2-Mercaptoethanol	Merck GmbH, Germany
Chloroform	Carl Roth GmbH, Germany
Isoamylalcohol	Carl Roth GmbH, Germany
NaOAc, ≥ 99%	Carl Roth GmbH, Germany
Phenol, Roti®Phenol	Carl Roth GmbH, Germany
Isopropanol	Sigma, Germany
Ethanol, 96%	Merck GmbH, Germany
RevertAid H Minus First Strand cDNA Synthesis Kit	Thermo scientific, USA

Instruments*:*

Centrifuge 5414R	Eppendorf AG, Germany
NanodropR ND-1000 Spectrophotometer	Nanotrop Technologies, USA
Thermomixer compact	Eppendorf AG, Germany
Centrifuge 5414R	Eppendorf AG, Germany
Thermomixer compact 5350	Eppendorf AG, Germany

3.2.3. Quantitative Real Time PCR

The qPCR measurements were performed on a Mastercycler® ep realplex 2, as described by Bischof *et al.* (ACIB protocol). 25 µl of reaction mix contained 12 µl SYBR® Green premix, 10 µl qPCR water, 1 µl of each primer dilution and the diluted cDNA sample. The used primers are shown in Table 1. Each sample was analyzed as a technical triplicate.

Table 1: Primers used for qPCR

Primer	Sequence	Target gene	Efficiency
qPCR_tef1_fw	CCACATTGCCTGCAAGTTCGC	*tef1*	0.87
qPCR_tef1_rv	GTCGGTGAAAGCCTCAACGCAC	(housekeeping gene)	
70349_1_fw	GCCTGTCGTTGAGGAACACTCG	*pap 1*	0.91
70349_1_rv	CAGCGATGACCCAGAGCATGAC	(pantothenate permease)	
70375_1_fw	CATTGGCAAGGGAACTCAGCAC	*pan1*	0.83
70375_1_rv	ACGGTGTTGAACGGCATGGTAG	(amidase)	
70334_2_fw	ACTCCAACAAGGCGACAGAGGG	*pan2*	0.9
70334_2_rv	AAGCTGCCAGGGCGATTCTC	(short chain dehydrogenase)	
70383_1_fw	CCTTGTTGTTCGTGCCACATCG	*pan3*	0.89
70383_1_rv	CCCTTTGCGAGAGCATCCTTG	(aldehyde dehydrogenase)	
112247_2_fw	CAACGGGATCTACGAGCCAGTG	*pan4*	0.81
112247_2_rv	GCAGCAGCATCGCCAGACTTAG	(dimethylmalate dehydrogenase)	

The program for qPCR analysis contains a two step protocol with 40 cycles for denaturing (95°C) and annealing (60°C). Thereafter a further denaturation and annealing step was performed and a melting curve was recorded (Table 2).

Table 2: qPCR program

Step	Temperature [°C]	Time [sec]
1	95	180
2	95	15
3	60	20, return to step 2, repeat 40x
4	95	15
5	60	15
6	60 → 95	During 20min (melting curve)
7	95	15

The housekeeping gene *tef1* was used for normalization to compensate the differences in the total amount of cDNA in the assay. Using the REST© Software tool the relative gene expression was calculated.

3.2.3.1. Chemicals & Instruments

Chemicals:

iQ™ Sybr® Green Supermix	Bio-Rad, USA
qPCR water	Carl Roth GmbH, Germany
Oligonucleotids	Sigma, Germany

Instruments:

iCycler iQ™ PCR plates 96 well	Bio-Rad, USA
iCycler iQ™ optical tape	Bio-Rad, USA
Mastercycler®ep realplex 2	Eppendorf AG, Germany

3.3. Complementation of the Δ*fen2 S. cerevisiae* strain

3.3.1. Strains

The used *S. cerevisiae strains* BY4741 and BY4741*Δfen2:KanMX* were provided by J. Stolz (TU Munich). The *pap1* transcript was amplified from cDNA of pantothenate induced cells and expressed under the *GAL1* promoter, using the SalI and XhoI sites for cloning into pESC-URA (Agilent), using the InFusion cloning kit (Clontech). The resulting plasmid pPE1982 was transformed into *S. cerevisiae* BY4741*Δfen2:KanMX* as described by Schuster *et al*. (2012). The strains were provided by Robert Bischof.

3.3.2. Cultivation

After thawing the strains from cryo-cultures, each sample was cultivated on YEDP-plates for 24 hours at 30°C. A minimal medium was prepared including YNB without amino acids & pantothenic acid (Formedium™, GB; Ref. No.: CYN 3301), the required amino acids and potassium phosphate buffer. After adjusting the pH to 6.5 with diluted H_3PO_4 the media was split into five large Erlenmeyer flask, each containing 190 ml, and into 5 small flasks, each containing 95 ml. Thereafter the flasks and the galactose were autoclaved separately. Pantothenic acid was sterile filtered. The pre-cultures were prepared by adding 5 ml of galactose (40% w/v) and 100 µl of pantothenic acid (1.68 µM) to each flasks containing 95 ml of media. Subsequently the media were inoculated and the cultures were cultivated in a rotary shaker at 220 rpm and 30°C for about 48 hours. The main cultures were prepared by addition of ten ml of galactose (40% w/v) and 200 µl of pantothenic acid (1.68 µM) to each flask containing 190 ml of media. They were inoculated with the pre-culture to an OD_{600} of 0.025. These main cultures were again cultivated in a rotary shaker at 220 rpm and 30°C for 2 days. Samples were taken and diluted to various concentrations (1; 1/2; 1/5; 1/20) to determine OD_{600} and dry cell biomass. Five ml of each sample were centrifuged for eight minutes and 2000 rpm. The supernatant was discarded, the pellet was washed once with dH_2O and was centrifuged again. After discarding the supernatant, the pellets were dried for 48 hours at 80°C. Thereafter the dry weight was determined.

3.3.3. Media:

YEDP: 1% yeast extract, 2% peptone, 50% (w/v) glucose stock, 1.5% Agar Agar

MM galactose: 5% galactose (40%w/v), 0.69% yeast nitrogen base (YNB) w/o AA & PA, 100 mg/l methionine, cysteine, histidine, leucine and uracil, 100mM potassium phosphate buffer (pH 6.5), 1.68 µM pantothenic acid

3.3.4. Chemicals & instruments

Chemicals:

Yeast Extract	Carl Roth GmbH, Germany
Peptone	Merck GmbH, Germany
Glucose	Carl Roth GmbH, Germany
Agar Agar	Merck GmbH, Germany
Galactose	Carl Roth GmbH, Germany
Yeast nitrogen base (YNB) without amino acids and Ca-Pantothenate (Ref. No.: CYN 3301)	Formedium™, GB
Methionine	Sigma, Germany
Cysteine	Sigma, Germany
Histidine	Sigma, Germany
Leucine	Sigma, Germany
Uracil	Sigma, Germany
D-Pantothenic acid hemicalciumsalt	Sigma, Germany

Instruments:

Rotary shaker, Infors HT Multitron Standard 230 V	Infor HT, Switzerland
CERTOclav Type CVII/1600	Kelomat, Austria
PHM 82 pH Meter	Radiometer, Copenhagen

3.4. Characterization of recombinant produced PAN1 & PAN2 in *E. coli*

3.4.1. *E. coli* strains & cultivation conditions

3.4.1.1. Strains

Strains were produced by Alexander Beinhauer and Robert Bischof (Beinhauer, 2014). Therefore, the *pan1* and *pan2* coding region was inserted into the NdeI/XhoI sites of pET-21 (provided by Oliver Spadiut, TU Vienna) by InFusion cloning. The proteins are expressed with a C-terminal His tag. The resulting pAB 9 expressing *pan1* and pAB 10 expressing *pan2* were transformed into competent *E. coli* BL21(D3) (Clontech), as described in the manufactures instructions.

3.4.1.2. Cultivation conditions

E.coli BL21D3 pAB9/Nr1. (PAN1) and *E.coli* BL21D3 pAB10/Nr2 (PAN2) were cultivated on LB_{amp} plates, containing 100 µl/ml Ampicillin. The plates were incubated over night at 37°C. For the subsequent protein production Super Broth (SB) medium was used, containing 100 µl/ml Ampicillin. The pre-cultures were prepared by inoculating ten ml of SB medium with a single *E. coli* colony. After 19 hours incubation in a rotary shaker at 37°C and 200 rpm OD_{600} was measured. The main cultures, containing 100 ml SB_{amp} medium, were inoculated with one ml of pre-culture (resulting in a start OD of about 0.1) and were again incubated for two hours at 37 °C and 250 rpm. Thereafter the samples were cooled down to 25°C and OD_{600} was measured again. Then the cultures were induced with one ml of a 100x IPTG stock solution. After 21 hours of incubation at 25°C and 250 rpm OD_{600} was measured again. One ml of cell culture was drawn for the screening test and subsequently the remaining cells were harvested.

3.4.2. Preparation of the *E. coli* cell lysate

The *E. coli* cells were harvested by centrifugation at 10 000 g and 4°C for four minutes. Thereafter the cell pellet was washed twice with buffer A and the wet cell weight was determined. The cell pellet was resuspended in enough buffer A to achieve a maximum dry weight concentration of 6 g/l *E. coli* cells (wet cell weight was assumed to be 4/5 times the dry cell weight).

The subsequent cell lyses was performed by using the French press system Emulsiflex©C-3 homogenizer. Before lysing the cells the homogenizer was flushed with 70% ethanol and distilled water. The resuspended cells were then recirculated for 12 - 15 minutes at a primary pressure of 4-6 bar and a main pressure of 1000 - 1500 bar. The cell lysate was immediately

placed on ice again. The homogenizer was cleaned with distilled water and was finally stored in 70% ethanol.

The cell lysates were again centrifuged twice at 4°C and 21 000 g for four minutes and subsequently sterile filtered using a 0.2 µm syringe filter.

3.4.3.Media:

LB^{amp}: 1% peptone, 0.5% yeast extract, 1% NaCl, 1.5% agar agar, adjust the pH to 7.0 with 5M NaOH, after autoclaving add 100 µl/ml 1000x Ampicillin stock solution

SB^{amp}: 3.2% peptone, 2% yeast extract, 0.5% NaCl, adjust the pH to 7.0 with 5M NaOH, after autoclaving add 100 µl/ml 1000x Ampicillin stock solution

1000x Ampicillin stock solution: 100 mg Ampicillin dissolved in 1 ml ethanol (96%)

IPTG stock solution: 100 mM IPTG dissolved in dH_2O

Buffer A: 50 mM KH_2PO_4, 500 mM NaCl, 20 mM imidazole

3.4.4. Chemicals & instruments

Chemicals:

Ampicillin sodium salt ≥ 99%	Carl Roth GmbH, Germany
Peptone	Merck GmbH, Germany
Yeast extract	Carl Roth GmbH, Germany
NaCl	Carl Roth GmbH, Germany
Agar Agar	Merck GmbH, Germany
NaOH	Carl Roth GmbH, Germany
IPTG, dioxane free	Thermo Scientific
KH_2PO_4	Merck GmbH, Germany
NaCl	Carl Roth GmbH, Germany
Imidazole for molecular biology, ≥ 99% (titration)	Sigma, USA

Ethanol	Merck GmbH, Germany

Instruments:

Rotary shaker, Infors HT Multitron Standard 230 V	Infors HT, Switzerland
CERTOclav Type CVII/1600	Kelomat, Traun, Austria
PHM 82 pH Meter	Radiometer, Copenhagen
Centrifuge 5414R	Eppendorf AG, Germany
Emulsiflex©C-3 homogenizer	Avestin, Germany

3.4.5. Purification of the recombinant proteins PAN1 and PAN2

Purification of the proteins was carried out with affinity chromatography. The basic principle of ÄKTA-FPLC protein purification is based on immobilized metal ion affinity chromatography (IMAC) using a Ni^{2+} sepharose high performance column. The system is operated by UNICORN 5 control software.

All buffers, solutions and samples, containing the target protein, were sterile filtered through 0.2 µm syringe filters to prevent the system from clogging. Before starting the program all pumps and tubings were washed with 100 ml 20% ethanol and subsequently flushed with the respective buffer solutions A and B. After the cleaning procedure the flow rate was adjusted to 1.5 ml/min and the system was again flushed with 100% buffer A for about five minutes. To install the column, the flow rate was reduced to 0.2 ml/min to avoid overpressure and to prevent damage to the Ni^{2+} sepharose bed by trapping air. The column was equilibrated with buffer A until a constant UV_{280} signal was visible. Then the crude cell extract, containing the target protein, was loaded by placing tubing A into the sample. The flow rate was adjusted to 2.5 ml/min. After loading the sample, the tubing A was placed back into buffer A, the flow rate was reduced again to 1.5 ml/min and the flow through was collected until the original UV_{280} signal was reached again. After flushing the column with about 5 CV (column volume) of buffer A, the elution with buffer B was started. Therefore the linear gradient was set to 100% buffer B in 15 minutes with a flow rate of 1.5 ml/min. One ml samples were collected. After the elution the system was flushed with buffer B for at least five minutes, washed with 20% ethanol and finally the column was stored under 20% ethanol.

The samples containing the target protein were pooled according to the UV_{280} signal. After protein concentration determination the sample was immediately rebuffered in 1x PBS buffer

for storage and further enzyme activity measurements. For buffer exchange Amicon®Ultra - 15 Centrifugal filters Ultracel® - 3K with a molecular weight cut-off of 3.000 were used. One ml sample was first concentrated to 200 µl by centrifuging at full speed at 4°C. Then the concentrate was reconstituted to the original sample volume (1ml) with 1x PBS buffer. This procedure was repeated five times.

3.4.5.1. Buffers

Binding buffer A (buffer A): 50 mM KH_2PO_4, 500 mM NaCl, 20 mM Imidazole

Elution buffer B (buffer B): 50 mM KH_2PO_4, 500 mM NaCl, 500 mM Imidazole

10x PBS buffer (PBS): 137 mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4 x $2H_2O$, 100 mM KH_2PO_4, adjust pH to 7.4 with diluted H_3PO_4

3.4.5.2. Chemicals & instruments

Chemicals:

KH_2PO_4	Merck GmbH, Germany
NaCl	Carl Roth GmbH, Germany
Imidazole for molecular biology, ≥ 99% (titration	Sigma, Life science, USA
Ethanol	Merck GmbH, Germany
KCl	Carl Roth GmbH, Germany
Na_2HPO_4 x $2H_2O$	Carl Roth GmbH, Germany
H_3PO_4	Merck GmbH, Germany

Instruments:

ÄKTAmicro™	GE Healthcare Life Science
HisTrap HP, 5x 1ml	GE Healthcare Life Science
Centrifuge	Eppendorf AG, Germany
PHM 82 pH Meter	Radiometer, Copenhagen

3.5. Screening test for the over expressed target enzymes

The screening test was based on a slightly modified version of a freeze-thaw protocol (https://www.embl.de/pepcore/pepcore_services/protein_purification/extraction_clarification/solubility_studies/). One ml sample of the *E. coli* culture broth was taken and centrifuged for five minutes at 6000 rpm and 4°C. To each cell pellet 100 µl of 1 x PBS containing one mg/ml lysozyme was added. The resuspended cells were lysed by freeze thawing. Therefore the sample was frozen for 40 seconds. in liquid nitrogen, immediately thawed at 42°C for one minute and vortexed well. This procedure was repeated four times.

The samples were then centrifuged at maximum speed and 4°C for five minutes. One ml acetone was added to the supernatant. After vortexing the sample was left on ice for 15 minutes to precipitate the protein. The samples were then centrifuged at maximum speed and 4°C for five minutes. The acetone was removed and the remaining pellet was dried at 37°C for about two minutes.

15 µl 1x loading dye was added to the acetone treated pellet and 25 µl 1x loading dye was added to the cell pellet. After heating the samples at 95°C for five minutes all were vortexed and centrifuged again at maximum speed and 4°C for five minutes.

Ten µl of the sample were then loaded onto a SDS-PAGE gel.

3.5.1. Media

10x PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 x $2H_2O$, 100 mM KH_2PO_4, adjust pH to 7.4 with diluted H_3PO_4

3.5.2. Chemicals & instruments

Chemicals:

Lysozyme	Boehringer Mannheim GmbH, Germany
NaCl	Carl Roth GmbH, Germany
KCl	Carl Roth GmbH, Germany
Na_2HPO_4 x $2H_2O$	Carl Roth GmbH, Germany
KH_2PO_4	Merck GmbH, Germany
H_3PO_4	Merck GmbH, Germany

Instruments:

Thermomixer compact 5350	Eppendorf AG, Hamburg, Germany
Centrifuge 5415R	Eppendorf AG, Hamburg, Germany
Magnetic stirrer Hei Standard	Heidolph Instruments, Germany

3.6. Enzymatic assays

3.6.1. Characterization of Pantoate 4-Dehydrogenase (PAN2)

To characterize the enzyme activity of pantoate 4-dehydrogenase (PAN2), an enzyme assay was performed as described by Goodhue *et al.* (1966). The respective components and their concentrations are listed below in Table 3.

All components (500 µl NaOH - glycine buffer, 10 µl NAD^+, 100 µl enzyme, fill up with dH_2O to 1 ml), excluding pantoate, were mixed together in a 1.5 ml semi-microcuvette. The reaction was started by adding the substrate pantoate (0.5 - 55 mM) to the reaction mixture. The reaction process was followed for four minutes at room temperature and the resulting NADH formation was measured photometrical at 340 nm.

Table 3: Components of the enzyme assay for pantoate 4-dehydrogenase

Components	Concentrations
NaOH - glycine buffer pH 9.7	100 mM
NAD^+ 100 mM stock solution	1 mM
Enzyme PAN2	50 - 100 µl depending on enzyme concentration
Pantoate	0.5 - 55 mM

ΔA/min were recorded by spectrophotometer and the specific activities were calculated using equation (3).

$$\frac{Units}{mg} = \Delta A/min \Big/ 6.22 * \frac{mg\ enzyme}{ml\ reaction\ mixture} \qquad (3)$$

3.6.1.1. Media

NaOH - glycine buffer: 270 mM glycine, adjust the pH to 9.7 with NaOH

3.6.1.2. Chemicals & instruments

Chemicals:

Glycine	Carl Roth GmbH, Germany
NaOH	Carl Roth GmbH, Germany
NAD, free acid, grade II approx. 98%	Roche Diagnostics GmbH, Germany
Pantoate	synthesized by Philipp Skrinjar

Instruments:

Spectrophotometer	Biolog, Germany

3.6.2. Characterization of Pantothenase (PAN1)

To characterize pantothenase (PAN1), an enzyme assay was performed as described by Nurmikko *et al.*(1966). The respective components are listed below in Table 4.

Table 4: Components of the enzyme assay described by Nurmikko *et al.* (1966)

Components	Concentrations
KH_2PO_4 buffer pH 7.5	50 mM
Potassium pantothenate 200x stock solution	10 mM
Enzyme PAN1	15 µg
dH_2O	

The substrate D-pantothenic acid hemicalciumsalt had to be pre-treated by precipitating Ca^{2+} with KH_2PO_4 buffer.

The assay was performed by mixing all components (375 µl KH_2PO_4 buffer pH 7.5, 150 µl potassium pantothenate, 15 µg enzyme and fill up to 1.5 ml with dH_2O) together in a 1.5 ml Eppendorf tube, incubating them at 25°C in the thermomixer for one hour. The reaction was stopped by heating the samples at 100°C for three minutes. The blank was performed

without enzyme. Then the samples were centrifuged and stored at -20°C for the further ninhydrin assay.

The further characterization of the PAN1 enzyme activity was based on a modified version of the ninhydrin colorimetric analysis for amino acids by Yemm and Cooking (1955), which determines the formed amount of ß-alanine. The components needed to perform the ninhydrin assay are listed below in Table 5.

Table 5: Ninhydrin assay components

Components	Volume [ml]
Ninhydrin reagent solution	1
Enzyme PAN1	1
dH_2O	1

All components are mixed together in a test tube, containing a boiling chip, and were placed in a boiling water bath for ten minutes. After heating the samples were cooled down to room temperature and 5 ml of 95% ethanol were added. Then the sample were vortexed again and the amount of ß-alanine formed was measured photometrical at 570 nm.

The standard curve was determined by measuring the respective absorbance at 570 nm of different ß-alanine concentrations. The blank was performed without the enzyme PAN1.

3.6.2.1. Media

KH_2PO_4 buffer: 200 mM KH_2PO_4, pH 7.5

3.6.2.2. Chemicals & instruments

Chemicals:

KH_2PO_4	Merck GmbH, Germany
D-Pantothenic acid hemicalciumsalt	Sigma, Germany
Ninhydrin reagent solution	Sigma, Germany
Boiling chips	Carl Roth GmbH, Germany

Instruments:

Spectrophotometer	Biolog, Germany
Thermomixer compact 5350	Eppendorf AG, Germany
IKA® Vortex	IKA, Germany

3.7. SDS-PAGE

SDS polyacrylamide electrophoresis was performed to estimate the protein content on the one hand and on the other to determine the purity of the over expressed enzyme after affinity chromatography. To determine the molecular weight, a PageRuler Prestained Protein Ladder (10 - 170 kDa) was used as reference.

The gel composition is listed below in Table 6. To characterize the enzyme PAN2, gels were used with a 4.5% stacking gel and a 15% separating gel. For PAN1 characterization gels were prepared containing 4.5% stacking gel and 12% separating gel.

Table 6: SDS-PAGE gel composition

Components	4.5% stacking gel	12% separating gel	15% separating gel
dH_2O	1.5 ml	2.1 ml	1.5 ml
Stacking/separating gel buffer	0,625 ml	1.5 ml	1.5 ml
30% Acrylamide-stock	0.375 ml	2.4 ml	3 ml
10% APS	12.5 µl	30 µl	30 µl
TEMED	2.5 µl	5 µl	5 µl

Samples containing the target protein were pre-treated before loading, by mixing them with 6 µl 5x loading dye and the appropriate amount of water. For analysis 15 µg total protein and 7 µg purified protein were necessary. The samples were heated to 97°C for seven minutes and then 25 µl of the pre-treated samples and 5 µl of protein ladder were loaded onto the gel. The electrophoresis was started at 80 V for 15 minutes to gather the protein sample in the stacking gel and subsequently the voltage was raised to 120 V. After completion the gels were first stained over night with Colloidal Coomassie stain, followed by destaining with distilled water.

3.7.1. Media

Stacking gel buffer (500 ml): 30.25 g Tris, 2 g SDS, adjust ph to 6.8 with HCl

Separating gel buffer (500 ml): 90.75 g Tris, 2 g SDS, adjust ph to 8.8 with HCl

30% Acrylamide stock solution (500 ml): 150 g acrylamide, 2 g bisacrylamide

10 % Ammoniumpersulfate (APS): 0.1 g/ml dH_2O

10x Running buffer (500 ml): 15 g Tris, 72 g glycine, 5 g SDS

Colloidal Coomassie stain (100 ml): 8 g $(NH_4)_2SO_4$ + 78 ml +2 ml 85% H_3PO_4, 1.6 ml Colloidal Coomassie Blue G250, 20 ml methanol

5x loading dye: 0.25% Bromphenol blue, 0.5 M DTT, 50% glycerol, 10% SDS, 0.25 M Tris HCl, 5% ß-mercaptoethanol, pH 6.8

3.7.2. Chemicals & instruments

Chemicals:

Acrylamide	Merck GmbH, Germany
Bisacrylamide 4K ultrapure	APPLICHEM, Germany
Sodiumdodecylsulfate	Merck GmbH, Germany
Colloidal Coomassie Blue G250	Merck GmbH, Germany
Bromphenol blue	Carl Roth GmbH, Germany
Glycerol	Carl Roth GmbH, Germany
PageRuler Prestained Protein Ladder 10 - 170 kDa	Thermo Scientific, USA
All other chemicals	Sigma, Germany

Instruments:

Gel electrophoreses chamber	Bio-Rad, Germany

4. Results

4.1. Regulation of the pantothenate gene cluster by the fungal Zn_2Cys_6 transcription factor PAA1

The different genes of the *T. reesei* pantothenate gene cluster are presumably regulated by the fungal Zn_2Cys_6 transcription factor PAA1, which is part of this group of genes (Bischof *et al.*, data not published). In order to demonstrate its activator role the transcription factor *paa1* was first knocked out in the QM9414 Δ*tku70* strain. Gene deletion was performed by replacing the coding region of *paa1* by the *hphB* expression cassette (Lindner, 2013). The deletion strain Δ*paa1* was grown for 24 hours on MA medium containing glycerol as carbon source, followed by a washing of the mycelia and further cultivation in a MA medium without carbon sources. Different pantothenic acid concentrations (0, 1, 50 mM) were then added and the expression of the genes of the pantothenate cluster was determined by means of qPCR (Fig. 9). The results show that all genes of the pantothenate cluster are induced by the addition of pantothenic acid.

To have a reference strain with a restored *paa1* locus, phenotype rescue was performed (Schuster *et al.*, 2012). Therefore a strain deleted in *paa1* was crossed with a sexual competent *MAT1-1* version of *T. reesei* QM6a. This strain RL1/A8-02 was obtained by crossing the CBS999.97 *MAT1-1* wild-type strain with QM6a (*MAT1-2*) followed by a backcrossing of a *MAT1-1* progeny with QM6a over eight generations (Linke *et al.*, 2015). The progenies of the cross between RL1/A8-02 and Δ*paa1* were then tested. Fruiting bodies ejected their ascospores onto the cover of the petri dish from where they were collected. The loss of the hygromycin resistance in the progenies was tested by a growth test on a medium containing hygromycin and by a PCR screening (Fig. 8).

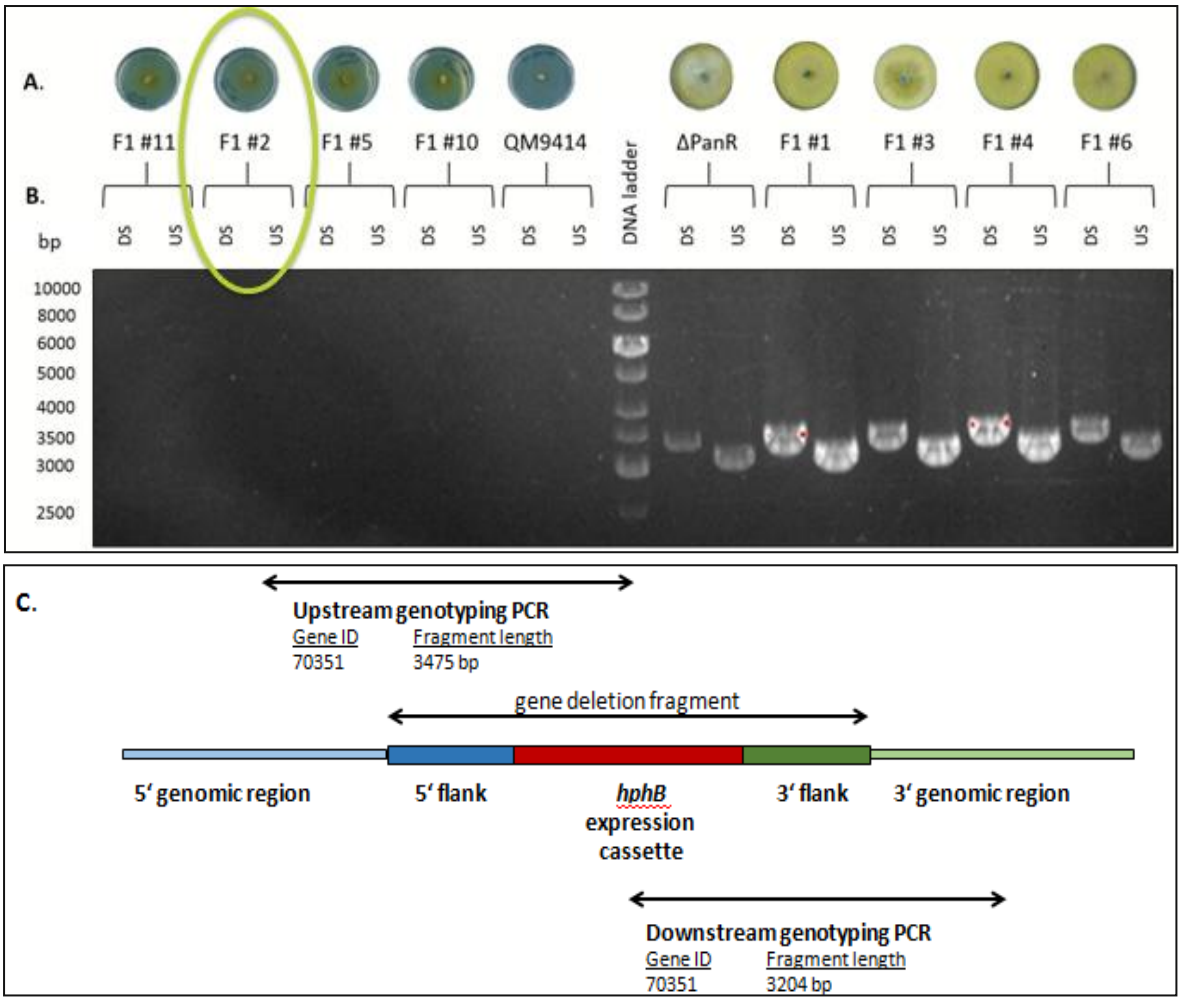

Figure 8: Growth and PCR screening of the first generation offspring (F1 #1-6, 10 and 11) from crossing the pantothenic acid regulator mutant (ΔPanR) and the sexually competent RL1/A8-02 of *T. reesei* as well as the QM9414 Δ*tku70* deletion strain. (A) Fungal growth on potato dextrose agar in the presence of 100 µg/ml hygromycin. (B) PCR screening using primers that either bind upstream of the gene deletion cassette and in the *hph* expression cassette (US) or in the *hph* expression cassette and downstream of the gene deletion fragment (DS). (C) Schematic representation of the *paa1* locus and lengths of the PCR fragments of the genotyping PCR reactions: US PCR fragment length of 3475 bp; DS PCR-fragment length of 3024 bp.

The growth and PCR screening tests (Fig. 8) show that the phenotype rescue was successful in the progeny F1 # 11, 2, 5 and 10, as they show neither growth on hygromycin nor a characteristic band at 3204 and 3475 bp in the PCR screening test. In contrast the screening tests of the offspring F1 # 1, 3, 4 and 6 are similar to the *paa1* knockout mutant (ΔPanR), which contains the hygromycin resistance cassette. Thus these progeny are able to grow on hygromycin and also show a characteristic band at about 3204 and 3475 bp as the *paa1* knockout mutant.

One of these strains (F1 # 2) containing the intact transcription factor *paa1* was then tested for restoration of inducibility of the genes of the pantothenate cluster similar as described above. The relative expression of the cluster genes is shown in Fig. 9 and was compared to the reference strains. Strains missing the transcription factor *paa1* show a significantly reduced transcript level (P = 0.05, onesided unpaired t-test) compared to the reference strain after induction with 1 and 50 mM pantothenic acid. In contrast, the restored mutant strains show no significant difference (P = 0.05, onesided unpaired t-test) to the reference strain QM9414 *Δtku70* and thus show a comparable transcript level.

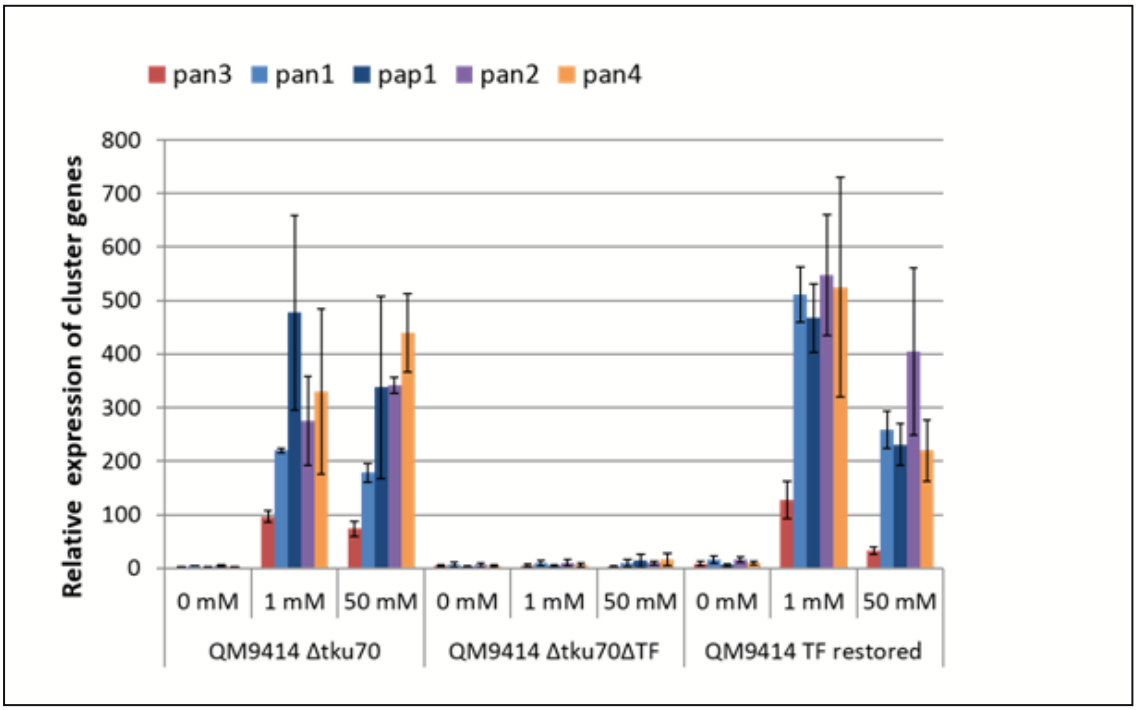

Figure 9: Relative expression of the pantothenic acid catabolic genes *pan1-4* and *pap1* at 0, 1 and 50 mM pantothenic acid compared between the reference strain QM9414*Δtku70* that contains the intact transcription factor *paa1* and the *paa1* knockout strain (QM9414 Δtku70ΔTF) and the strain with the restored *paa1* (QM9414 Δtku70 TF restored). Mean and standard error between 3 and 7 biological replicates are shown.

4.2. Complementation of the *S. cerevisiae* Δ*fen2* strain with the *T. reesei pap1*

To learn whether *T. reesei pap1* indeed encodes a pantothenate permease a functional complementation approach was followed. In *S. cerevisiae* a MFS transporter encoded by *FEN2* mediates the uptake of pantothenate from the medium which is impaired in a *fen2* mutant (Stolz *et al.*, 1999). Therefore a *S. cerevisiae fen2* mutant was transformed with an expression plasmid carrying the *T. reesei pap1* gene under the control of the galactose inducible promoter of *GAL10*.

The transformants were cultivated on a galactose minimal medium containing 1.68 µM pantothenic acid. To exclude the possibility of passive transport by diffusion, the medium was buffered to allow a constant pH of 6.5 during cultivation. Cultures were sampled at regular intervals for the following 48 hours of incubation at 30°C to determine the biomass formation.

As shown in Fig. 10, growth of *fen2* cells is limited by the amount of pantothenic acid biosynthesis leading to a substantial elongation of the lag phase as compared to the parental strain BY4741 from which the *fen2* mutant was derived. In contrast growth of cells harboring the *T. reesei pap1* containing plasmid start biomass formation simultaneously with that of strain BY4741 albeit at a slightly slower rate. The results show that growth was only observed in strains expressing *T. reesei pap1*, thus demonstrating that the gene in fact encodes a functional *FEN2* homologue.

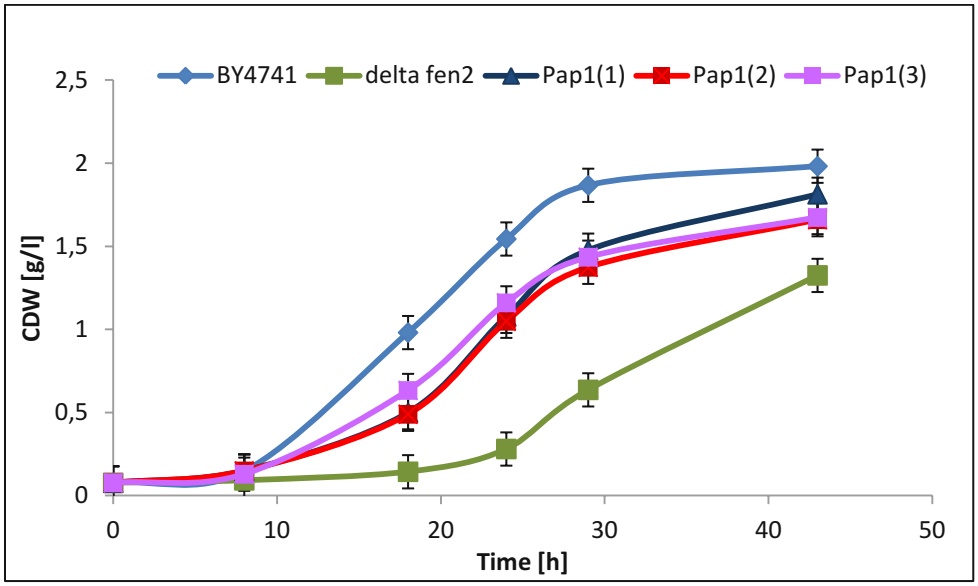

Figure 10: Complementation of the pantothenate permease (*FEN2*) deficient *S. cerevisiae* strain BY4741*ΔFEN2* with the *T. reesei* pantothenate permease *pap1*. Growth is compared between the *pap1* transformed *S. cerevisiae* strains (red, purple and dark blue squares) Pap1 (1-3), the non-transformed *fen2* mutant (BY4741*ΔFEN2*, green squares) and its parental strain BY4741 (blue dots) on galactose minimal medium containing 1.68 µM pantothenic acid. Mean values and standard deviation are shown.

4.3. Characterization of recombinantly produced Pantoate 4-Dehydrogenase PAN2

4.3.1. PAN2 recombinant production and purification

To characterize the enzymatic properties of pantoate 4-dehydrogenase PAN2 three *E.coli* strains, containing plasmid pAB10 (Beinhauer, 2014), were reactivated on LB_{amp} plates at 37°C. Pre-cultures were grown in 10 ml SB_{amp} medium for 19 hours at 37°C and 200 rpm and 100 ml SB_{amp} main cultures were then inoculated with one ml of the pre-culture. After two hours of growth at 37°C and 250 rpm the cultures were cooled down to 25°C in about 15 minutes. Thereafter the cultures were induced with one mM IPTG and were again incubated at 25°C and 250 rpm for 21 hours. After incubation the cells were harvested and stored at -20°C.

A reference strain was cultured at the same conditions, but without IPTG induction.

OD_{600} was measured before and after 21 hours of induction. The results are listed below in Table 7.

Table 7: OD_{600} results of *E. coli* strains carrying the plasmid pAB 10 before and after 21 h of IPTG induction.

E. coli strains	OD_{600} before induction	OD_{600} after 21h of induction
pAB10 Nr.1	0.5	8.78
pAB10 Nr.2	0.5	9.3
pAB 10 Nr.3	0.5	8.64

For characterizing the recombinant produced PAN2, the target protein has first to be isolated from the cell extract. Isolation and purification was performed by means of ÄKTA-FPLC (Fig. 11) using a 1ml Ni^{2+} sepharose high performance column (Histrap™ HP; 1ml; GE Healthcare). After loading the crude extract containing the target protein on the column the flow through was collected until the original UV_{280} signal was reached again. Thereafter elution with buffer B was started using a linear gradient. The first small peak shows contaminants followed by a big peak at about 30 - 45% buffer B. This peak reflects the purified protein PAN2.

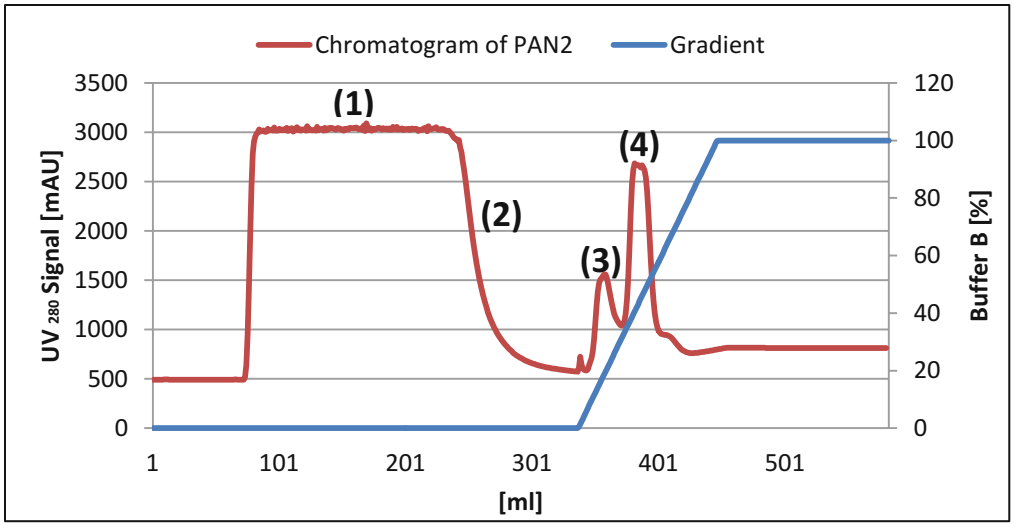

Figure 11: Purification of the His-tagged *T. reesei* pantoate 4-dehydrogenase PAN2 (red line). The first big peak (1) shows the loaded crude cell extract followed by the flow-through (2). After starting the linear gradient (blue line) with buffer B the first small peak (3) shows different contaminants followed by a larger peak (4) that represents the isolated target enzyme PAN2.

The fractions containing a high protein concentration were pooled and analyzed together with the crude extract and the flow through by SDS-PAGE.

15 µg crude cell extract, flow through and not induced cell extract and 7 µg of the purified fraction were loaded onto the gel. The theoretical molecular weight of PAN2 was calculated by an online tool from the EXPASY homepage (http://web.expasy.org/compute_pi/) and equals 26 kDa. The SDS gel (Fig. 12) shows a pronounced band around 27 kDa in the purified fraction (lane 2) as well as in the crude cell extract (lane 4) which reflects a successful over expression and purification of PAN2. In contrast the not induced reference strain does not show a similarly prominent protein band at around 27 kDa.

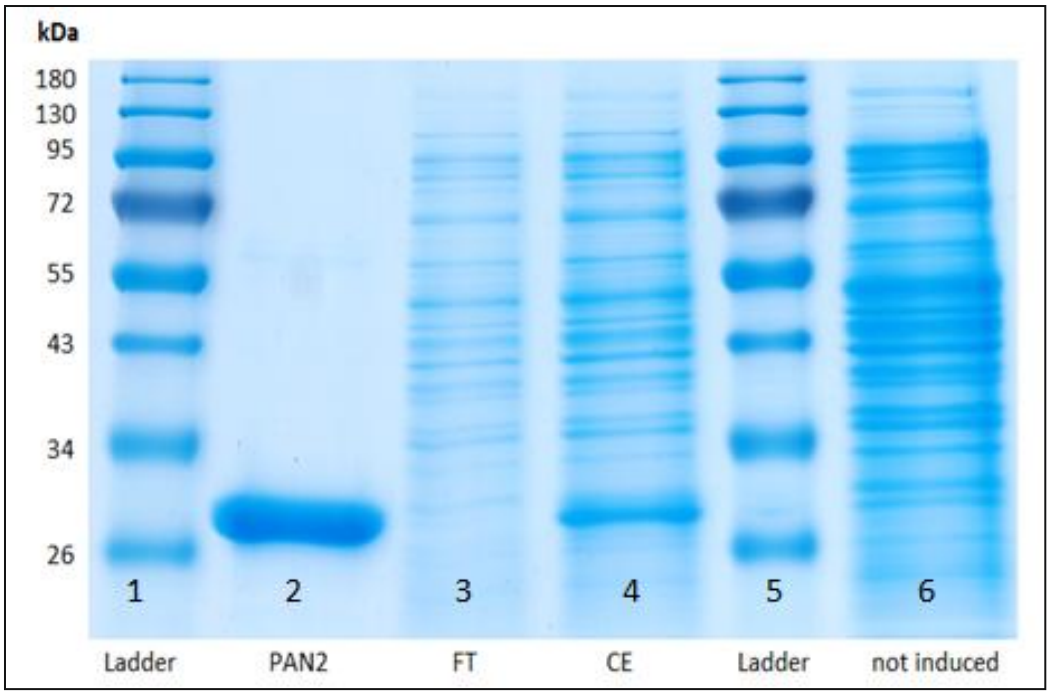

Figure 12: SDS PAGE of *T. reesei* PAN2 producing cells and fractions from the protein purification. Lane 2 (PAN2) shows the purified fraction from the affinity chromatography, lane 3 shows (FT) the unbound fraction of the cell lysate from the affinity chromatography, lane 4 (CE) shows the extracted intracellular protein from induced *E. coli* cells, lane 6 (not induced) shows the extracted intracellular protein from non-induced *E. coli* cells. Lane 1 and 5 show the prestained molecular weight marker.

To determine the exact molecular weight of PAN2 in the SDS-PAGE gel, non linear regression was performed by using SigmaPlot 13 as shown in Fig. 13. The best fitting function was found to be an inverse 2nd order fit with parameters a = 57.14, b = 65.32, y0 = 2.82, R^2 = 0.999. The molecular weight of PAN2 was calculated at 27 kDa which is in good accordance with the theoretical size (26 kDa).

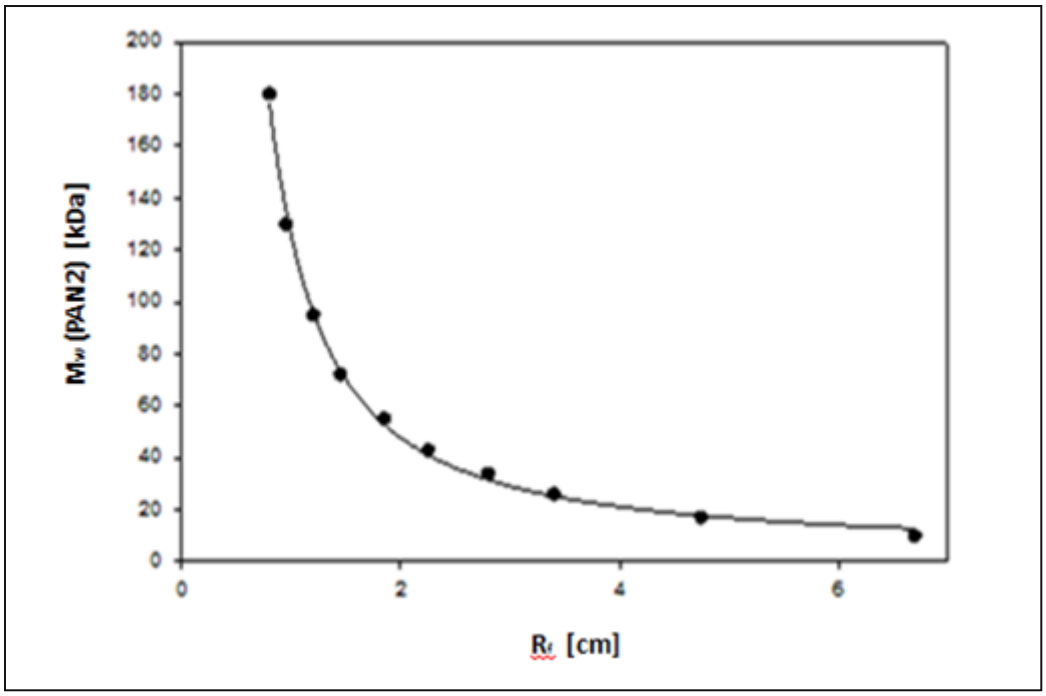

Figure 13: The exact molecular weight (M_W) of *T. reesei* PAN2 was calculated by Sigma Plot 13 using non linear regression. Model: inverse 2nd order; Parameters: a= 57.14, b= 65.32, y0 = 2.82, R^2= 0.999.

4.3.2. *T. reesei* PAN2 enzymatic properties

To further characterize the enzyme activity of *T. reesei* pantoate 4-dehydrogenase PAN2, an enzyme assay was performed as described in the methods section, using the purified and rebuffered enzyme fraction. The activity was measured by following the $NADH^+$ formation photometrical at 340 nm.

Different buffer systems, covering a wide pH - range, and different substrate and NAD^+ concentrations were tested, to determine the respective Michaelis Menten constant. The results are shown in the following graphs below (Figs. 14 and 15).

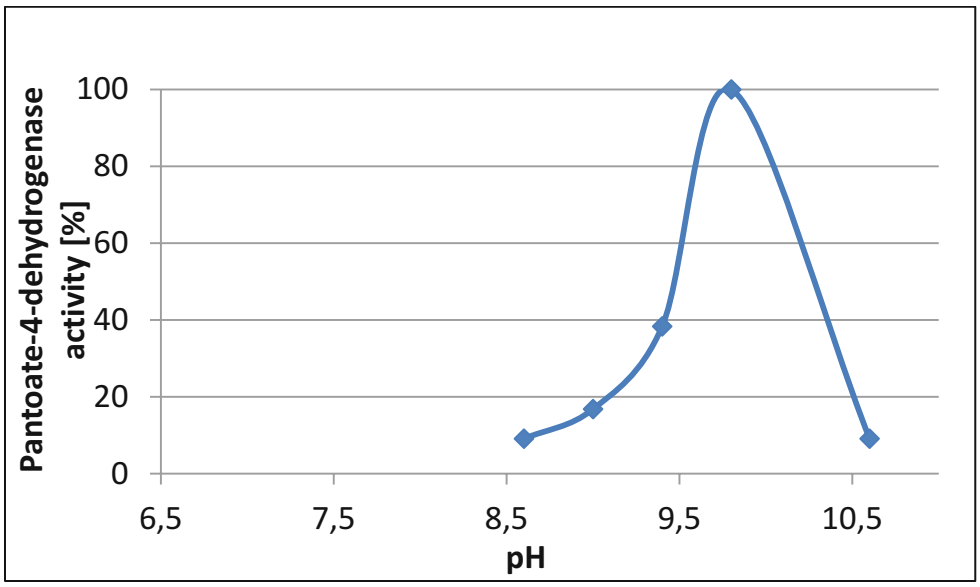

Figure 14: Pantoate 4-dehydrogenase assay using a NaOH glycine buffer (100 mM) to determine the pH-optimum. The pantoate-4-dehydrogenase activity increases up to the pH of 9.7 and then it strongly decreases again.

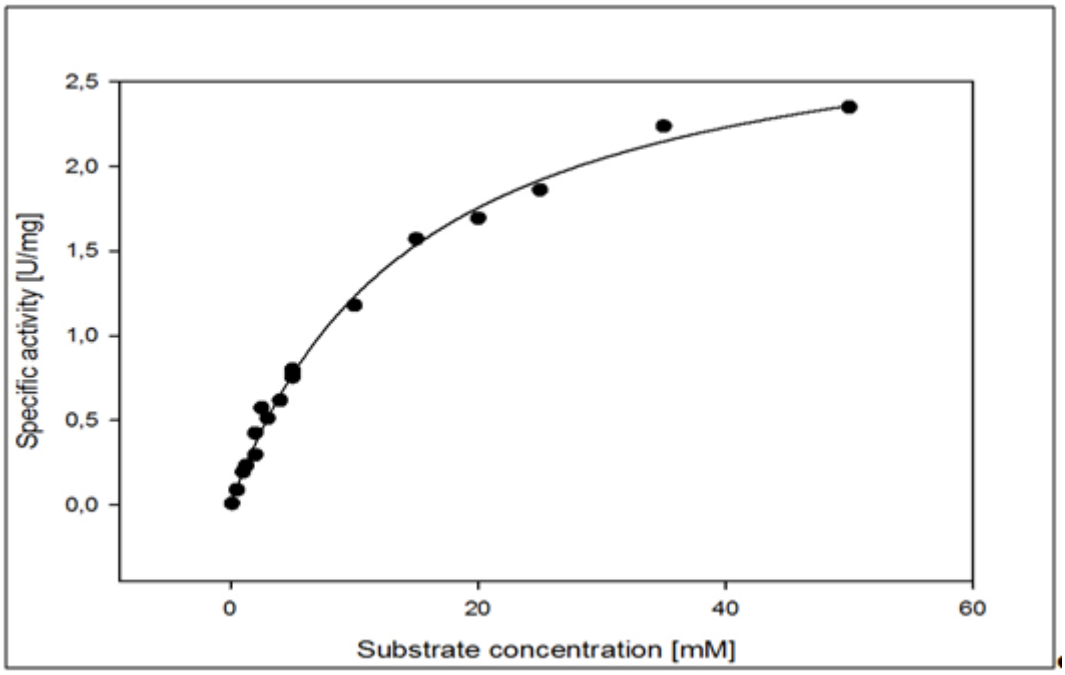

Figure 15: Pantoate 4-dehydrogenase assay: The concentration of pantoate was varied between 0,5 and 50 mM. The NADH formation was followed for 4 min. The Michaelis Menten constant was determined by using the enzyme kinetics tool of Sigma Plot 13.

Fig. 14 shows that the pantoate 4-dehydrogenase activity increases gradually up to a pH of 9.7 and then it decreases strongly again. Fig. 15 shows the specific activity (U/mg) in dependence of the substrate concentrations (mM). The resulting K_M (pantoate) of 19.51 mM ± 1.57 and the v_{max} of 3.33 ± 0.12 U/mg was calculated by Sigma Plot 13 using the enzyme kinetics tool.

4.4. Characterization of recombinant produced Pantothenase PAN1 in *E.coli*

4.4.1. PAN1 recombinant production and purification

For characterizing the enzymatic properties of PAN1, *E. coli* transformants, carrying the plasmid pAB9 (Beinhauer, 2014), were grown on LB_{amp} plates at 37°C. 10ml of SB_{amp} pre-culture were inoculated with a single *E. coli* colony and then cultivated for 19 hours at 37°C and 200 rpm. The main culture medium was then inoculated with the pre-culture as illustrated below. To obtain the optimum yield of target protein the following IPTG induction and different cultivation conditions were tested (Table 8 - 10). After the respective cultivation time the cells were harvested and stored at -20°C. Reference strains were cultured at the same conditions but without IPTG induction.

Table 8: OD_{600} values of *E.coli* strains carrying the plasmid pAB 9 before and after IPTG induction at 37°C*

E.coli strains	Induction	OD_{600} before induction	OD_{600} after 3h of induction
pAB9 Nr.1	40 µM	0.8	5,04
pAB9 Nr.1	400 µM	0.8	4,34
pAB9 Nr.1	not induced	0.8	4,7

* The main culture was inoculated with 200µl pre-culture and grown to an OD_{600} = 0.8 followed by IPTG induction.

Table 9: OD_{600} results of *E.coli* strains carrying the plasmid pAB 9 before and after 21 h of IPTG induction at 25°C*

E.coli strains	Induction	OD_{600} before induction	OD_{600} after 21h of induction
pAB9 Nr.1	1mM	0.4	9,41
pAB9 Nr.1	Not induced	0,48	8.85

* The main culture was inoculated with 1 ml pre-culture and grown to an OD_{600} = 0.4 followed by IPTG induction.

Table 10: OD_{600} results of *E.coli* strains carrying the plasmid pAB 9 before and after 21 h of IPTG induction at 15°C*

E. coli strains	Induction	OD_{600} before induction	OD_{600} after 21h of induction
pAB9 Nr.1	40 µM	0.432	3,42
pAB9 Nr.1	400 µM	0.432	2,44
pAB9 Nr.1	1 mM	0.412	2,82
pAB9 Nr.1	not induced	0.398	3,7

* The main culture was inoculated with 1 ml pre-culture and grown to an OD_{600} = 0.4 followed by IPTG induction.

Expression of PAN1 in *E. coli* turned out to be difficult in initial experiments. To find conditions suitable for PAN1 expression and to check, whether it is produced in a soluble form (rather than in the form of inclusion bodies) a screening test was performed. One ml of culture broth was centrifuged and the resulting pellet was resuspended in 1x PBS - lysozyme buffer. The cells were lysed by means of freeze thawing and thereafter the intracellular proteins were precipitated in acetone on ice. The cell pellet and the acetone treated pellet were analyzed with SDS-PAGE. The results are shown in the Figs 16 - 18.

The theoretical molecular weight of PAN1 was calculated by an online tool from the EXPASY homepage (http://web.expasy.org/compute_pi/) and equals 62 kDa.

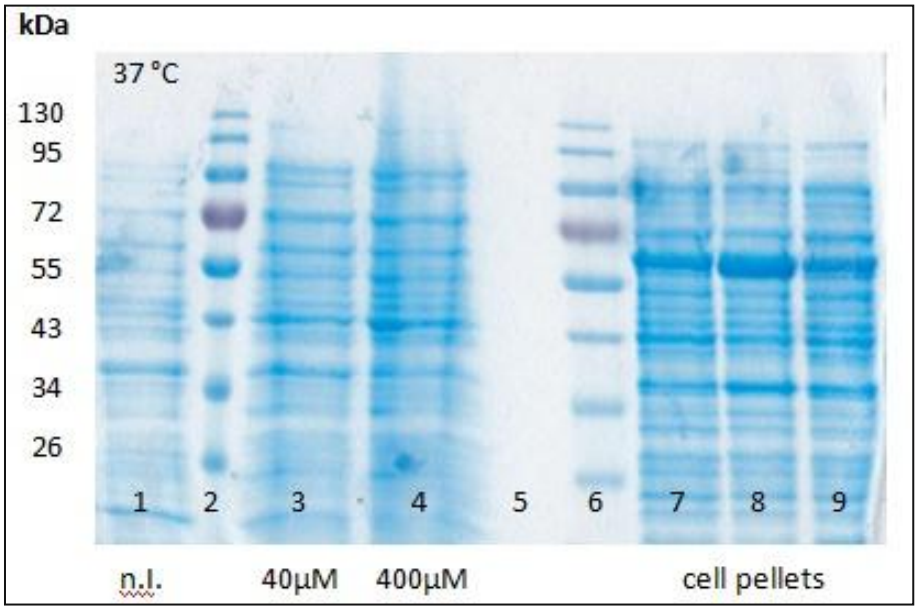

Figure 16: SDS PAGE screening test of PAN1 producing cells at 37 °C: Lane 1 shows the not induced (n.I.) *E. coli* strain; Lane 3 & 4 show the 40 µM and 400 µM IPTG induced *E.coli* strains; Lanes 7 -9 show the respective cell pellets and Lane 2 & 6 show the prestained molecular weight marker.

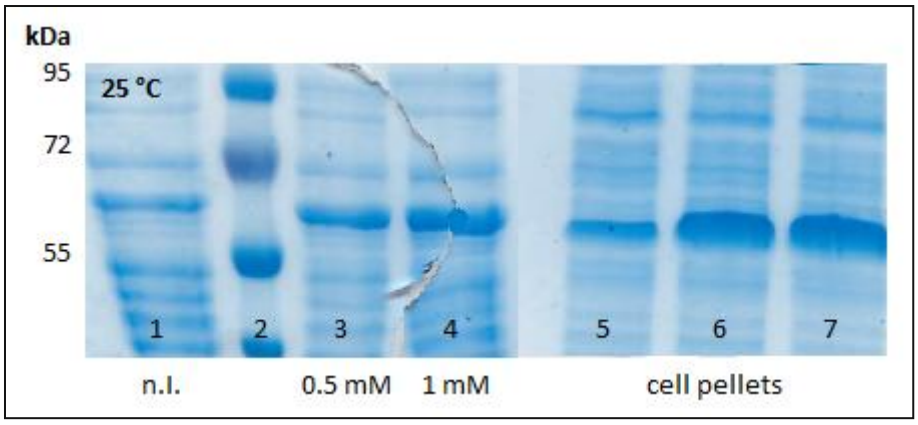

Figure 17: SDS PAGE screening test of PAN1 producing cells at 25 °C: Lane 1 shows the not induced (n.I.) *E.coli* strain; Lane 3 & 4 show the 0.5 mM and 1 mM IPTG induced *E.coli* strains; Lanes 5 - 7 show the respective cell pellets and Lane 2 shows the prestained molecular weight marker.

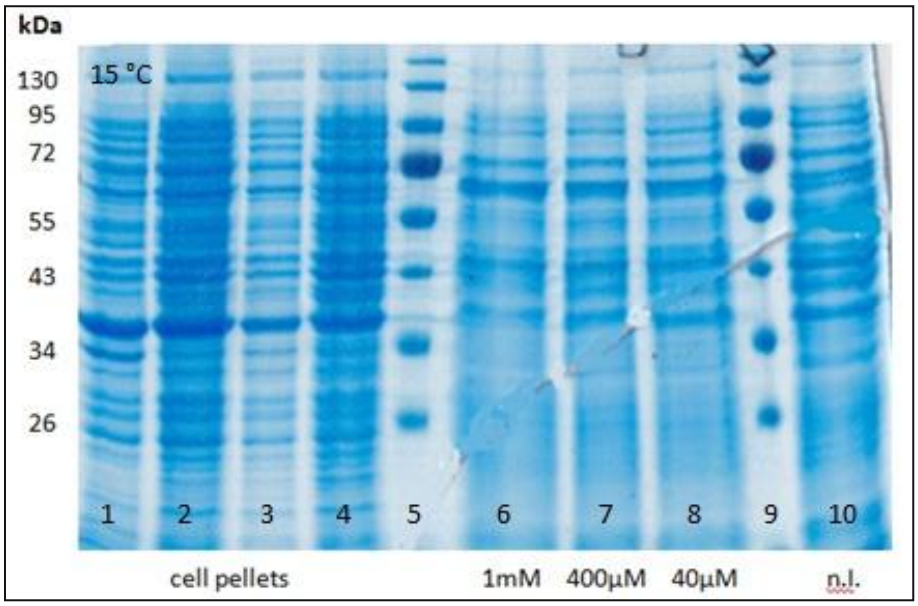

Figure 18: SDS PAGE screening test of PAN1 producing cells at 15°C: Lanes 6 - 8 show the 1mM, 400µM and 40µM IPTG induced *E. coli* strains; Lane 10 shows the not induced (n.I.) *E. coli* strain; Lanes 1 - 4 show the respective cell pellets and Lane 9 & 5 show the prestained molecular marker.

Fig. 16 shows the SDS-PAGE screening of PAN1 producing cells at a cultivation temperature of 37°C after IPTG induction. The gel shows that the high temperature after induction leads to the formation of highly aggregated PAN1 (~ 62 kDa) in both induced *E. coli* strains and almost no PAN1 was produced in soluble form. Also the not induced reference strain showed a slight presence of PAN1 inclusion bodies. A similar pattern is seen in Fig. 17 showing the 25°C cultivation after IPTG induction but with a slightly higher concentration of the soluble form of PAN1. Fig. 18 shows the cultivation at 15°C after induction and demonstrates quite

well that the formation of PAN1 containing inclusion bodies decreases significantly with decreasing temperature.

For characterizing the recombinant produced PAN1, the target protein was isolated and purified by means of ÄKTA-FPLC, using a Ni^{2+} sepharose high performance column (Histrap™ HP;1ml; GE Healthcare). The purification of the his-tagged enzyme PAN1 was challenging as the target protein was probably sensitive to the used elution buffer A containing imidazole. Possible improvements of the purification quality could be obtained by first lysing the *E.coli* cells in Buffer A without imidazole and shortly before loading the crude cell extract onto the column imidazole is added.

4.4.2. PAN1 pH optimum

The degradation of (R)-pantothenate into (R)-pantoate and ß-alanine is catalyzed by the enzyme pantothenase. The enzyme activity was analyzed by an enzyme assay described in the methods section, using the purified and rebuffered enzyme fraction. The ß-alanine formation was measured by a ninhydrin assay at 595 nm.

Different buffer systems (Mc Ivain buffer, HEPES and KH_2PO_4 buffer) were tested. The KH_2PO_4 buffer showed the best results as it spans the pH range of 6 - 8.5 (pH optimum of PAN1 = 7.5) but because of precipitation problems with the substrate, D-Pantothenic acid hemicalciumsalt had to be pre-treated as described in the method section. Fig. 19 shows an increasing ß-alanine concentration up to a pH of 7.5 and then it decreases again.

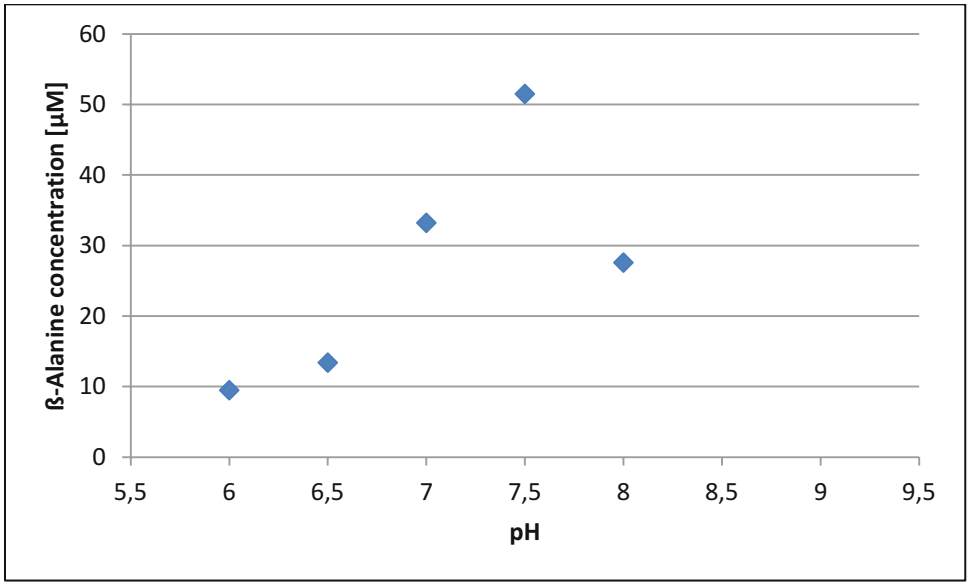

Figure 19: Pantothenase assay using a KH_2PO_4 buffer (100mM) to determine the pH-optimum of PAN1. The ß-alanine concentration increases up to a pH of 7.5 before it decreases.

5. Discussion

Pantothenic acid also known as Vitamin B_5 is essential for life as it is needed for the proliferation of nearly every cell (Higdon, 2003). In contrast to mammals eukaryotic pathogens and some bacteria which must obtain the vitamin from exogenous sources, many bacteria, fungi and plants are capable of producing pantothenic acid de novo (De Villiers *et al*., 2015). The obligatory necessity of pantothenic acid is based on its function as the biosynthetic precursor of coenzyme A, which is required for about 9% of all known enzyme activities (Spry *et al*., 2007) for instance in fatty acid biosynthesis and the citric acid cycle (De Villiers *et al*., 2015). CoA is also required as a cofactor for polyketide synthases as well as for non-ribosomal protein synthases (Kelly, 2011). Further, Brambl *et al.* (1986) determined in *Neurospora crassa* the presence of pantothenic acid derivates in cytochrom c oxidase and ATP synthases.

The metabolic pathway of pantothenate biosynthesis and the respective genes are already well characterized in many bacteria and fungi. In contrast to that is the situation in pantothenate catabolism where the amino acid sequences of the enzymes and their corresponding nucleotide sequences are not known. Only recently Bischof *et al.* (unpublished data) reported the existence of five clustered pantothenate inducible genes (*pap1*, *pan1*, *pan 2*, *pan 3* and *pan4*) and the fungal $Zn(II)_2Cys_6$ transcription factor *paa1* in the *T. reesei* genome which are presumably involved in pantothenate catabolism.

The aim of this thesis was to further characterize components of this pantothenate inducible gene cluster in *T. reesei*. The first step was to prove the hypothesis that the fungal $Zn(II)_2Cys_6$ transcription factor PAA1 is responsible for the regulation of the genes of this cluster. Therefore the effect of the *paa1* gene deletion was studied by comparing the relative expression of the cluster genes in response to 0, 1 and 50 mM pantothenic acid in the QM9414 *Δtku70 Δpaa1* knockout strain and in the parental strain QM9414 *Δtku70*. The results of the investigation demonstrated a positive correlation between the presence of *paa1* and the induction of the other five genes found in the cluster. In order to be able to further substantiate these results *paa1* was reintroduced by crossing into the *Δpaa1* strain. The results showed that the initial expression pattern of the five cluster genes was fully restored under inducing conditions in this backcrossed strain. Therefore, we conclude that PAA1 is the positive regulator of the pantothenate cluster. Further studies will be needed to determine the DNA binding site of PAA1 in the promoter regions in this cluster.

One of the genes of the cluster encodes a putative permease with strong sequence similarity to *FEN2* of *S. cerevisiae* (Stolz *et al.,* 1999) and *Liz1* of *S. pombe* (Stolz *et al.*, 2004). To prove its function as a pantothenate permease, a *S. cerevisiae* complementation assay was

performed. Our results demonstrate that the permease gene *pap1* indeed encodes a pantothenate permease as the *T. reesei pap1* was able to complement a *S. cerevisiae fen2* mutant, defective in pantothenate transport, albeit this complementation was not complete as indicated by a slower rate to the *pap1* complemented strain compared to the parental strain. Low growth is also found in *Δfen2* cells, however it lags behind that of its parental strain BY4741 or the *pap1* complemented BY4741 strain, as it is limited by the pantothenate biosynthesis which is possible due to the ß-alanine production from spermine. In 2001 W. H. White and co-workers proved that *S. cerevisiae* is capable of producing pantothenic acid de novo involving a novel pathway of ß-alanine production from spermine. The pantothenate biosynthesis pathway includes three enzymatic steps starting with 3-methyl-2-oxobutanoate hydroxymethyltransferase (EC 2.1.2.11) which catalyzes the degradation of 2-keto-isovalerate to 2-dehydropantoate, which is degraded by gluconate 5-dehydrogenase (EC 1.1.1.169) to form pantoate. The final step is the condensation of pantoate and ß-alanine (formed from spermine by means of the amine oxidase encoded by *FMS1* (White *et al*., 2001)) to form pantothenate, catalyzed by the pantothenate synthases (EC 6.3.2.1) (https://www.pathway.yeastgenome.org/YEAST).

The first two genes *pan1* and *pan2* in the pantothenate catabolic pathway were expressed in *E. coli* in order to characterize the recombinant produced enzymes PAN1 and PAN2. PAN1 catalyzes the degradation of pantothenate into pantoate and ß-alanine. Its characterization was challenging as the target protein on the one side was often aggregated into inclusion bodies and on the other side PAN1 activity was lost during the purification step. However, decreasing temperature (37°C to 15°C) after IPTG induction of the *E. coli* strains showed an increasing production of soluble PAN1 (Figs. 16 - 18). In recombinant protein biotechnology the accumulation of denatured protein molecules into inclusion bodies is usually a undesirable aspect reported in several studies (Büssow *et al*., 2005; Pacheco *et al*., 2012). *E. coli* is the dominant and most convenient host for high level expression of recombinant proteins as it has several advantages, e.g. a fast and cheap high yield protein production and the tools for recombinant protein expression are also well characterized. Nevertheless, these bacterial cells also have their limitations in producing more complex proteins as they do not possess a sophisticated posttranslational modification machinery which often results in the aggregation of the foreign proteins into inclusion bodies. In order to counteract these stressful situations (high yield protein production) in the *E. coli* cell different strategies are possible to contribute to a soluble protein production und purification: (I) low expression temperatures slow down the protein synthesis rate and the folding kinetics which leads to a decrease of the hydrophobic interactions that are responsible for protein self-aggregation; (II) special *E. coli* engineered strains, developed through the introduction of DNA mutations (e.g. Arctic Express strain which shows an improved protein processing at low temperatures;

Rosetta strain and the BL21 Codon Plus strain which supply extra copies of rare tRNAs and the C41 and C43 BL21 (DE3) mutant strain which were developed for a better synthesis of membrane proteins); (III) high density culture systems like batch (limited control of cell growth) and fed batch (real time optimization of the cell growth); (IV) co production of chaperons and foldases; (V) fusion tags mediate the solubility of expressed target proteins as they are stable and soluble expressed molecules in *E. coli* and thus they can be genetically linked with the target proteins. The novel Fh8 fusion tag is one of the best enhancer partners for a soluble protein overproduction and purification (Costa *et al.*, 2014). The small protein Fh8 (8kDa) is secreted by the parasite *Fasciola hepatica* in the early stages of infection and belongs to the calmodulin-like EF-hand CaBP family (Costa *et al.*, 2014). By using recombinant produced Fh8 a fast and simple immunodetection of *Fasciola hepatica* was developed (Silva *et a*l., 2004). Furthermore, it was revealed that rFh8 is highly soluble and it demonstrates an unusual thermal stability (T_m = 74°C ± 0.3) compared to other members of the EF-hand family. The stability even rises in the Ca^{2+} - loaded state because Fh8 is stabilized by Ca^{2+} ligand interactions (Silva *et al.*, 2004, Fraga *et al.*, 2010). Thus Fraga *et al.* (2010) characterized Fh8 as a Ca^{2+} sensor protein.

Further investigations will be necessary to prevent the target protein PAN1 from denaturing and thus obtaining sufficient results for its characterization. In *Pseudomonas P-2* Nurmikko *et al.* (1966) also reported the loss of pantothenate hydrolase activity (~ 97 %) after the purification steps. Therefore it will be necessary to optimize the purification and isolation steps (buffers) during affinity chromatography. Furthermore, a different fusion protein strategy which increases solubility and stability of the target protein could be an effective solution for finally characterizing PAN1.

The enzyme PAN2 catalyzes the oxidation of pantoate to R-4-dehydropantoate. It was possible to characterize the recombinant produced PAN2 in *E. coli*: M_w = 27 kDa; pH of 9.7; K_M (NAD^+) = 0.8 mM and K_M (pantoate) = 19.51 mM. The relative high pH of 9.7 (Fig. 14) is quite surprisingly as the cytosol normally has a pH of about 7.4. A reason for this high pH could be that the hydroxyl group of pantoate is more easily deprotonated at a higher pH. Compared to the our received results C.T. Goodhue *et al.* (1966) also reported that the best yields for aldopantoate formation were obtained at a pH setting of 10 throughout the reaction. The reason for the high pH optimum results from the removal of the hydrogen ion formed and from the partial non enzymatic reversal of the aldol reaction at high pH values which removes a part of the formed aldopantoate. In *Pseudomonas p-2* the K_M for pantoate was determined as 33 µM at a pH of 10 which is much lower compared to our achieved result (K_M (pantoate) = 19.51 mM, pH of 9.7) in *T. reesei*.

Challenging future targets in this research field will be the characterization of the other two enzymes PAN3 and PAN4 to fully characterize the pantothenate catabolic pathway in *T. reesei.*

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